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Ruminant nutrition and function: understanding methane mitigation routes and impacts

Irene Cabeza Luna

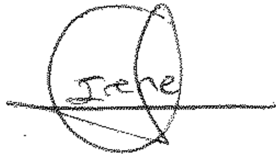
Thesis submitted for the degree of Doctor of Philosophy

August 2017

Declaration

I declare that this thesis is my own composition, and the research contained within it is my own work, except where acknowledged. The work that has been described in this thesis has not been submitted for any other degree or professional qualification.

Irene Cabeza Luna



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"*Cuando finalmente aceptas que esta bien no tener respuestas y*

que esta bien no ser perfecta,

te das cuenta de que sentirse confundida

es una parte normal del ser humano"

Table of contents

Declaration

Acknowledgements ii

Table of contents iv

List of tables x

List of figures xiv

Publications xvii

Abbreviations xviii

Abstract xx

Lay summary xxii

Chapter 1 General introduction 1

1.1 Ruminant production 1

1.1.1 The role of ruminants in the global supply of food 1

1.1.2 Ruminant industry: place in the world and within EU 3

1.2 Methane emissions: world and UK 4

1.3 Methane production by ruminants 7

1.3.1 Rumen fermentation 7

1.3.2 Methanogenesis in the rumen 9

1.3.3 Microbial population involved in methanogenesis 11

1.4 Strategies to reduce methane emissions from ruminants 13

1.4.1 Animal manipulation 16

1.4.2 Rumen manipulation 18

1.5 Aims of the thesis 32

Chapter 2. *In vitro* screening of biochar compounds as antimethanogenic feed additives for ruminants 34

2.1 Introduction 34

2.1.1 Biochar 34

2.1.2 Aim of the study 36

2.2 Material and methods 37

2.2.1 Biochar production 37

2.2.2 Experimental design 39

2.2.3 Rumen fluid inocula 39

2.2.4 *In vitro* gas production test 40

2.2.5 Analytical methods 42

2.2.6 Calculations and statistical analyses 44

2.3 Results 45

2.3.1 Rumen inocula 45

2.3.2 Overall effects of biochar compounds 47

2.3.3 Effect of inclusion level of biochar on rumen fermentation 49

2.3.4 Effects of preparing biochar at different temperatures on rumen gas production and fermentation 49

2.3.5 Effect of preparing biochar from different feedstocks on rumen gas production and fermentation 50

2.4 Discussion 52

2.4.1 *In vitro* gas fermentation technique 52

2.4.2 Effects of biochar on gas production 54

2.4.3 Effects of biochar compounds on rumen fermentation 54

2.4.4 Properties of biochar compounds and mechanism of action 56

2.5 Conclusions and implications 58

2.6 Future work 59

Chapter 3. Effect of encapsulated nitrate and elemental sulphur on methane production, rumen fermentation and nitrogen metabolism in beef cattle 60

3.1 Introduction 61

3.1.1 Nitrate 62

3.1.2 Urea 71

3.1.3 Sulphur compounds 72

3.1.4 Aim of the study 74

3.2 Material and methods 75

3.2.1 Experimental design, Animals and Diets 75

3.2.1.1 Experimental design 75

3.2.1.2 Animals 75

3.2.1.3 Diets 76

3.2.2 Sampling and Measurements procedures 80

3.2.2.1 Feed sampling and intakes 80

3.2.2.2 Blood sampling 80

3.2.2.3 Methane gas collection 82

3.2.2.4 Faeces and urine collection 84

3.2.2.5 Rumen content sampling 85

3.2.3 Laboratory analysis 86

3.2.4 Calculations and statistical analysis 87

3.2.4.1 Statistical analysis 89

3.3 Results 90

3.3.1 Adaptation period 90

3.3.1.1 Blood methaemoglobin 90

3.3.1.2 Nutrient intake, digestibility and feed consumption rates 91

3.3.2 Sampling period 95

3.3.2.1 Feed intake and refusal composition ... 95

3.3.2.2 Enteric methane emissions ... 95

3.3.2.3 Nitrogen metabolism ... 98

3.3.2.4 Rumen fermentation ... 101

3.4 Discussion ... 107

3.4.1 Effect of dietary nitrate and sulphur on methane production ... 107

3.4.2 Nitrate toxicity ... 111

3.4.3 Effect of nitrate and sulphur on nitrogen utilization and microbial protein synthesis of steers ... 113

3.4.4 Effect of nitrate and sulphur on rumen fermentation ... 115

3.5 Conclusions and implications ... 117

3.5.1 Implications ... 117

3.6 Future work ... 119

Chapter 4.Assessment of the effects on the rumen microbial community of adding nitrate to diets containing different proportions of concentrate ... 122

4.1 Introduction ... 122

4.1.1 Relationships between the rumen microbiome and CH4 production ... 123

4.1.2 Microbial populations involved in nitrate-nitrite metabolism ... 125

4.1.3 Molecular biology techniques to study the rumen microbiome ... 127

4.1.4 Experiment setup and aim of the study ... 129

4.2 Material and methods ... 130

4.2.1 Animal study ... 130

4.2.1.1 Experiment 1. Experimental setup ... 131

4.2.1.2 Experiment 2. Experimental setup ... 132

4.2.2 DNA extraction from rumen fluid ... 133

4.2.3 PCR amplification of 16S rDNA and amplicon library preparation ... 134

4.2.3.1 Amplicon library submission and sequencing 137

4.2.4 Bioinformatics and statistical analysis 140

4.3 Results 142

4.3.1 Experiment 1 142

4.3.1.1 CH_4 yield (g/kg DMI) 142

4.3.1.2 Analysis of Microbial composition (16S rRNA) 143

4.3.1.3 Effect of basal diet on microbial community composition. 146

4.3.1.4 Effect of nitrate on microbial community composition 154

4.3.1.5 Taxonomic populations correlated with methane production 160

4.3.2 Experiment 2 166

4.3.2.1 CH_4 yield (g/kg DMI) 166

4.3.2.2 Analysis of Microbial composition (16S rRNA gene)................ 167

4.3.2.3 Effect of nitrate on microbial community composition 167

4.3.2.4 Taxonomies correlated with methane production 175

4.3.3 Effects of nitrate: Results Summary 179

4.3.3.1 CH_4 and H_2 production (g/kg DMI) 179

4.3.3.2 Bacterial population affected by nitrate 180

4.3.3.3 Archaeal population affected by nitrate 180

4.3.3.4 Correlations between bacterial and archaeal populations and CH_4 across different basal diets 183

4.3.3.5 Correlation between H_2 released from the rumen, CH_4 production, main bacterial genus and archaeal populations 183

4.4 Discussion 186

4.4.1 Effect of basal diet on CH_4 production and microbial community structure.. 187

4.4.2 Effect of nitrate on CH_4 production and microbial community structure ……………………………………………………………………..191

4.4.3 Correlation between CH_4 production, H_2 production and microbial community structure 196

4.5 Conclusions and implications 199

4.5.1 Implications of the study 200

4.6 Future work. 202

Chapter 5. General discussion 204

5.1 Summary of main findings 205

5.2 Practical considerations of methodologies 208

5.2.1 Sampling of rumen fluid 208

5.3 General implications 212

5.3.1 Strategies to reduce methane emission from ruminants 212

5.3.2 Practical implications of nitrate and biochar addition to beef cattle diets. 213

5.3.3 Microbial community analysis 217

5.4 Future work 218

5.4.1 Biochar 218

5.4.2 Nitrate 219

5.4.3 Microbial community analysis. 221

5.5 Concluding remarks 223

Reference List 224

Appendices 253

List of tables

Table 1.1 Animal management strategies to reduce CH_4 emission intensity from ruminants (adapted from Hristov 2013) 17

Table 2.1 Physicochemical composition of biochar samples used for *in vitro* incubations 38

Table 2.2 Individual content of serum bottles present in each day incubation with one unique rumen fluid source 41

Table 2.3 Average, Maximum, Minimum and Standard deviation (SD) of all parameters studied 45

Table 2.4 Values from different sources of rumen fluid used on *in vitro* incubations 46

Table 2.5 Parameters expressed as proportion of control samples and results from T-tests (SEM and P values) with the hypothesis that values from samples which included biochar were different from 1 (control) 47

Table 2.6 Effects of different levels of biochar inclusion on *in vitro* gas production and fermentation parameters after 24h incubation 49

Table 2.7 Effects of inclusion of biochar compounds prepared at different temperature in gas production and fermentation parameters *in vitro* after 24 h incubation 50

Table 2.8 Effects of inclusion of biochar compounds from 5 different feedstocks in gas production and fermentation parameters *in vitro* after 24 h incubation 51

Table 3.1 Effect of nitrate addition on *in vivo* CH_4 emission (summary of studies).. 65

Table 3.2 Toxicity effects of nitrate addition (MetHb and DMI) (summary of studies) 69

Table 3.3 . Effects of nitrate on rumen fermentation (summary of studies) 70

Table 3.4 Experimental treatments 77

Table 3.5 Chemical composition of diet ingredients (g/kg DM) 78

Table 3.6 Experimental diets formulation and chemical composition (g/kg DM) 79

Table 3.7 Experimental timeline used to gradually increase dietary encapsulated nitrate (EN) fed to steers with ad libitum intake 81

Table 3.8 Blood MtHb values (% total Hb) on days 7, 10 and 14 of adaptation. Animals were fed 15 g/kg of EN on day 7 and 20 g/kg of EN on days 10 and 14 90

Table 3.9 Nutrient intakes and refusals (DM, expressed as a proportion of daily feed offered) from steers fed the different diets: Control (True protein), Urea, Urea + S, Nitrate or Nitrate + S, during adaptation to experimental diets 93

Table 3.10 Intake the first 3 h after fresh feeding was offered as total intake and as a proportion of the total daily intake by steers fed the different diets: Control (True protein), Urea, Urea + S, Nitrate or Nitrate + S, during adaptation to experimental diets 94

Table 3.11 Diets vs refusals composition. Diet composition analysed at the beginning of the experiment. Samples of refusals obtained from day 15 to day 21, composited for animal and period 96

Table 3.12 Methane produced by steers fed the different diets: Control (True protein), Urea, Urea + S, Nitrate or Nitrate + S (g/day and g/kg DMI) 97

Table 3.13 Nitrogen utilization and excretion by steers fed the different diets: True protein (control), Urea, Urea + S, Nitrate or Nitrate + S 99

Table 3.14 Urinary excretion of purine derivatives and Microbial Protein Synthesis (MPS) by steers fed the different diets: Control (True Protein), Urea, Urea + S, Nitrate or Nitrate + S. 100

Table 3.15 Rumen fermentation parameters before feeding of steers fed the different diets: Control (True protein), Urea, Urea + S, Nitrate or Nitrate + S 102

Table 3.16 Interaction between treatments and time points for rumen parameters measurements for the different contrasts studied 103

Table 3.17 Methane production from steers fed different dietary treatments (combining studies) 108

Table 4.1 Animal and samples selected for the experiment 130

Table 4.2 Ingredients composition of Mixed and Concentrate diets (g/kg DM) 131

Table 4.3 Ingredients composition of experimental diets (dry matter basis; g/kg) 132

Table 4.4 Shannon index values of samples from steers fed the mixed and concentrate with and without addition of nitrate 143

Table 4.5 Mean relative abundance of main phyla across mixed and concentrate diets with and without nitrate 144

Table 4.6 Phyla significantly different ($P<0.05$) between mixed and concentrate diets (Relative abundance %) 147

Table 4.7 Correlation between diet, CH_4 yield and interactions with PC scores..... 147

Table 4.8 Relative abundance of families in samples from steers fed concentrate and mixed diets 150

Table 4.9 Relative abundance of genera in samples from steers fed concentrate and mixed diets 151

Table 4.10 Differences in relative abundances of archaeal populations between mixed and concentrate diets 153

Table 4.11 Main genus relative abundance on samples from steers fed the concentrate and mixed diets with and without nitrate addition 157

Table 4.12 Families, genus and some OTUs archaeal populations from samples from steers fed the concentrate and mixed diets with and without nitrate addition . 158

Table 4.13 Relative abundances (%) of genus *Campylobacter* and OTU 686 identified as Campylobacter genus 159

Table 4.14 Relative abundance of taxonomic populations potentially correlated with CH_4 production from concentrate and mixed basal diets with and without addition to nitrate. 161

Table 4.15 OTUs correlated with CH_4 in both mixed and concentrate diets 162

Table 4.16 Correlation between archaea population and CH_4 production within mixed and concentrate diets 163

Table 4.17 Mean relative abundance of phyla most abundant and A: B ratio on samples from steers fed mixed diets with and without addition of nitrate. 168

Table 4.18 Correlation between CH_4 yield, diet and interaction with PC scores 168

Table 4.19 Relative abundance of main genera in samples from mixed diet with and without nitrate 171

Table 4.20 Relative abundance of archaeal populations with and without addition of nitrate 173

Table 4.21 Relative abundance of the genus Campylobacter, OTU 686 and OTU 567 in the presence or absence of supplementary nitrate 174

Table 4.22 Relative abundance of taxonomies potentially correlated with CH_4 production from mixed diets with and without addition of nitrate 176

Table 4.23 Correlation between main genera and CH_4 production across all samples 177

Table 4.24 Correlation between archaea population and CH_4 production 177

Table 4.25 Relative abundances of taxonomies with significant different abundances with nitrate addition to control mixed diets from Exp 1 and Exp 2 179

Table 4.26 Spearman correlation between archaeal populations and CH_4 production across all samples and within concentrate and mixed (combining data from the two experiments) basal diets. 184

Table 5.1 Mean relative abundance of archaea taxonomies and correlation with CH_4 (g/kg DMI) in *in vivo* and *post-mortem* samples 210

List of figures

Figure 1.1 Global ruminant numbers from 1961 to 2011 (Ripple et al., 2014) 3

Figure 1.2 Current estimated annual anthropogenic emissions from major sources of CH_4. Error bars represent ±standard deviation (Ripple et al., 2014) 5

Figure 1.3 Metabolism of glucose in the rumen. Electron sinks are highlighted with white boxes 8

Figure 1.4 Methanogenesis in the rumen 9

Figure 1.5 Potential targets of decreasing CH_4 emissions from rumen 15

Figure 1.6 Rumen manipulation strategies to reduce CH_4 production....................... 19

Figure 2.1. Incubation images... 41

Figure 2.2 *In vitro* incubation images 43

Figure 2.3 Linear regression between electrical conductivity (dS/m) of biochar compounds versus CH_4 produced (ml/g substrate) and total VFA produced (mmol/g substrate) from *in vitro* incubations 48

Figure 3.1 Angus x Nelore steer in its corresponding experimental unit 76

Figure 3.2 Feed ingredients of diets.. 79

Figure 3.3 Experimental timetable.. 81

Figure 3.4 a) Steer wearing halter and canisters 83

Figure 3.5 Blood MetHb values (% total Hb) for individual steers receiving the nitrate and nitrate +S diets on days 1, 4, 7 and 10 of adaptation period 91

Figure 3.6 Daily rumen NH_3-N concentration pattern (mg/dl) (hours after feeding) of steers fed the different diets 101

Figure 3.7 Daily pattern (hours after feeding) of rumen VFA production from steers receiving nitrate containing diets vs urea diets 105

Figure 3.8 Propionate molar proportions during the day (hours after feeding) from steers receiving sulphur treatments vs no sulphur containing ones 106

Figure 3.9 Daily pattern of NH_3-N rumen concentration (hours after feeding) from steers receiving nitrate diets vs urea diets 106

Figure 4.1 PCR reaction (0:00 minutes:seconds).. 135

Figure 4.2 PCR products from a single PCR reaction 135

Figure 4.3 1% agarose gel image of the two pools 136

Figure 4.4 Tape Station image. F1Pool 1; B2 Pool 2 138

Figure 4.5 Electropherogram of amplicon libraries (Ed. Genomics) 138

Figure 4.6 Process workflow from rumen fluid collection to Amplicon for sequencing 139

Figure 4.7 Boxplot of CH_4 produced (g/kg DMI) by steers fed the mixed and concentrate diets with and without the addition of nitrate 142

Figure 4.8 Plot of relative abundances at phylum level for individual rumen samples for mixed and concentrate diets with and without nitrate 145

Figure 4.9 Correlation between CH_4 yield and PC-1 score for mixed and concentrate diet 148

Figure 4.10 PCA overview of 16S rRNA gene analysis of concentrate and mixed diets (a) scores plot for PC-1 vs. PC-2 (b) scores plot for PC-3 vs. PC-2 (c) scores plot for PC-1 vs PC-3 (d) explained variance plot 149

Figure 4.11 Relative abundances of OTUs 964 (genus *VadinCA11*) and OTU 26 (genus *Methanobrevibacter*) in samples from steers fed the concentrate and mixed diets 152

Figure 4.12 PCoA showing the relationship of samples from mixed diet based on all OTUs with or without addition of nitrate to diets (a) scores plot for PC-1 vs. PC-2 (b) scores plot for PC-3 vs. PC-2 (c) scores plot for PC-1 vs PC-3 (d) explained variance plot. 155

Figure 4.13 PCoA showing the relationship of samples from concentrate diets based on all OTUs with or without addition of nitrate to diets (a) scores plot for PC-1 vs. PC-2 (b) scores plot for PC-3 vs. PC-2 (c) scores plot for PC-1 vs PC-3 (d) explained variance plot. 156

Figure 4.14 Relative abundance of genus *Campylobacter* from steers fed the concentrate and mixed diets with and without nitrate addition 160

Figure 4.15 Linear regression between Total Archaea with CH_4 (g/kg of DMI) within mixed and concentrate basal diets 164

Figure 4.16 Linear regression between *Methanobrevibacter* genus and OTU 9 with CH_4 (g/kg of DMI) within mixed and concentrate basal diets 165

Figure 4.17 Boxplot of CH_4 produced (g/kg DMI) by steers offered mixed diets with and without nitrate addition 166

Figure 4.18 Plot of relative abundances for individual rumen samples at phylum level from steers fed mixed diets with and without addition of nitrate 169

Figure 4.19 PCoA for relative abundance of all OTUs in samples from steers fed mixed diets with and without nitrate (a) scores plot for PC-1 vs. PC-2 (b) scores plot for PC-3 vs. PC-2 (c) scores plot for PC-1 vs PC-3 (d) explained variance plot 170

Figure 4.20 Relative abundance of the genus Campylobacter in the presence or absence of supplementary nitrate 174

Figure 4.21 Linear regression between Total Archaea, *Methanobrevibacte*r genus and OTU 9 (*Methanobrevibacter*) with CH_4 (g/kg of DMI) in mixed diets........ 178

Figure 4.22 Venn diagram showing bacterial taxonomies of interest affected by nitrate across different diets and experiments. 181

Figure 4.23 Venn diagram showing archaeal OTUs affected by nitrate across diets and experiments 182

Figure 4.24 Plot of relative abundance of total archaea, against CH_4 yield within mixed diet and concentrate diet in Exp 1 (conc, mix 1) and within mixed diet in Exp 2 (mix 2). Regression equations and P-values for each group diet are presente 185

Figure 5.1 Number of OTUs with different relative abundance between mixed control and mixed nitrate diets in chamber and slaughter samples........ 209

Figure 5.2 Correlation between CH_4 emissions of steers in respiration chambers and archaea relative abundance in ruminal digesta in live animals and at slaughter 211

Publications

Research articles

Yang, C., Rooke, J.A., **Cabeza, I**., Wallace, R.J., 2016. "Nitrate and Inhibition of Ruminal Methanogenesis: Microbial Ecology, Obstacles, and Opportunities for Lowering Methane Emissions from Ruminant Livestock." Frontiers in microbiology 7. Jamuary 2016

Cabeza I, Waterhouse T, Sohi S, Rooke JA. "Effect of biochar produced from different biomass sources and at different process temperatures on methane production and ammonia concentrations in vitro". Animal Feed Science and Technology 2018 Jan 237, 1-7

Conference contributions (oral presentations and posters)

SRUC Postgraduate Conference 2015. 19th and 20th March 2015. Poster Presentation "*In vitro* screening of different biochar compounds as antimethanogenic additives for ruminants".

I Cabeza, R Dewhurst, T Waterhouse, S Sohi, J Rooke. *In vitro* screening of different biochar compounds as antimethanogenic additives for ruminants. BSAS Annual Conference 2015 (oral presentation).

Cabeza Luna I, John Rooke, Telma Teresinha Berchielli, Juliana Duarte Messana, Lucas Rocha Rebelo, Izabelle Texeira. Effect of encapsulated nitrate (EN) and elemental sulphur (S) on methane (CH_4) production and microbial protein synthesis in beef cattle. BSAS Annual Conference 2017 (oral presentation).

Abbreviations

ADG – Average Daily Gain

BW – Body Weight

C – Carbon

CH_4 - Methane

CHO - Carbohydrate

CO_2- Carbon dioxide

CP – Crude Protein

DM – Dry Matter

DMD – Dry Matter Digestibility

DMI – Dry Matter Intake

DOM – Digestible Organic Matter

DUP- Digestible Undegraded Protein

EN- Encapsulated Nitrate

FCE – Feed Conversion Efficiency

FCR – Feed Conversion Ratio

G - Free energy

H_2 – Hydrogen

Hb- Haemoglobin

MetHb – Methaemoglobin

MP- microbial protein

MPS- microbial protein synthesis

N-Nitrogen

NDF - Neutral detergent fibre

NGS - Next generation sequencing

NH_3-N - Ammonia

NRB- Nitrate reducing bacteria

O_2 – Oxygen

OM – Organic Matter

OMD – Organic Matter Digestibility

OTU – Operational Taxonomic Unit

PCA – Principal Component Analysis

PCR – Polymerise Chain Reaction

PD- purine derivatives

S-sulphur

SF6- sulphur hexafluoride

SED- Standard error of differences

SRB- Sulphur reducing bacteria

UDP - Undegradable Dietary Protein

VFA - Volatile fatty acid

WGS - Whole Genome Sequencing

16S ribosomal RNA- 16S rRNA

Abstract

Methane is a potent greenhouse gas with a global warming potential 21 times that of carbon dioxide. Globally, ruminants are the main anthropogenic contributors to methane release to the atmosphere. Methane is produced in the gastrointestinal tract of ruminants, mostly within the rumen by methanogenic archaea. However, methane production represents a loss of 2 to 12% of dietary gross energy for the animal, which could otherwise be available for growth or milk production. Therefore, mitigation of methane production by ruminants could produce both economic and environmental benefits, with more sustainable and energy efficient livestock, and offering a promising way of slowing global warming. Despite extensive research undertaken to find ways of reducing methane emissions from ruminants, progress has been relatively limited. Furthermore, there is still a lack of studies linking rumen microbiology and ruminant nutrition and production.

The central purpose of this research was to investigate feed additives to reduce methane emissions and to understand associated changes that occur in the rumen microbiota. For the first experiment (Chapter 2), biochar was evaluated as an antimethanogenic compound for beef cattle. The *in vitro* gas production technique was used to study the effects of biochar on rumen fermentation and methane production. Overall, methane production was reduced by 5% by the addition of biochar compounds (10 g/kg of substrate). The observed reduction in methane produced was not associated with a change in volatile fatty acid profile suggesting biochar primarily inhibited fermentation. Ammonia concentration was significantly reduced with biochar inclusion. Because different biochars had different effects on methane production, further investigation of relationships between the physicochemical properties of biochars and antimethanogenic effects are necessary. However, due to the small reduction in methane production recorded, research with biochar was discontinued. Encapsulated nitrate was then explored as an antimethanogenic additive and as an alternative non-protein nitrogen source to urea (Chapter 3). The effect of using encapsulated nitrate as a replacement for urea or dietary protein, plus the addition of inorganic sulphur, on enteric methane emissions, nutrient digestibility, nitrogen utilization and microbial protein synthesis from

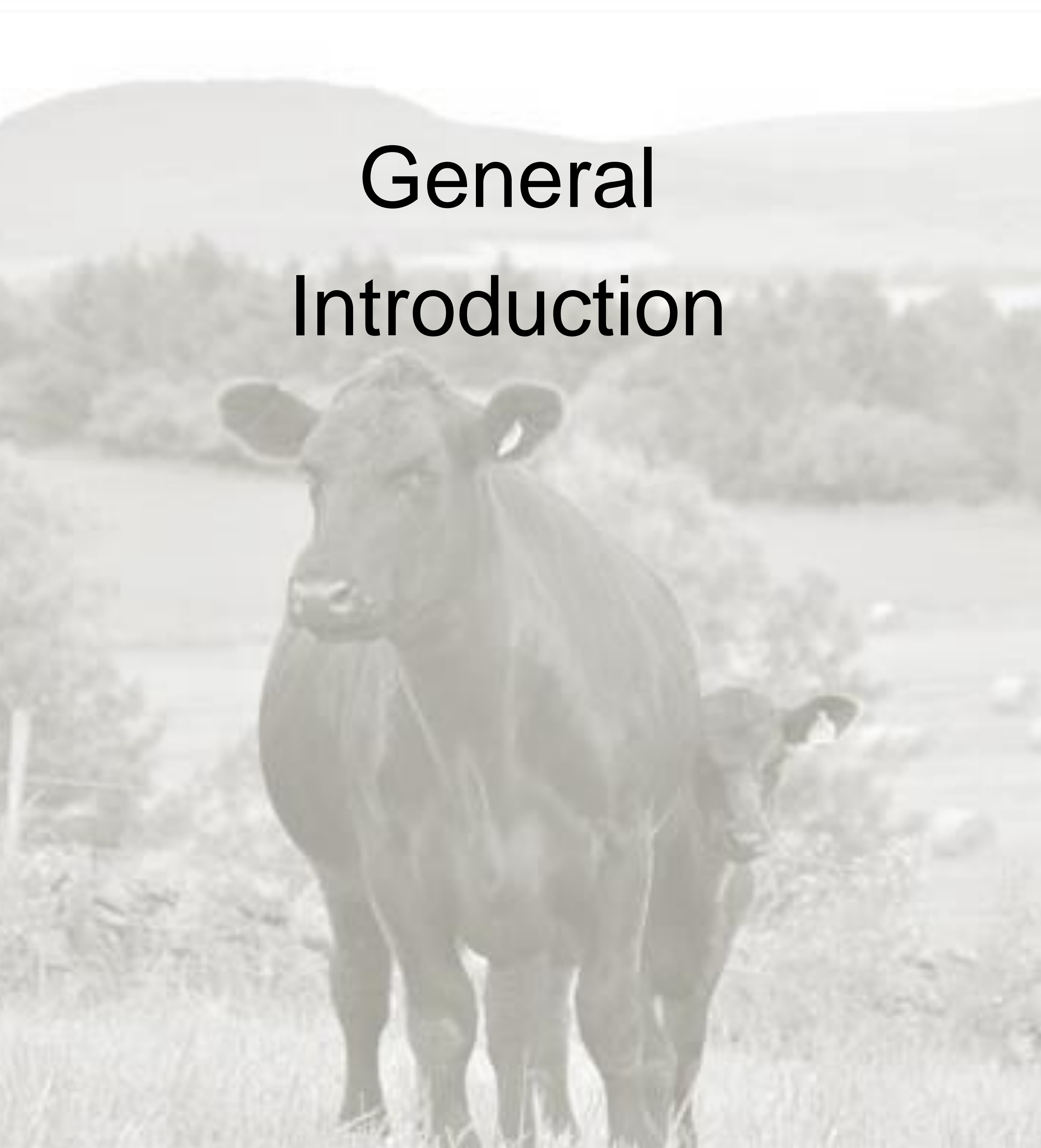
crossbred beef steers were studied. In addition, nitrate toxicity and eating behaviour were investigated. The inclusion of encapsulated nitrate reduced methane production compared to urea and a true protein source, with no adverse effects on rumen fermentation or nitrogen metabolism and no effects with the inclusion of elemental sulphur. The level of addition of encapsulated nitrate (14.3 g nitrate /kg DM) and the time of adaptation chosen for this study (14 days) were adequate to avoid nitrate toxicity. Finally, the effects of adding nitrate inclusion to different basal diets on rumen microbial populations and relationships of these populations with methane production were investigated (Chapter 4). The V4 hypervariable regions of the bacterial and archaea 16S rRNA genes were amplified and sequenced. Effects on microbial population induced by nitrate were dependant on the basal diet but nitrate altered specific archaeal and bacterial OTUs consistently between studies. A direct and strong correlation between some archaea taxonomic groups and OTUs with methane production was observed.

Lay summary

Ruminants occupy an important biological niche as they are capable of transforming poor quality feedstuff, such as roughage forages and food by-products into high quality protein and other sources of human food nutrients. However, ruminant production contributes significantly to total methane emissions from human sources. Therefore, research is needed to provide new effective methane reduction strategies and refine existing technologies to increase their applicability. This project aims to benefit the society, both because of the environmental implications of methane emissions from ruminants and, by providing guidelines to the agricultural sector to improve the profitability of farms.

In trial 1 carbon rich material produced from a burning controlled process known as biochar was tested as a novel additive to reduce methane production from ruminants. The compounds were incubated in laboratorial conditions with rumen content from beef cattle. A small (5%) but significant reduction in methane production was observed. However, the mechanism of action of biochar is still poorly understood and the effectiveness of biochar as a feed additive to reduce methane production from ruminants remains unclear. For the next experiments the focus was on the study of nitrate as a promising additive to reduce methane production from ruminants. A source of protected nitrate was tested as an additive for beef cattle diets and a source of sulphur was included with nitrate and urea treatments as a possible methane inhibitor and to decrease nitrite toxicity. Methane production was reduced with nitrate addition but no improvement in microbial protein synthesis compared with urea was observed. For the Experiment 3 (Chapter 4), the effects of nitrate addition to beef cattle diet in rumen microbial population and correlations with methane produced were studied. Microbial population were more altered by nitrate addition in high forage diets compared with high concentrate diets. Nitrate was confirmed to affect specific microbial populations consistently between studies. A direct and strong correlation between some microbial populations with methane production was observed.

CHAPTER 1



General Introduction

Chapter 1.

1.1 RUMINANT PRODUCTION

1.1.1 The role of ruminants in the global supply of food

The human population is projected to reach 9 billion by 2050 (McAllister et al., 2011). Following the increase in meat and dairy consumption in the developing world it is unlikely that the global supply of food can be fulfilled by vegetable sources alone. Therefore, it has been forecast that meat production will need to double by 2030 to meet the demand from world population increase and change in dietary patterns (Steinfeld et al., 2006). In this context, it is important to provide scientific based options for increasing food for human population, without negative environmental consequences.

Ruminant livestock are an important source of meat and dairy products, supplying 51% of all protein from the livestock sector: 67% from milk and 33% from meat. Within ruminant species, cattle supply 81% of protein, while buffalo, sheep and goats contribute 11%, 5% and 4%, respectively (Gerber et al., 2013).

The important role of domestic ruminants in the global supply of food is justified by their ability to utilize fibrous feedstuffs not readily utilised by monogastric animals to provide human food nutrients (Morgavi et al., 2010a). This capacity is due to a diverse and complex rumen microbial population that converts poor quality feedstuff into high quality protein and energy in form of volatile fatty acids.

Grazing lands would not provide food for the human population if they were not grazed by ruminants, with more than one billion cattle and 1.5 billion sheep and goats supported from this resource (Bradford, 1999). By-product feedstuff is another resource to feed ruminants that would be otherwise useless. It has been estimated that as a weighted average, 100 kg of human food provides 37 kg of animal feed by-product. Surprisingly, in some crop production system the use of food by-products

by ruminants surpasses the monetary inputs of the crop itself (*i.e.*, soya bean meal as an animal feed exceeds the value of soya bean oil for human use) (Bradford, 1999). In addition, ruminants contribute to reducing costs associated with waste disposal in food chain production through recycling of feed by-products. Ruminants can be an important contributor to the global supply of food at the expense of fibrous feedstuff produced in non-arable areas or using food by-products.

In developing countries, the use of grains for livestock feeding has controversial implications as the direct use of grains for human consumption may increase the global supply of food, with more food provided by vegetable sources. However, the proportion of grain used for ruminants is small compared with its use within monogastric livestock production systems. Therefore, ruminant livestock represent an opportunity to build up sustainable systems of animal production that do not compete directly with human demand for foodstuff.

It is widely recognised that ruminants are essential for the livelihood of millions of people in the world. Ruminants support rural communities and help to maintain ecosystems (Kipling et al., 2016) and in some developing areas, ruminants are the only way of feeding the population and to use non arable land. Opportunities for increasing the efficiency of production are greater for ruminants compared with monogastric animals because there is yet more room for improvements. An increase in feed efficiency by ruminants for the production of food product is a desirable and achievable goal.

1.1.2 Ruminant industry: place in the world and within EU

The global domestic ruminant population was estimated to be 3.9 billion in 2014 (1.5 billion cattle, 1.2 billion sheep, 1 billion goats and 0.2 billion buffalo). In Europe the domestic population is around 270 million, with beef cattle accounting for nearly half (122 millions) (http://www.fao.org/faostat/en/#data/QL). Ruminant production in Europe is strongly influenced by the Common Agricultural Policy through agricultural subsidies with single farm payments. Since 1950, the world ruminant population has increased greatly (Figure 1.1). Ruminant industry in the world is important regarding food production and use of grassland and/or crop residues and by-products that would be otherwise useless. In fact, the increase in livestock population is predicted to occur primarily in developing countries.

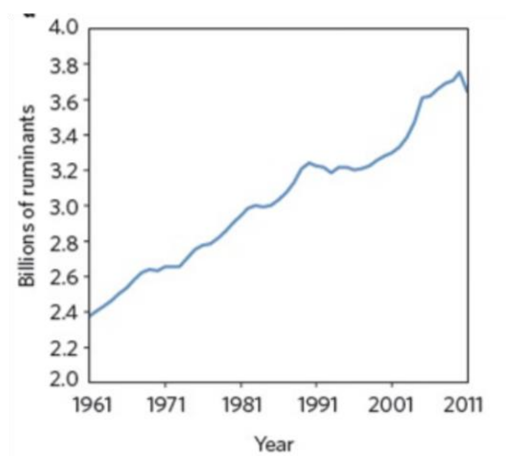


Figure 1.1 Global ruminant numbers from 1961 to 2011 (Ripple et al., 2014)

1.2 METHANE EMISSIONS: WORLD AND UK

Methane is the most abundant non-carbon dioxide (CO_2) greenhouse gas (GHG) and the second major contributor to global warming with a global warming potential 21 times that of CO_2 (Patra et al., 2014). The Earth's atmospheric CH_4 concentration has increased by about 150% since 1750, and CH_4 accounts for 20% of the long-lived and globally mixed GHGs. The rising concentration of CH_4 is correlated with increasing human population and currently about 70% of CH_4 production arises from anthropogenic sources (Moss et al., 2000). A life cycle assessment of GHG emissions indicated that livestock contribute about 18% to the global anthropogenic GHG emissions accounting for 37% of anthropogenic CH_4 (Steinfeld et al., 2006). Regional emissions and production are very variable, due to differences in the distribution of ruminant and monogastric species and emission intensities. Ruminants contribute significantly more to GHG emissions (5.7 gigatonnes (Gt) CO_2 equivalent (eq) per year) than monogastric livestock (1.4 Gt CO_2 eq per year). Globally, ruminants contribute 11.6 % and cattle 9.4% of all GHG emissions from anthropogenic sources (Ripple et al., 2014). The major contribution to GHG emissions from ruminants arises from enteric CH_4: about 2.7 Gt CO_2 eq. of enteric CH_4 annually, or about 5.5% of total global GHG emissions from human activities (Figure 1.2). Cattle account for 77% of these emissions (2.1 Gt), buffalo for 14% (0.37 Gt) and small ruminants (sheep and goats) for the remainder (0.26 Gt). When expressed as emissions on a unit protein basis, beef meat is the product with the highest emission intensity, with an average of 342 kg/CO_2-eq per kg of protein. Meat and milk from small ruminants present the second and third highest emission intensities with averages of 165 and 112 kg CO_2-eq per kg of protein (http://www.fao.org/gleam/results/en/).

In the UK, approximately 18% of the CH_4 currently released to the atmosphere arises from the agricultural sector where the main sources are enteric fermentation by domestic ruminant (16%) and the anaerobic break down of stored manures and slurries (2%) (Milne et al., 2014). In 2014 in the United Kingdom,

enteric CH_4 emissions were estimated to account for 23.8 Mt CO_2-eq or 48% of total GHG emissions from the agriculture sector (Brown et al, 2016). Nonetheless, concerning the environmental significance of ruminant production, attention should be paid in recalculating the amount of feed provided by ruminants from sources not readily available for human consumption. When efficiency was calculated on an edible input/output basis in UK production systems, spring-calving/grass finishing upland suckler beef and low land lamb production were more efficient than pig and poultry meat production (Wilkinson, 2011).

Nevertheless, the estimated total annual emissions of CH_4 into the atmosphere in the UK have declined since the early 1990s. This decline is due to reform in Common Agriculture Policy (CAP) and impacts of epidemics (BSE and FMD) that have led to a fall in the number of livestock (Change, 2007).

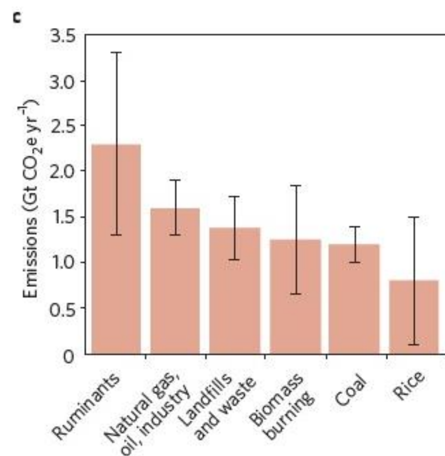


Figure 1.2 Current estimated annual anthropogenic emissions from major sources of CH_4. Error bars represent ±standard deviation (Ripple et al., 2014)

In 2014, agriculture was responsible for 17% of Scotland's total GHG emissions. Nearly half of GHG emissions were attributed to CH_4 (4.7 Mt CO2e or 44%) and the major proportion of CH_4 was derived from enteric fermentation. Beef cattle accounted for the greatest share and therefore reducing CH_4 emissions from enteric fermentation is needed and part of Scottish government call at the moment. (http://www.climatexchange.org.uk/reducing-emissions/emissions-livestock-production).

Reducing enteric CH_4 emissions would help reduce the rate of warming in the near term and, if reductions in emissions are sustained, can also help limit peak warming. Relative to other global greenhouse gas abatement opportunities, enteric CH_4 is among the lowest cost options and has a direct economic benefit to farmers.

It is a big challenge to meet the growing demand for food while decreasing GHG emissions. However, there are opportunities to decrease emission intensity and improve production efficiency via the implementation of technologies that result in greater yields per animal and per unit of feed. Methane emission intensities vary greatly between ruminant products because of differences in agro-ecological conditions, farming practices and supply chain management. In this context, many opportunities to decrease CH_4 production in the rumen are currently receiving a great deal of attention (Hristov et al., 2013).

1.3 METHANE PRODUCTION BY RUMINANTS

1.3.1 Rumen fermentation

The ruminant's stomach is formed by rumen, reticulum, omasum and abomasum. The rumen is the main fermentation chamber, containing a large and diverse microbial population consisting of bacteria, protozoa, fungi and archaea which perform specific metabolic functions that are essential for host animal performance, health and nutrition. The rumen is not homogeneous with solid and liquid fractions. The conditions in the rumen allow the growth and interactions between the microbial populations. The normal and essential conditions are a constant temperature of roughly 39ºC; pH of 5-7.2, osmolarity, 300 mOsm/L, redox potential of 200-450 mV and mixing caused by rumen contractions, primary and secondary.

The ingested feed components remain for variable times in the rumen, depending on feed structure and size, fermentation intensity and quantity of hemicellulose and cellulose (Ramin and Huhtanen, 2013b).The process of rumination facilitates the microbial colonization of substrates and the breakdown of plant structures. The metabolism of carbohydrates (CHOs) occurs in anaerobic conditions with an oxidative pathway (the Embden-Meyerhof-Parnas pathway) (Moss et al., 2000). This pathway produces reduced co-factors such as NADH, that have to re-oxidised to NAD^+ to complete the fermentation of sugars. NAD^+ is regenerated by electron transfer to acceptors (Leng and Preston, 2010). Carbon dioxide is the main electron acceptor against hydrogen (H_2) as an electron donor within the rumen. One mole of glucose produces two moles of pyruvate which are metabolised to the different volatile fatty acids (VFAs; Figure 1.3). VFAs are the main source of energy for ruminants and are largely absorbed across the rumen wall. The stoichiometry of the main anaerobic fermentation pathways within the rumen can be summarised as follows (Moss et al., 2000):

- 2H producing reactions:

  Glucose → 2 pyruvate + 4H (Embden-Meyerhof-Parnas pathway)

  Pyruvate + H_2O →acetate (C2) + CO_2 + 2H

- 2H using reactions:

  Pyruvate + 4H→propionate (C3) + H_2O

  2 C_2 + 4H→butyrate (C4) + $2H_2O$

The proportion of different VFAs formed in the rumen depends on the diet. With high forage diets, the fermentation of CHOs is slower, the pH remains high and the development of cellulolytic genera of bacteria is promoted producing mainly acetate (70%) and less propionate (15%). In contrast, with high concentrate diets the passage rate is faster, the pH decreases and amylolytic bacteria are predominant producing higher concentrations of propionate (35%) and less acetate (50%) and butyrate (15%) (Allison and Reddy, 1984; Baldwin and Allison, 1983).

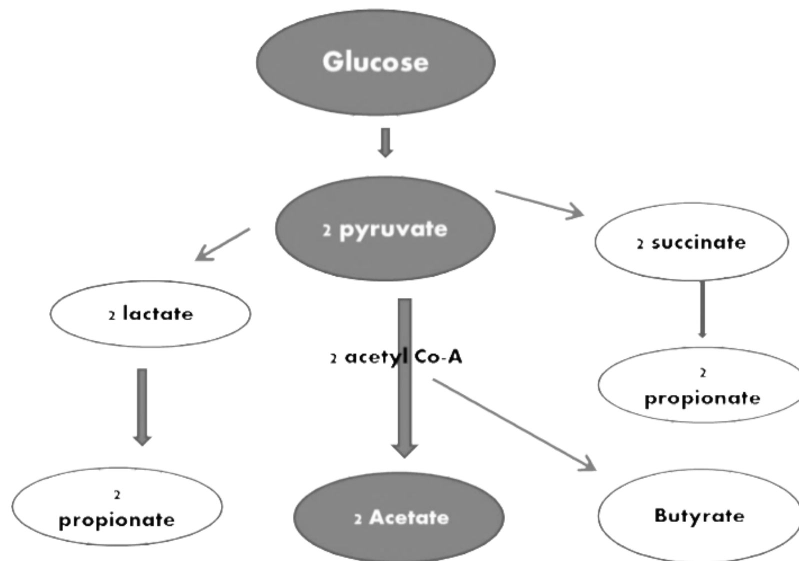


Figure 1.3 Metabolism of glucose in the rumen. Electron sinks are highlighted with white boxes

1.3.2 Methanogenesis in the rumen

Methane is produced in the gastrointestinal tract of ruminants, mostly within the rumen by methanogenic archaea and is synthesised from CO_2 and H_2, produced by the degradation of feeds by primary anaerobic fermenters (Morgavi et al., 2010b) (Figure 1.4). Methane emitted from ruminants is produced in the rumen (89%) and exhaled through the mouth and nose (Hook et al., 2010). Methane formation in the rumen depends on both the supply of H_2 from acetate and butyrate-producing bacteria and on the conversion of H_2 and CO_2 to CH_4 by methanogenic archaea that obtain the energy for their growth by this reducing pathway.

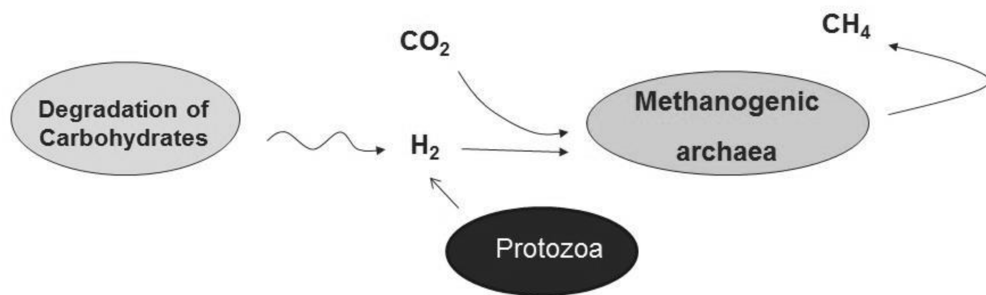


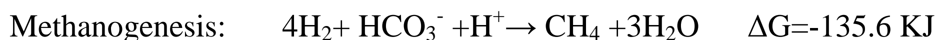
Figure 1.4 Methanogenesis in the rumen

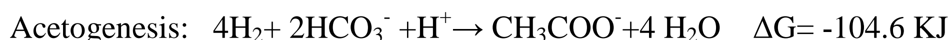
The formation of CH_4 avoids an increase in the partial pressure of H_2 and contributes to the efficiency of the system, as accumulation of H_2 produces a thermodynamic inhibitory effect on microbial enzymes that carry out electron transfer reactions and reduce rumen fermentation and fibre breakdown. The rate of rumen CH_4 production is directly proportional to the concentration of dissolved H_2 (Czerkawski et al., 1972; Hegarty, 1999). On the other hand, there are other electron acceptors rather than CO_2 which can use H_2 such as sulphate, nitrate and fumarate (Moss et al., 2000).

The amount of biomass formed from a substrate is determined by the free energy (G), the G change between reactants and products. A given population of cells, metabolising a substrate under conditions with a defined G, will produce more biomass than another population metabolizing the same substrate via a pathway with

a less negative G (Janssen, 2010). Free energy change thermodynamics under standard conditions determines the three major competing processes for the safe removal of H_2 from the rumen: sulphate reduction, reductive acetogenesis and methanogenesis (Oren, 2012). The thermodynamics of all the three major processes are as follows:

Sulphate reduction: $4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4\ H_2O$ $\Delta G = -152.2 KJ$


Methanogenesis: $4H_2 + HCO_3^- + H^+ \rightarrow CH_4 + 3H_2O$ $\Delta G = -135.6\ KJ$


Acetogenesis: $4H_2 + 2HCO_3^- + H^+ \rightarrow CH_3COO^- + 4\ H_2O$ $\Delta G = -104.6\ KJ$

When sulphate is limited, methanogens will dominate the role of H_2 removal from the rumen. The lower energy yield of acetogenesis is probably accountable for making the reductive acetogenesis a less favourable pathway (Malik et al., 2015). The total amount of CH_4 formed in the rumen varies between diets and animals and depends mostly on feed type and quality and thus on the different molar proportions of VFAs. Propionate formation is an alternative to CH_4 production (Janssen, 2010), decreases CH_4 production and improves energy efficiency (Baldwin and Allison, 1983). In contrast, acetate and butyrate promote CH_4 formation in the rumen. Therefore, high forage diet increases the production of CH_4.

Methanogens could use alternative substrates to produce CH_4 other than CO_2 and H_2. Acetate is used by some members of methanogens via the aceticlastic pathway, and formate is an important electron donor used by many hydrogenotrophic methanogens, contributing to 18% of rumen CH_4 (Hungate et al., 1970).

The amount of enteric CH_4 expelled by the animal is related to the level of intake, the type and quality of feed, the amount of energy it consumes, and environmental temperature. Sheep and goats produce 10 to 16 kg CH_4/yr and cattle 60 to 160 kg CH_4/yr, depending on their size and dry matter intake (Brown et al., 2011).

1.3.3 Microbial population involved in methanogenesis

Traditionally, microbial populations have been identified using culture based studies, but difficulties in isolation and maintenance of many cultures have limited progress. The development of culture-independent techniques and new DNA sequencing tools have allowed identification of the rumen microbiome and main populations involved in CH_4 production (Zhou and Hernandez-Sanabria, 2009).

Methanogens belong to the phylum Euryarchaeota of the domain Archaea, and they are associated with rumen fluid, solid phases and with rumen epithelium. Archaea are the main rumen microorganisms that produce CH_4. They are estimated to comprise between 0.3 to 3% of the total rumen microbiome (Leahy et al., 2013). The methanogens classified as archaea have a distinctly different cell wall structure from rumen bacteria (Aluwong et al., 2011). Archaea diversity in the rumen has been extensively studied (Janssen and Kirs, 2008; Liu and Whitman, 2008; Morgavi et al., 2010a; Ross et al., 2013). Surveys of archaeal 16S ribosomal RNA (16S rRNA) gene sequences from ruminants around the world show that three methanogen groups dominate (Leahy et al., 2013); *Methanobrevibacter* spp., *Methanomicrobium* spp. and Rumen Cluster C (RCC), also known as *Thermoplasmatales* make up 92% of the rumen methanogen. Remaining groups of methanogens include representatives of the genera *Methanosphaera, Methanimicrococcus* and *Methanobacterium*, and uncultured organisms designated Qld26 and rumen crenarchaeota (Attwood et al., 2011).

Most methanogenic archaea (Fonty et al., 2007) use H_2 to reduce CO_2 to produce CH_4, being the hydrogenotrophic pathway the main mechanism to produce CH_4 in the rumen (Hook et al., 2010). This process keeps the partial pressure of H_2 low and allows the correct function of the rumen (Moss et al., 2000). Formate is also utilised by all the most abundant archaea, and is equivalent to $H_2 + CO_2$, so formate is included in the hydrogenotrophic pathway (Tapio et al., 2017). Others methanogens (methylotrophs) use other substrates, such as methyl-containing

compounds (methanol, trimethylamine or dimethyl sulphide) to form CH_4 (Liu and Whitman, 2008; Poulsen et al., 2013). Few species can dissimilate acetate using the aceticlastic pathway that converts acetate to carboxyl and methyl groups and uses them to form CH_4 and CO_2, respectively. However, this mechanism is present to a limited extent in the rumen because the slow growth rate of this group of methanogens compared with the high rate of passage of rumen contents in the rumen (Attwood and McSweeney, 2008b; Janssen and Kirs, 2008).

There are other microbes that influence CH_4 production, either because they are involved in H_2 metabolism or, because they affect the numbers of methanogens or other members of the microbiota. The transfer of H_2 from fermenting organisms to methanogens is known as interspecies H_2 transfer. The fermenting organisms contain hydrogenase enzymes releasing H_2 which can be taken up by methanogens (Leng, 2014). Protozoa harbour significant numbers of archaea favouring inter-species H_2 transfer, playing a catalytic role in rumen methanogenesis (Moss et al., 2000) and a direct correlation between the number of protozoa and CH_4 production has been observed (Morgavi et al., 2010a). Most of the fibrolytic microorganisms in the rumen produce H_2 as a main end product (*e.g.*, *Ruminoccocus albus* and *Ruminoccocus flavefaciens*), which is used by methanogens. On the other hand, there are non-H_2 producers within fibrolytic community such as *Fibrobacter succinogenes* what could be promoted to reduce methanogenesis, without reducing fibre degradation in the rumen (Morgavi et al., 2010a).

1.4 STRATEGIES TO REDUCE METHANE EMISSIONS FROM RUMINANTS

There has been extensive research to find effective and sustainable strategies to reduce rumen enteric CH_4 production. Many reviews of enteric CH_4 mitigation practices have been published in the last decades (Beauchemin et al., 2008; Cottle et al., 2011; Eckard et al., 2010; Gerber et al., 2013; Hegarty, 1999; Hristov et al., 2013; Patra, 2012a; Ribeiro Pereira et al., 2015). Beforehand, it is important to assess whether the implementation of a CH_4 mitigation strategy would encounter an increase in any other GHG from the animal up to the farm scale (Eckard et al., 2010; Martin et al., 2010). In addition to direct emissions, livestock supply chains release GHG through animal feed production and post-harvest activities.

The emissions of GHG by beef cattle on the whole farm basis would include the burning of fossil fuel to produce mineral fertilizers for food production, CH_4 production from animal manure and fertilizers, land use changes for grazing and feed production, land degradation, and transport (Gill et al., 2010). For example, on the whole production system, permanent pasture produces little change in soil carbon with lower net carbon emissions from soils than with changes in land use that would be needed to increase the animals fed under concentrate diets. However, it is well known that more CH_4 is produced from animals fed high forages diets (Gill et al., 2010). Farm-scale modelling provides information for policymakers to predict GHG from livestock farms and should be include in any study that aims to implement any GHG mitigation strategy (Kipling et al., 2016). Moreover, the trade-off between nitrogen (N) excretion and enteric CH_4 production needs to be understood at the animal scale (Dijkstra et al., 2011). In this sense, when life cycle assessment and estimation on the whole farm based were used, beef cattle production share of GHG emission vary between production systems. For example, in Irish grass-based systems the share of GHG emission between gases shows, as in dairy systems, a major contribution of CH_4: 50% to 70% (Casey and Holden, 2006); on the contrary, nitrous oxide (N_2O) is the main GHG in feedlot system (Phetteplace et al., 2001). Therefore, any mitigation approach based on changes in production system is more likely to change the emission of all GHG on-farm and off-farm. On the contrary, feed

additives seem to be less prone to change the global GHG emission of the farm (Martin et al., 2010a).

Another acute aspect of all mitigation practices is the likelihood of adoption (Hristov et al., 2013). Farmers would adopt any practice if there is a clear positive economic impact on animal production and farm profitability. Any practice that requires additional cost and has a chance to reduce productivity would be rejected by the producer. To summarize, when assessing the implementation of any CH_4 mitigation practice, any method should:

- Have evidence of decreased CH_4 excretion *in vivo*.
- Not be toxic to the animal at the level needed to have significant effects on CH_4 production.
- Have no adverse effect on animal performance, digestibility or intake.
- Long lasting and persistent effect (productive life of the animal).
- Economically viable.
- Practical in on farm settings.

One way to classify all mitigation strategies studied is the main target. All strategies could fall in one of the following categories: animal manipulation, dietary composition, modulation of rumen fermentation and inhibition of methanogenic archaea (Patra, 2016). Dietary strategies mainly act by one of the following mechanisms: direct inhibition of methanogens, lowering the production of H_2 in the rumen or providing alternative pathways for the use of H_2 in the rumen (Martin et al., 2010b) (Figure 1.5).

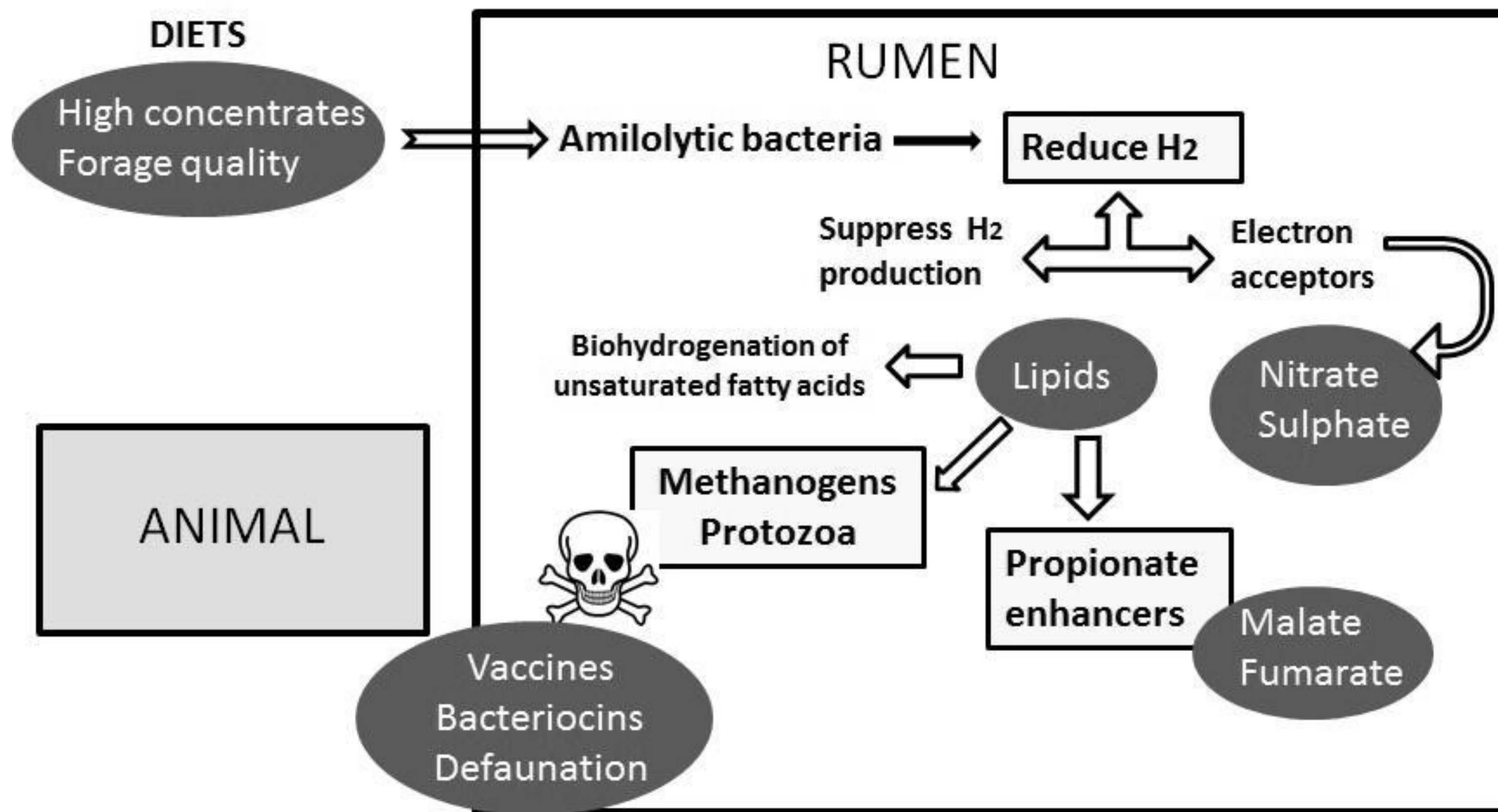


Figure 1.5 Potential targets of decreasing CH_4 emissions from rumen

1.4.1 Animal manipulation

Improving productivity through breeding, increasing fertility and/or health could lead to a reduction in CH_4 production per unit of product (Table 1.1). Individual variation between animals in CH_4 emissions per unit of feed intake has been observed, under the same feeding and handling conditions (Eckard, 2010, Pinares-Patiño et al., 2013) suggesting a genetic component on CH_4 production from animals. Therefore, genetics provide an opportunity to select livestock with lower CH_4 production (Clark, 2013; Patra, 2012; Pinares-Patiño et al., 2003). However, selection directly for CH_4 production traits is currently impractical because CH_4 is difficult and costly to measure in a large number of animals (Cottle et al., 2011). Feed intake is directly correlated to CH_4 emission and feed use efficiency has been suggested to be, to some extent, also correlated with CH_4 emissions (Hegarty et al., 2007). Therefore, the use of feed conversion ratio (FCR) or residual feed intake (RFI) as a criteria for ***animal selection*** would likely select for lower CH_4 emitters (Eckard et al., 2010; Hegarty and Gerdes, 1999), without compromising production traits (Patra, 2012). However, the direct effect on CH_4 emission with selection for productivity is difficult to predict (Hristov et al., 2013) and it would be desired to find direct markers for CH_4 emission before this strategy become feasible (Clark, 2013). In addition, the genome of the animal could affect its rumen microbial population which are directly responsible for CH_4 emissions (Cottle et al., 2011). Rumen microbial population are influenced by diet, mother and environment and there is an additive genetic influence of the host on its microbiome. Some researchers are trying to identify methanogenic populations or particular species or genotypes that could be associated with feed efficiency and/or CH_4 production in cattle (Khiaosa-ard and Zebeli, 2014; Roehe et al., 2016; Zhou and Hernandez-Sanabria, 2009). Therefore, methagenomics offer a new challenge for genetic selection for low CH_4 emissions based on the abundance of specific genes in the ruminal metagenome associated with the trait (Herd et al., 2014; Pickering et al., 2015; Roehe et al., 2016).

On a whole farm basis, strategies directed towards ***increasing animal productivity***, such as a reduction in the number of unproductive animals, and earlier finishing of beef cattle, would reduce the total emissions of CH_4 with less CH_4 produced per unit of product (meat or milk) (Eckard et al., 2010). Any strategy adopted at farm level should carefully assess the effect on the emissions of the whole production system, to confirm the effectiveness (Hristov et al., 2013).

In developing countries, where the animals have been poorly selected, enhancing the genetic potential of the animal would lead to an increase in production and concomitant decrease in CH_4 production. However, high genetic potential animals should not be imported into these areas to achieve this goal, as these breeds may not achieve their potential under adverse climatic and nutritional condition, being less productive than the native breeds. Especially in these areas, a reduction in disease incidence and reproductive problems, and an improvement in feed quality would lead to an increase in productivity and can decrease greatly CH_4 emissions in herd (Gerber et al., 2013).

Table 1.1 Animal management strategies to reduce CH_4 emission intensity from ruminants (adapted from Hristov 2013)

Category	Species	Production	Potential CH_4 mitigation	Recommended
Increase productivity	All	Increase	High	Yes
Fertility	All/ Dairy cows	Increase	Low/Medium	Yes
Genetic selection	Beef and dairy cattle	?	Low?	Yes(developed countries)
Animal health	All	Increase	Low?	Yes
Reduce mortality	All	Increase	Low?	Yes
Reduce days on feed	Meat animal	None	Medium	Yes

1.4.2 Rumen manipulation

There are several strategies to target the rumen to reduce CH_4 production (Figure 1.6). Methane is produced during the fermentation of substrate and it is strongly correlated with DMI intake (Brown et al., 2011) and the starch content of the diet (Cottle et al., 2011). Therefore, ***feeding strategies*** have been among the main methods investigated for reducing CH_4 emissions from ruminants. The addition of grain to forage diets can reduce CH_4 production (Beauchemin et al., 2008). The main reason is that high starch content in the diet reduces rumen pH and favours the production of propionate rather than acetate with a reduction in H_2 produced and consequently in CH_4 production (Johnson and Johnson, 1995; Rooke et al., 2014). The low ruminal pH might also inhibit the growth and/or activity of methanogens. However, the response to increase in concentrate for CH_4 production is not linear. The relationship between CH_4 emissions and the proportion of concentrates in the diet has been shown to be curvilinear, with emissions remaining relatively constant from diets containing (DM basis) up to 400 g/kg concentrate, and then decreasing rapidly as concentrate is increased to 900 g/kg (Martin et al., 2010b; Troy et al., 2015). For example, when comparing diets with 500 g of concentrate/kg of DM with diets with 100 g of concentrate/kg DM, the yield of CH_4 was not significantly different and neither the proportions of acetate and propionate (Danielsson et al., 2012). An increase in protozoa population may explained the increase in butyric acid observed and may protect somehow methanogens (Danielsson et al., 2012). Concerning the effect of the type of concentrate on methanogenesis, grain concentrates rich in starch lead to lower CH_4 productions compared with fibrous concentrates (beet pulp), and barley grain have been reported to produced greater amounts of CH_4 compared with maize grain (Beauchemin and McGinn, 2005; Rooke et al., 2014).

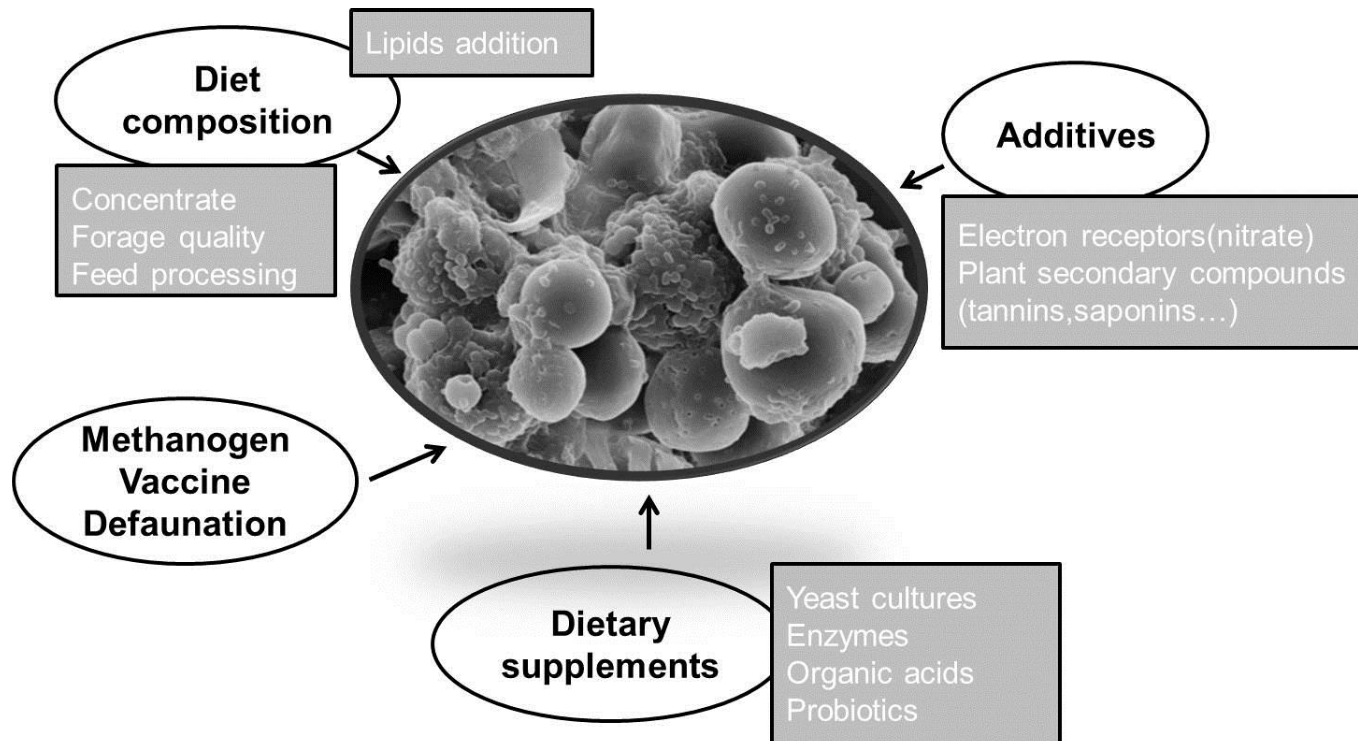


Figure 1.6 Rumen manipulation strategies to reduce CH_4 production

However, increasing the concentrate proportion in the diet above a certain level, might have a negative effect on fibre digestibility and animal health (Hristov et al., 2013). Moreover, the proportion of concentrate in the diet needed to observe a reduction in CH_4 production is relatively large, being not always feasible in the developing countries because of economic constraints (Kumar et al., 2014) and is not a socially acceptable mitigation option in many parts of the world. Furthermore, increased concentrate content in ruminant diet sometimes result in additional releases from feed production or manure stored, incurring in an increase in total net emissions (Beauchemin et al., 2008; Eckard et al., 2010; Hristov et al., 2013). The use of forages with higher soluble CHO concentrations can be a better option than feeding high concentrate based-diets (Kumar et al., 2014) because it is less costly, enviromental friendly and better for rumen health. Improved pasture management, and feeding starch rich maize or cereal silages instead of fibre rich grass silages may decrease CH_4 emissions per unit feed without competition with human food supply (Beauchemin et al., 2008). Increased fibre content of forages is negatively correlated with digestibility, suggesting that an increase in the digestibility of forages would decrease CH_4 production because of lower fibre content. No direct correlation has been found between chemical composition of forages and CH_4 production (Hammond et al., 2013; Sun et al., 2012) but specific characteristics of forages could reduce CH_4 emissions, such as concentrations of plant secondary compounds, starch fibre ratio, and stage of maturity (McAllister and Newbold, 2008; McAllister et al., 2011). For example, the use of species with condensed tannins and legumes compared to grass forages have shown a reduction in CH_4 production per unit of DMI (Clark, 2013; Eckard et al., 2010). Some studies have also demonstrated that diets with legumes silages versus grass silage had lower proportion of propionate and lower production of CH_4 (Waghorn et al., 2002) and that maize silage produce less CH_4 compared with grass or legume silages (Dewhurst, 2013). Nevertheless, some studies have demonstrated that the major part of the variability in CH_4 emissions under different forages feeding is due to the level of intake and the passage rate. An increase in feed intake and reduction in rumen retention time results in increased production with a concomitant reduction in CH_4 yield (Hammond et al., 2014;

Pinares-Patiño et al., 2003). Therefore, forage preservation and processing through its effect on digestibility and passage rate could affect enteric CH_4 production but the information is still limited (Yang et al., 2012). Increasing feeding frequency and feeding complete rations are associated with decreased CH_4 formation per unit of feed (Janssen, 2010). Nevertheless, caution should be taken in application of any of these feeding systems under particular production systems. For example, increasing the quality of forages is desirable where feeding high concentrate diets is not feasible. Without doubt, it is important that science based feeding systems and feed analysis are introduced in all systems with special emphasis in developing countries. This would lead to maximized production, feed utilization, reduced GHG livestock emissions and economic benefits for the farmers.

In UK beef production system, forage have a higher relevant role in animal diet than in other production systems, and permanent pastures represent most of the grassland (Wilkinson, 2011). Moreover, most of beef (and dairy) production systems are sustained by higher amount of forage, with only 6% of beef cattle fed diets based in cereal grains. From all the forage fed, 70% is grazed and 30% is fed as conserved forage (22% grass silage, 4% maize silage and 5% hay). Therefore, there is a need to find additives able to reduce CH_4 production from beef cattle fed forage based diets without changing the production system.

Lipid supplementation of the diet seems a promising nutritional strategy to depress ruminal methanogenesis. Vegetable and animal lipids are generally used in ruminant diets to increase the energy density. It has been estimated that an increase of 10g/kg DM in dietary fat would decrease CH_4 yield by 1.7–6.7% (Grainger and Beauchemin, 2011). The modes of action of lipids decreasing CH_4 production are multiple including: suppression of methanogens or protozoa, decreases in ruminal organic matter fermentation, increased the production of propionic acid and to some extent through biohydrogenation of mono- and polyunsaturated fatty acids (Beauchemin and McGinn, 2006b; Chuntrakort et al., 2014; Johnson and Johnson, 1995; McGinn et al., 2006). Consequently, CH_4 production could be declined due to production of less H_2 per unit of feed when higher levels of fats are included in the

diets. The effects on CH_4 emissions per animal are dependant on the fat source, level of supplementation and type of diet. Considerable variations in the CH_4 reduction among fat sources have been observed, with lauric acid (C12:0), linolenic acid (C18:3), and polyunsaturated fatty acids more effective compared with saturated long chain fatty acids (Patra, 2013). The use of fatty acids such as myristic and lauric acids has been investigated *in vitro* and *in vivo* and has shown a synergistic effect supressing methanogenesis (Odongo et al., 2007; Soliva et al., 2004), but have shown to decrease DMI in some cases (Fiorentini et al., 2014). There are lipid sources commercially available such as sunflower oil, canola oil, rapeseed oil and coconut oil that have the potential to reduce CH_4 emissions with a level of addition of 3% of DM (Machmuller et al., 2006). Nevertheless, edible oils might not be economical for the producers if there is not a direct effect in productivity. In practice, the use of seeds as a source of lipid instead of refined oil are easier to apply and less expensive (Martin et al., 2010). As an alternative, the by-products from the biofuel industries containing high concentration of lipids (up to 17% DM basis) are promising for decreasing CH_4 and drop feed cost (Hristov et al., 2013). Examples of by-products that contain fat and are suitable for adding to ruminant diets are whole cottonseed, brewer grains, cold pressed canola, and hominy meal (Dewhurst, 2013). The inclusion of lipids in the diet of ruminants at certain level could have negative effects on fibre digestion and feed intake, depressing animal productivity. These negative effects could be reduced by feeding high-concentrate diet low in fibre and total dietary fat must not exceed 6 to 7% of dietary DM (Beauchemin et al., 2008; Beauchemin et al., 2007). For pasture based diets, options for increasing the fat level in the diet are more limited (Grainger and Beauchemin, 2011) as supplementation is not always possible.

Researchers have also been investigating feed additives that could inhibit methanogenesis pathways or stimulate other pathways competing with methanogenesis. The application of nutritional additives for CH_4 mitigation must consider animal welfare, feasibility, profitability and regulatory issues. Among the approaches tested, ***plant bioactive compounds*** have been of great interest as a natural

alternative to chemical additives. *Saponins* are natural detergents found in many plants, that possess membranolytic properties, causing protozoa cell lysis (Newbold and Rode, 2006). Saponins damage protozoa by forming complexes with sterols in the protozoal membrane surface and selectively inhibit rumen bacteria and methanogens (Goel et al., 2008; Wina et al., 2005). The antiprotozoal effect of saponins may also provide nutritional benefits enhancing the amount of microbial protein (MP) leaving the rumen and increasing efficiency of feed utilization (Makkar et al., 1998; Patra and Yu, 2013). The effects of saponins on methanogenesis are dependent on the composition of the diets and levels of saponins used and the growth-promoting effect of saponins has been more evident in high roughage diets. Therefore, the level used, the interaction with the basal diet and the active compound should be studied in detail. However, the anti-protozoal activity of saponins was shown to be transient and did not always results in CH_4 inhibition (Goel and Makkar, 2012; Patra and Saxena, 2010; Patra et al., 2010; Ramos-Morales et al., 2017). The transient effect of saponins is explained because saponins are deglycosylated in the rumen to sapogenins by rumen microorganisms and sapogenins are inactive. A promising alternative is the development of saponin analogues that are protected against bacteria degradation by small polar residues. It was demonstrated *in vitro* a persistent antiprotozoal activity of sapogen-like analogs, shifting the fermentation pattern toward higher propionate and lower butyrate (Ramos-Morales et al., 2017). There is still a need for further research *in vivo* with different saponins and theirs analogues and possible interactions with diets for practical use as antimethanogenic additives.

Tannins are polyphenolic secondary metabolites of diverse molecular weights and of variable complexity (Goel and Makkar, 2012). The mode of action of tannins has been proposed to be a direct effect on methanogens and lowering feed degradation (Martin et al., 2010). The direct effect of tannins on methanogens depends upon the chemical structure of the compound. Despite the fact that hydrolysable tannins had a greater effect in reducing CH_4 and a less adverse effect in terms of digestibility compared with condensed tannins (Jayanegara et al., 2015), most research has focused on condensed tannin rich plants or extracts because of the lower risk of toxicity to the animal than hydrolysable tannins (Beauchemin et al.,

2008). Nevertheless, the effect of condensed tannins is through lowering fibre degradation and hydrolysable tannins act directly against methanogens. A strategy to avoid the toxicity of hydrolysable tannins and any detrimental effects on production parameters while obtaining their potential beneficial effects is the administration of hydrolysable tannins at levels below 20 g/kg (Toral et al., 2011). An increase in tannins compounds above 20 g/kg could greatly decrease nutrient digestibility, palatability and intake, and affect performance. Therefore, it is important to take into account the effects of tannins compounds especially in diets low in crude protein (CP). The risks of impaired rumen fermentation are greater with tannins than with saponins. Since the effects of tannins and saponins vary from source to source, generalization of the dose for antimethanogenic effects must be avoided (Goel and Makkar, 2012). Although extracts of tannin and saponin are commercially available, the high prices of such products renders them impractical for use in ruminant production systems (Eckard et al., 2010).

Essential oils *(EO)* such as eugenol and limonene, are a group of plant secondary metabolites obtained from volatile fractions of plants (Patra and Saxena, 2010). The main EO are terpenoid and phenylpropanoid compounds that develop their action against bacteria through interacting with the cell membrane (Calsamiglia et al., 2007). The antimethanogenic properties of EO have been described *in vitro* (Benchaar and Greathead, 2011; Kim et al., 2013; Klevenhusen et al., 2012; McAllister and Newbold, 2008; Soliva et al., 2011) but have been scarcely studied *in vivo* (Beauchemin and McGinn, 2006a; Khiaosa-ard and Zebeli, 2013). For example, Khiaosa-ard and Zebeli, (2013) reported a decrease of 12% in CH_4 production with doses of 0.25 g/kg DM in beef cattle, being the antimethanogenic effect more significant in beef cattle than in small ruminants or dairy cattle. Contrastingly, 13 g/kg of DM of EO included in a forage based diet did not reduce CH_4 production in beef cattle (Beauchemin and McGinn, 2006a). EO can increase rumen protozoa, acetate: propionate ratio, and decrease rumen NH_3-N concentrations and CH_4 production (mmol/100 mol VFA), but the effect is highly dependent on the diet (Khiaosa-ard and Zebeli, 2013). The increase observed in acetate: propionate ratio with EO addition may imply that EO will be more favourable for beef production than dairy cattle. However, high doses required to reduce CH_4 emissions (0.25 g/kg

DM) could have adverse effects on feed digestion, fermentation and overall inhibition of total VFA production (Benchaar and Greathead, 2011). Overall, although the potential for use of EO to supress CH_4 emissions appear interesting, EO have not been extensively studied *in vivo* and their effectiveness differed between studies. Palatability of these compounds could represent another practical issue (Martin et al., 2010). *In vivo* application of essential oil may be limited by adaptation of rumen microbes to these compounds and effects on organoleptic properties of animal products require further research to ensure that EO can be safely used in livestock production (Benchaar and Greathead, 2011).

Dietary additives that can consume H_2 using electron acceptors other than CO_2 have been studied, increasing the utilization of H_2 by organism other than methanogens. ***Reductive acetogenesis*** is another pathway for H_2 utilization in the rumen, but less favourable energetically than methanogenesis and in a functional and developed rumen, rumen methanogens out-compete acetogens. Acetate has the additional advantage of being a source of energy for the animal. To promote acetogenesis, inhibition of methanogens would be needed. Promotion of acetogenic pathway is a desired strategy, but further effort should be made to identify ruminal acetogens. The use of acetogens as probiotics has been tested with no conclusive results (Lopez et al., 1999; Martin et al., 2010b). Rumen ecosystem modification presents a promising alternative to promote reductive acetogenesis in the rumen (Attwood et al., 2011; Fonty et al., 2007). However, some works made to increase the natural rumen population of acetogens were unsuccessful (Demeyer et al., 1996). On the other hand, inoculation of lambs with a functional rumen microbiota lacking methanogen was demonstrated to promote reductive acetogens and replace methanogens as a sink for H_2 in the rumen (Fonty et al., 2007).

Increasing the production of propionate would also redirect H_2 from methanogenesis (Ungerfeld, 2013). There are intermediates in propionate production that could be used to enhance the process. Malate and fumarate are ***propionate precursors*** in the succinate-propionate pathway, and can act as alternative H_2 sinks in the rumen (Carro and Ranilla, 2003; Newbold et al., 2005; Ungerfeld and Kohn, 2006). Malate is dehydrated to fumarate and fumarate to succinate stimulating

propiogenesis against acetogenesis and decrease the availability of H_2 for CH_4 production (Attwood and McSweeney, 2008b; van Zijderveld et al., 2010). The reduction of fumarate and malate produces variable proportions of acetate and propionate (Ungerfeld and Kohn, 2006) and it is important to determine the conditions that favour propionate rather than acetate (Attwood and McSweeney, 2008a). The responses to dietary supplementation with malate and fumarate *in vivo* have been highly variable and appear to be influenced by diet, with greater reduction of CH_4 with high concentrate diets (Hook et al., 2010). Malate may have a beneficial effect on rumen health as it increases pH, decreases lactate concentrations and CH_4 production (Foley et al., 2009b). Wallace et al., (2006) observed a reduction in CH_4 production in lambs when malate was fed and Foley et al.(2009a) reported similar reductions in beef cattle with malate, but in both studies a reduction in DMI was detected and this could potentially decrease animal performance. Also, organic acids are problematic in practice because their acidic properties restrict the quantity which can be fed (Wallace et al., 2006). In conclusion, the results of adding organic acids to ruminants diets are inconsistent and suggest that the doses of organic acids required for CH_4 mitigating effect are high, which makes the use of organic acids as an additive difficult to be affordable in the near future (Carro and Ranilla, 2003; Kumar et al., 2014).

Sulphate and nitrate compounds decreases the availability of H_2 for methanogens (Malik et al., 2015). Sulphate reducers obtain more energy from H_2 oxidation and their affinity for H_2 is much higher than that of the methanogenic archaea, therefore sulphate could act as an H sink in the rumen. Also, sulphate reduction has been reported to be coupled with CH_4 oxidation (Stams and Plugge, 2009). However, the product of sulphate reduction, hydrogen sulphide is toxic at high concentration for both microorganisms and the animal (Dewhurst et al., 2007), which limits the amount that could be included in diets. Nitrate lowers CH_4 production due to the presence of nitrate reducing bacteria in the rumen that use nitrate as a terminal electron acceptor and out-compete methanogens for H_2 produced in fermentation. Nitrate and sulphate will be discussed in more detail in Chapter 3.

For a long time, ***halogenated compounds*** such as chloroform, bromochloromethane (BCM), cyclodextrin, chloral hydrate and 2-bromochloromethane sulphonate were tested for a specific inhibitory effect on rumen archaea (Hristov et al., 2013). The antimethanogenic effect of BCM has been proved *in vitro* (Goel et al., 2009; Martinez-Fernandez et al., 2015) and *in vivo* (Abecia et al., 2012; Tomkins et al., 2009). The effects on methanogen populations have been variable: a reduction in the total number of methanogens was observed in some studies (Denman et al., 2007; Goel et al., 2009; Martinez-Fernandez et al., 2015) whereas no effect in total number of methanogens was found in others (Abecia et al., 2012). All previous studies reported a reduction in the acetate:propionate ratio in the rumen which is common to many antimethanogenic compounds. Although halogenated compounds did not affect the total number of methanogens in some studies, they decreased CH_4 production, and promote a change on microbial archaea composition (Abecia et al., 2012). However, BCM is an ozone depleting agent and is banned for the use for livestock. Therefore, efforts have been made to develop compounds with similar effects on methanogenesis, but not toxic for the host and the environment. Examples of these novel inhibitors are ***nitrooxy compounds,*** such as 3-nitrooxypropanol (3NOP) and ethyl- 3-nitrooxypropanol (E3NP) (Anderson et al., 2008; Brown et al., 2011; Kumar et al., 2014). 3NOP and E3NP have been demonstrated to decrease CH_4 production from sheep *in vitro* and *in vivo* (100 mg/d) without changes in total number of any microbial group populations. A shift in fermentation pattern was observed, with significantly lower acetate:propionate ratio with nitro-compounds inclusion (Martinez-Fernandez et al., 2013b). 3NOP tested at higher doses (2,500 mg/d) in lactating dairy cows produced a consistent decrease in CH_4 production (Reynolds et al., 2014). Nevertheless, the reduction in CH_4 production in sheep (16%) at the lower dose applied was higher than the reduction achieved in lactating cows at higher dose (Klug and Reddy, 1984). In addition, the high dose fed to cows produced a decrease in digestibility. Further, 3NOP inhibited methanogenesis *in vitro* and *in vivo* by 30% (60 mg/kg DM). The mode of action of the nitro-compounds studied is through target the methyl-Coenzyme M reductase (MCR) which catalyses the final step of CO_2 reduction to CH_4 (Duin et al., 2016).

These additives have shown potential as CH_4 inhibitors in the rumen with no negative effects on rumen fermentation at low doses (Haisan et al., 2013; Martinez-Fernandez et al., 2013b).

Ionophore antibiotics, such as monensin, have been used in beef production to improve feed efficiency, showing a potent antimethanogenic effect. Monensin is routinely used in beef production and dairy cattle in North America but ionophore antibiotics are banned in the European Union for possible antibiotic resistance (Hristov et al., 2013). No effects on CH_4 production were reported from dairy cattle fed forage based diets with monensin (Grainger et al., 2010). However, a long term reduction on CH_4 production from cows fed forage based diets with monensin inclusion (6 months) was reported by Odongo et al. (2007). The most likely mode of action of monensin is affecting microbes other than methanogens, including protozoa, inducing a shift in fermentation towards propiogenesis (Guan et al., 2006). The anthimethanogenic effect has been inconsistent across studies and species dependant, showing stronger methanogenic effects in beef cattle than in dairy fed forage based diets (Beauchemin et al., 2008; Hristov et al., 2013). In addition, it has been observed that ciliate protozoal can adapt to ionophore compounds, independently of the basal diet, being the anthimethanogenic effect of monensin transient (Guan et al., 2006).

Enzymes such as ***cellulases and hemicellulases*** improve ruminal fibre digestion and typically lower the acetate-to-propionate ratio in rumen fluid which could bring a decrease in CH_4 production (Beauchemin et al., 2008). Therefore, it may be possible to develop commercial enzyme additives to reduce CH_4 emissions. However, little research has been published to confirm this hypothesis (Grainger and Beauchemin, 2011)

Direct-fed microbials (DFM or probiotics) are feed additives that contain microbial species that are considered to be non-pathogenic normal flora. Probiotics could replace antibiotics and chemical substances that have a risk of antibiotic resistance and residues in animal products. Prebiotics are non-digestible food ingredient that promotes the growth of beneficial microorganisms in the rumen. Galacto-oligosaccharides are non-digestible CHOs in non-ruminants and have a long

history of research as a prebiotic (Mwenya et al., 2004). Probiotics and prebiotics may either enhance specific microbial groups able to use excess H_2, or stimulate microbial growth leading to a higher H_2 consumption for microbial biomass synthesis (Jeyanathan et al., 2013). The most common DFM used in ruminant nutrition are yeast cultures to improve rumen fermentation and animal performance (Hristov et al., 2013). Yeast increases bacterial activity due to the removal of oxygen (O_2) from the rumen making the rumen more anaerobic. Yeast cultures stabilize rumen pH by reducing lactic acid concentrations and it is suggested that they stimulate acetogenic microbes (Jeyanathan et al., 2013). *Saccharomyces cerevisiae* is the most commonly used yeast and the one that has been most extensively studied for its effect on rumen methanogenesis (Lila et al., 2004). *Saccharomyces cerevisiae* as doses between 0.5-20 g/day increases MP flow, increases intake and fibre degradation, and have been reported to decrease CH_4 production in some studies (Chung et al., 2011; Mwenya et al., 2004). Lactate-producing and lactate-utilizing bacteria have been studied to improve rumen microflora composition or to stabilize PH, respectively. The increase in *Megasphaera elsdenni*, one of the most abundant lactate utilizing bacteria in the rumen, has led to an increase in fibre utilizing bacteria and decrease in amylolytic bacteria, but the effects on ruminal pH and fermentation have been variable between strains (Henning et al., 2010). The inclusion of lactate-producing bacteria should be done carefully because the possibility to promote acidosis, especially if it is included in high starch diets. Other examples of combination of DFM could be used to decrease CH_4 production: the combination of lactate-producing bacteria with *M. elsdenii* increased propionate production and animal productivity, prevented rumen acidosis, decreased pathonogenesis in young animals and potentially decreased CH_4 production. A culture of the yeast *Trichosporom sericeum* was effective in modifying ruminal fermentation patterns by increasing the pH and lowering CH_4 production (Mwenya et al., 2004). However, the production of DFM is expensive and technical advancements for the preparation of anaerobic bacteria are essential to increase their feasibility. Furthermore, there is a need for more comparative *in vitro-in vivo* studies feeding DFM to have consistent results and prove long lasting effects (Jeyanathan et al., 2013)

Protozoa are metabolically very active and contribute to rumen fermentation in different ways: protozoal numbers are positively related to fibre digestion and negatively with microbial protein synthesis (MPS) in the rumen (Eugene et al., 2004). Protozoa have been reported to be responsible for 25% of total CH_4 production. Therefore, ***defaunation techniques***, that consist of the total or partial removal of protozoa from the rumen may reduce CH_4 production. Treatments normally used to partially or completely defaunate the rumen include: chemicals that are toxic to protozoa (copper sulphate, dioctyl sodium sulphosuccinate, alcohol ethoxylate or alkanates, calcium peroxide), ionophores, lipids, and saponins. Elimination of the ciliate protozoa has been reported to increase MP supply by up to 30% and reduces CH_4 production by up to 11% (Newbold et al., 2015), without affecting cellulolytic activity (Yanez-Ruiz et al., 2009). A meta analysis of the effects of protozoa numbers on CH_4 emissions (Guyader et al., 2014) showed that 31% of the experiments reported a concomitant reduction of both protozoa populations and CH_4 emissions (g/kg DMI) with a significant linear relationship between CH_4 emission and protozoa concentration (Guyader et al., 2014). The results support defaunation as a promising technique to decrease CH_4 production in the rumen.

Recently, ***biochar*** has been included in ruminant diets with the aim to modify rumen microbiota and further decrease CH_4 production. Biochar compounds in the rumen seem to increase the inert surface area that allows closer association of both methanotrophs and methanogens (Leng et al., 2012) and stimulate direct electron transfer through electrical conductance(Leng et al., 2014). However, more research is needed to better understand the mode of action of biochar in rumen environment and its possible antimethanogenic properties.

Microbial intervention is a relatively new approach to reduce CH_4 production. Genome sequencing (methagenomic, metratranscriptomic and chemogenomics) is providing information directly applicable to CH_4 mitigation strategies based on vaccine and small molecule inhibitor approaches, and offering information relevant to other CH_4 mitigation strategies (Attwood et al., 2011; Di Bella et al., 2013; Kumar et al., 2014; Leahy et al., 2013; Sirohi et al., 2012). For example, ***bacteriophages*** are

obligate microbial viruses that infect both bacteria and archaea and lyse their hosts during the lytic phase of their development (McAllister and Newbold, 2008). Phages are very specific and therefore could be used without affecting other microbes in the rumen. However, this specificity could be also a limiting factor for their anthimethanogenic effectiveness, due to the high diversity of methanogens in the rumen. Host immunization with ***vaccines*** may be an environment and animal friendly antimethanogenic strategy (Williams et al., 2009). Some vaccines against specific methanogens have been developed targeting different species/ strains of methanogens present in the rumen (Wright et al., 2004). However, results have been variables and not correlated with the number of strains targeted. For example, Williams et al. (2009) targeted 52% of the different strains of methanogens present in the sheep expecting a decrease in CH_4 output. However, although a change in methanogen composition was observed, CH_4 output was not decreased. It seems that vaccines reduction in CH_4 production is obtained only when certain undefined conditions are met. In addition, the diversity of methanogens in the rumen may be influenced by both diet and geographic location, which increases the challenge in developing a broad-spectrum methanogen vaccine that will be effective across production conditions and over geographically diverse regions (Zhou and Hernandez-Sanabria, 2009). A better understanding of methanogen structure and function is needed to target all the rumen methanogens without affecting other microbes present in the rumen. For example, identifying genes encoding specific membrane-located proteins for *Methanobrevibacter ruminantium* has been done, being this methanogen identified as one of the major CH_4 producers within the rumen (Leahy et al., 2013). More genome sequences will allow the identification of the cellular mechanisms that define the methanogens (Kumar et al., 2014).

Despite the extensive research already undertaken on feed additives to decrease CH_4 emissions from ruminants, there are factors that are not yet well understood and there is a need of further research in this area to develop feasible sources of additives to adopt at a farm level. For example, the time of adaptation to dietary changes of methanogens is longer than from bacteria, around 4 weeks compared with 10-15 days for the bacterial community which could affect the action

of the additives (Williams, 2000). Long-term *in vivo* experiments are required to confirm the effects of different strategies on methanogens and on CH_4 emissions.

1.5 AIMS OF THE THESIS

1. *In vitro* evaluation of the effects of biochar compounds addition on rumen gas and CH_4 production, and fermentation.

2. To investigate the effect of encapsulated nitrate plus the addition of inorganic sulphur on enteric CH_4 production, nutrient digestibility, N utilization and MP synthesis from cross-bred beef heifers.

3. To investigate correlations between rumen microbial community structure and CH_4 yields in response to nitrate addition to basal diets fed to steers.

CHAPTER 2

In vitro screening of biochar compounds as antimethanogenic feed additives for ruminants

Chapter 2.

2.1 INTRODUCTION

Methane emissions arising from the enteric fermentation of feed by ruminant livestock contribute significantly to GHG emissions. Many feed additives have been tested to reduce ruminant enteric CH_4 production, but with differences in effectiveness. Research is still needed to find new, safe, and practical strategies to manipulate ruminal fermentation and reduce CH_4 emission from farmed ruminants without impairing livestock production. Biochar produced from different feedstocks has been proposed as a potential additive to reduce enteric CH_4 production from ruminants, but the information is still scarce.

2.1.1 Biochar

Biochar is the solid carbon rich material resulting from controlled heating of biomass at high temperature under oxygen-limiting conditions (pyrolysis). The characteristics, quality and potential use of biochar product will vary depending on the extent of pyrolysis (peak process temperature), and the nature of the feedstock the biochar was produced from (Joseph et al., 2007 ; Sohi et al., 2010). Biochar has been used as a soil amendment to sequester carbon and is reported to improve nutrient retention and suppress GHG emissions from soil (Lehmann and Joseph, 2009; Shackley et al., 2009; Sohi et al., 2010). These responses have been attributed to specific properties of biochar: carbon-rich and porous with a large surface area which is negatively charged and mostly associated with its insoluble components and stability. The chemical composition of biochar is characterized by the presence of polycondensed aromatic compounds, which provide long-term stability against microbial degradation. The chemical properties which have been measured for biochars include total carbon (C), total nitrogen (N), and pH (Gurwick et al., 2013) Physical composition of biochar is characterized by a large surface area, porosity and cation exchange capacity.

Recently, it has been noted that biochar changes the composition and abundance of the soil microbiological community (Farrell et al., 2013; Feng et al, 2012; Lehmann et al., 2011). Feng et al. (2012) focused on the effects of the addition of biochar to paddy soils on CH_4 emissions and the underlying mechanism, and they found a decrease in CH_4 emission from soils due to an increase on methanotrophic proteobacterial abundance and a decrease in the ratio of methanogenic to methanotrophic abundances. Biochar in soil ecosystems seems to act as a support to microbial activity thereby increasing substrates degradability. Mechanisms proposed for the stimulatory effect of biochar on soil microbiota include high sorption capacity , which may change organic matter availability, high porosity , which may provide a suitable microhabitat and alkaline pH, which may increase the nutrient retaining capacity of soils. The effects of biochar in soils will be influenced by the soil composition itself (*e.g*, greater effects in soils with low fertility and/or low pH (Lehmann et al, 2011).

More recently, the inclusion of biochar in ruminant diets has been investigated for two reasons. First, biochar may reduce enteric CH_4 emissions (Leng et al., 2012; Hansen et al. 2012; Calvelo Pereira et al. 2014) and secondly, faecal excretion of dietary biochar may provide an effective means of applying biochar to pasture (Calvelo Pereira et al. 2014; Joseph et al., 2015). Responses to the inclusion of biochar in rumen *in vitro* assays have been variable ranging from 0% (Calvelo Pereira et al., 2014) to a 13% reduction (Leng et al. 2012a). Leng et al. (2012b) evaluated biochar for the first time *in vivo*. Biochar (6 g/kg DM) and/or nitrate (60 g/kg DM) were included in a forage-based diet fed to cattle. Both biochar and nitrate reduced CH_4 production and the effects of biochar and nitrate were additive. Feed conversion efficiency was also improved when biochar was added to the diet. The above studies suggested that biochar may decrease CH_4 production from ruminants, but the evidence is limited and biochars with different physicochemical properties have not been explored in detail. As the properties of biochar are dependant on both the temperature of pyrolysis and the feedstock from which it was prepared, then the above variation in response with different biochar compounds is not surprising.

2.1.2 Aim of the study

The objective of the current work was therefore to determine *in vitro* whether biochar reduced CH_4 production and by using a range of biochars with defined chemical and physical compositions to investigate the attributes of biochar responsible for reducing CH_4 *in vitro*.

The hypothesis of the present study was that the inclusion of biochar in the diet of ruminants would lead to a reduction in enteric CH_4 emissions and the working hypotheses were that:

- Biochar reduces CH_4 production in *in vitro* rumen fluid incubations.
- Biochars with different chemical and physical composition will have different effects on CH_4 production in *in vitro* fermentation.

2.2 MATERIAL AND METHODS

This experiment was conducted at Scotland's Rural College (SRUC) Beef and Sheep Research Centre, Easter Howgate, Edinburgh during 2014. The experimental protocol was approved by SRUC's Animal Welfare and Ethical Review Body, the Animal Experiments Committee and was conducted in accordance with the requirements of the UK Animals (Scientific Procedures) Act, 1986.

2.2.1 Biochar production

The ten different biochars used in the experiment were produced by the UK Biochar Research Centre, University of Edinburgh from five different feedstocks: miscanthus straw (MSP), oil seed rape straw (OSR), rice husk (RH), soft wood pellets (SWP) and wheat straw (WSP) by pyrolysis in a gas-fired, rotating-drum kiln up to peak temperatures of either 550 or 700 ºC. The ten different biochars produced in the Biochar Research Centre were identified with the following codes indicating starting material and pyrolysis temperature: MSP550, MSP700, OSR550, OSR700, RH550, RH700, SWP550, SWP700, WSP550, WSP700 (Table 2.1). Biochar physicochemical properties data were obtained from Charchive database. The database contains material information on biochar products as well as the feedstock material, production and storage conditions. Full details of biochar production and composition can be found at (http://www.biochar.ac.uk/standard_materials.php; accessed 07/02/2017). To ensure that particle size was small enough for inclusion in assays and to avoid gross differences between biochars, material that passed through a 2 mm screen was used for the assay.

Table 2.1 Physicochemical composition of biochar samples used for *in vitro* incubations
Retrieved from (http://www.charchive.org/record.php) (Accessed March 17)

Biochar	Moisture[0]	Ash[1]	C[1]	N[1]	H[1]	P[1]	K[1]	pH	Elect. conduct[2]	Stable C[3]	Volatile C [4]	Fixed C[4]
MSP550	1.83	12.1	754.1	7.8	24.2	1.9	9.5	9.8	0.8	88.4	11.6	76.2
MSP700	2.28	11.5	791.8	10.3	12.6	1.1	13.0	9.7	1.9	96.3	7.7	*
OSR550	2.81	17.9	669.1	17.7	16.8	0.4	3.2	9.8	2.3	94.7	12.9	69.1
OSR700	2.94	22.2	677.4	12.6	10.9	2.6	29.8	10.4	3.1	*	8.9	68.9
RH550	1.50	47.8	486.9	10.9	12.4	1.7	6.5	9.7	0.5	99.3	7.5	44.7
RH700	*	53.5	473.2	8.5	6.3	1.3	5.7	9.8	0.7	91.1	*	*
SWP550	*	1.2	845.1	1.0	28.0	0.1	0.7	7.9	0.1	96.6	14.2	*
SWP700	1	1.7	902.1	<0.1	18.3	0.1	1.1	8.4	0.2	96.5	6.9	91.4
WSP550	1.92	21.2	682.6	13.9	21.0	1.5	18.7	9.9	1.7	97.2	10.5	68.2
WSP700	2.30	23.2	690.4	13.2	11.8	1.5	16.2	10.0	1.5	98.3	7.7	69.2

% weight
[1](mg/g) on dry basis
[2]Electrical conductivity (H_2O) decisiemens/metre (dS/m)
[3] % total C
[4] % total mass dry basis
*Not recorded

2.2.2 Experimental design

The *in vitro* cumulative gas production technique was used to incubate biochar samples anaerobically with rumen fluid (Menke et al., 1979). A 2 (biochar inclusion) x 2 (process temperature) x 5 (feedstuff) factorial design was used where the factors were: biochar addition (10 or 100 g biochar/kg substrate); biochar process temperature (550 or 700 ºC) and feedstock (miscanthus straw, oil seed rape straw, rice husk, soft wood pellets or wheat straw). Each of the 20 individual treatments was incubated in triplicate in each of four replicates. Within each replicate, control samples which contained substrate but no added biochar and blank samples without substrate or biochar were also included in triplicate giving a total of 66 incubations per replicate.

2.2.3 Rumen fluid inocula

Rumen samples were obtained from a group of cross-bred beef cattle (approximately 16 months in age) fed *ad libitum* a diet consisting of 500 g forage and 500 g concentrate/ kg DM (DM basis). The steers were fed once daily and rumen samples were obtained at approximately 08.00 h before fresh feed was offered. Rumen samples were obtained using a stomach tube (16 × 2700 mm) introduced into the oesophagus via a nostril and then passed down to the rumen. Samples were immediately strained through two layers of muslin and transported in insulated flasks under anaerobic conditions to the laboratory and used as inocula within one hour of collection.

For each replicate of the experiment, three different rumen inocula were prepared. Where possible each inoculum was derived from an individual animal but where sample volume from an individual animal was inadequate, a composite sample was produced by mixing samples from two animals. Rumen fluid from an individual steer was not included in incubations on more than one occasion. Each of the triplicate incubation noted above therefore contained rumen fluid from three different inocula: that is of the 66 incubations per replicate, 22 each contained rumen fluid

from different rumen fluid inocula. Thus 12 different inocula were used in total for the four replicates.

10 biochar types x 2 level of inclusion x 1 RF source + 1 blank + 1 control = 22
10 biochar types x 2 level of inclusion x 3 RF sources + 3 blank + 3 control = 66

2.2.4 *In vitro* gas production test

Incubations took place in 160 ml serum bottles which contained 400 mg feed substrate (343 mg DM) and biochar (4 or 40 mg) as appropriate (Figure 2.1.a). This was achieved by mixing 1 g biochar with 10 g feed substrate to obtain a 10 g biochar/ kg mixture. This initial mixture was further used to include the desired biochar inclusion; to include 4 mg of biochar for the 10 g/kg inclusion, 40 mg of the 100 g/kg mixture was added to 360 mg feed substrate. For the 100g/kg inclusion, 440 mg of the 100 g/kg mixture was placed in the bottles.

The feed substrate consisted of a mixture of hay, barley and rapeseed meal (5:4:1 w:w). Feed substrate was analysed for DM, CP, acid hydrolysed ether extract and neutral cellulose and gamanase digestibility according to Ministry of Agriculture Fisheries and Food (1992). Chemical composition was DM, 857 g/kg and (g/kg DM). CP, 105; acid hydrolysed ether extract, 19; neutral cellulase plus gamanase digestibility, 760 and estimated metabolisable energy, 11.2 MJ/kg DM.

The solutions for the buffer-mineral medium were prepared beforehand (1 L) as described by Menken ad Steingass (1988) and stored at 4 ºC: buffer solution, macromineral solution, micromineral solution and resazurin (redox indicator). The reducing solution was prepared just before the incubation and added to the medium. A stream of CO_2 was applied to the solution until the blue colour turned to pink and then colourless which indicated that the medium was reduced (Figure 2.1.b). The pH of the medium was measured to guarantee that was around seven before use. The rumen fluid was mixed with buffer-mineral solution at a ratio 1:3 (v/v), rumen fluid: buffer. Rumen fluid: buffer mixture (40 ml / bottle) was dispensed under a stream of CO_2, and the bottles were closed with a butyl rubber stopper (Figure 2.1.c) and placed in a water bath at 39 ºC for 24 h. Contents were thoroughly mixed periodically throughout the 24 h (Table 2.2; Figure 2.1.d).

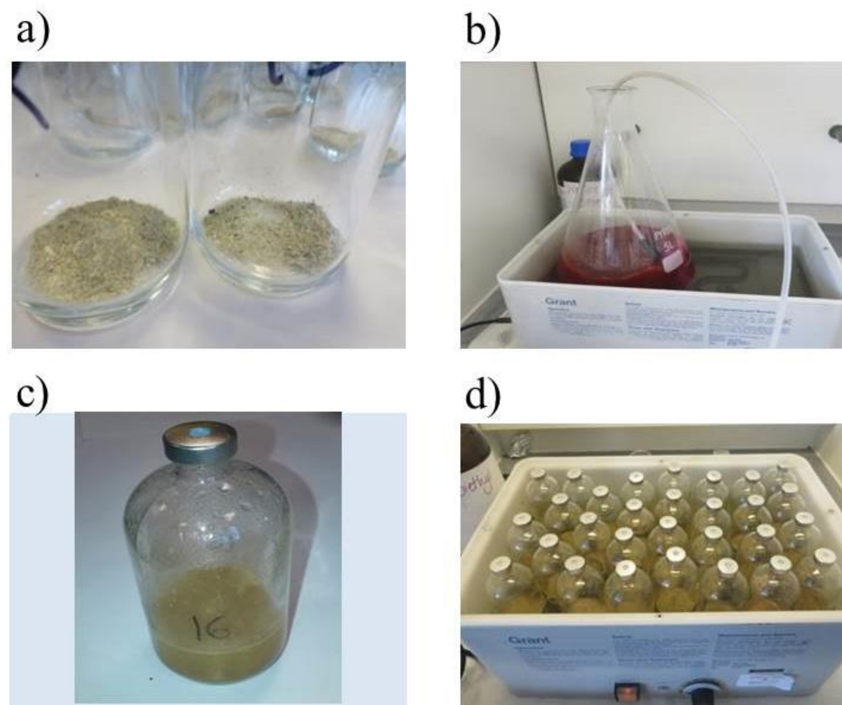


Figure 2.1. Incubation images

a) Bottles with substrate and biochar samples
b) Buffer-mineral solution into the water bath and under a stream of CO_2
c) Incubation bottle with medium and substrate
d) water bath at 39 C fill in with bottles for an incubation

Table 2.2 Individual content of serum bottles present in each day incubation with one unique rumen fluid source

Samples	RF(ml)	Buffer (ml)	Substrate(mg)	100g/kg biochar mix	RF*
10 g/kg biochar	10	30	360	40	1
100 g/kg biochar	10	30	0	440	1
Control	10	30	400		1
Blank	10	30			1

*unique rumen fluid source for each batch of 22 bottles

2.2.5 Analytical methods

Cumulative gas production during the 24 h incubation was measured by pressure using a manual pressure transducer (Digitron 2023P, Digitron, Torquay, Devon, UK). The pressure values were converted to the volumes of gas produced using the equation below determined for local laboratory conditions.

$V = (P - 11.58) / 7.55$ where V = gas volume (ml) and P = pressure (mbar)

The gas produced due to fermentation of the feed substrate was corrected for gas produced in appropriate blank incubations. After measurement of pressure, 20 ml gas samples were transferred in duplicate to head-space vials (Figure 2.2.a). and CH_4 was analysed by gas chromatography (Agilent 7890, Agilent Technologies**,** Cheshire, UK) using a HayeSep Q (80/100), 0.25m x 1mm internal diameter column with helium as carrier gas and detection by flame ionisation. At the end of the incubation, the bottles were uncapped and pH measured immediately (Figure 2.2. b). Samples of the medium were taken to determine VFA (1 ml) and NH_3-N concentration (0.6 ml) (Figure 2.2.c). Samples for VFA analysis (5 mL) were de-proteinized by adding 1 mL metaphosphoric acid (215 g/L) and 0.5 mL methylvaleric acid (10 g/L) as an internal standard. These samples were stored at -20 °C between collection and analysis. VFA concentrations were determined by HPLC as described in Rooke et al. (1990). Samples for analysis for NH_3-N were diluted 1:1 (v/v) with 1 M-HCl and analysed using the phenol-hypochlorite method of Weatherburn (1967) adapted for 96 well plates with absorbance measured at 625 nm.

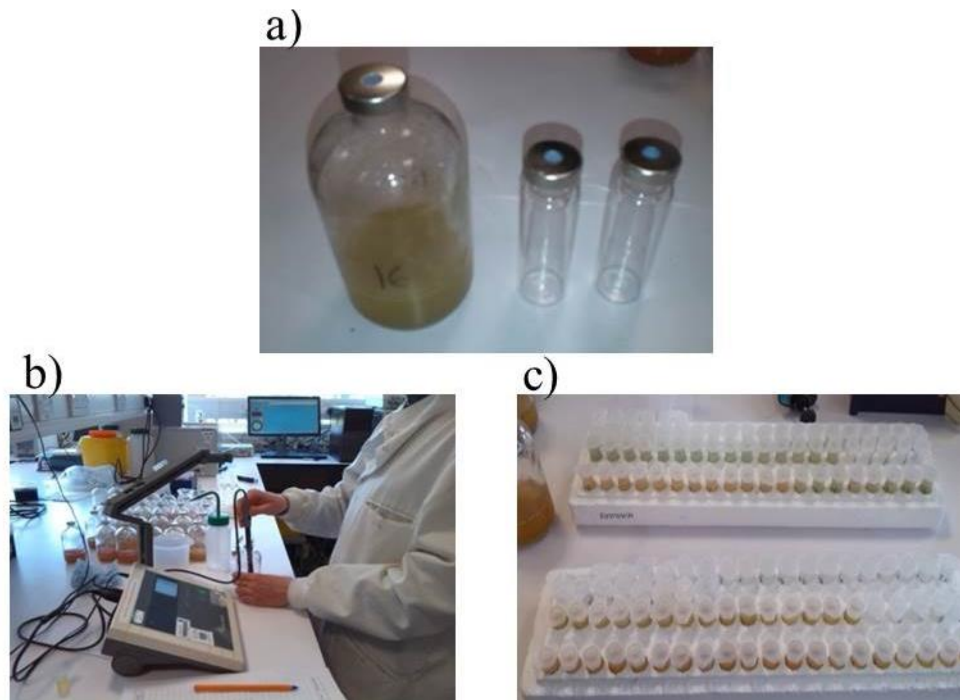


Figure 2.2 *In vitro* incubation images

a) Bottle after incubation and 20 ml sample vials with gas samples
b) Measure of pH after incubation
c) Samples of medium for VFA and NH3-N analysis

2.2.6 Calculations and statistical analyses

Amounts of total gas, CH_4, VFA, and NH_3-N produced were corrected for amounts produced in blank incubations and expressed either as total amount produced or amount produced or per g fresh weight substrate incubated. To assess the overall effect of biochar inclusion, values were expressed as a proportion of the control value for each of the 12 rumen fluid inocula and a single sample t-test used to determine if the overall mean value differed from one (control value). Differences between biochar treatments were analysed according to a factorial design using the Linear Mixed Models procedure of GenStat (version 11.1 for Windows; VSN International Limited). The model included the fixed effects of the type of starting material (i.e., MSP, OSR, RH, SWP and WSP), the pyrolysis temperature of biochar production (550 and 700 ºC), and the level of biochar addition (10 or 100 g/kg substrate), and the interaction between the three factors. The different replicates and rumen fluid inocula (within replicate) were included as random factors. Where significant differences ($P<0.05$) were detected between feedstocks, differences between means were identified using least significant differences. In addition, Spearman correlation were done to assess the relationship between the physicochemical properties of biochar compounds and the parameters studied (Minitab Software).

2.3 RESULTS

2.3.1 Rumen inocula

Gas production (Table 2.3) ranged from 220 to 184 ml/g substrate, CH_4 production ranged from 21.7 to 15.0 ml/g substrate; VFA production from 8.43 to 2.12 mmol/g substrate and NH_3-N concentration from 2.21 to 0.44 mmol/g substrate.

Using rumen fluid inocula obtained from different animals to inoculate the *in vitro* incubations achieved the objective of producing fermentations differing not only in the extent (amounts of total gas, CH_4 and VFA produced) but also in the type of fermentation (VFA molar proportions and NH_3-N) (Table 2.4).

Table 2.3 Average, Maximum, Minimum and Standard deviation (SD) of all parameters studied

	Mean	Maximum	Minimum	SD
Gas production (ml/g substrate)	184.5	220.1	146.9	11.6
CH_4 production (ml/g substrate)	39.2	55.3	31.9	3.1
Total VFA (mmol/g substrate)	1.90	4.21	1.10	0.4
VFA (mmol/mol)				
Acetate	558.4	678.0	473.4	41.3
Propionate	301.1	421.8	197.9	54.6
Butyrate	115.5	182.2	65.1	23.8
NH_3-N (mmol/g substrate)	1.21	2.21	0.44	0.38

SD, standard deviation

Table 2.4 Values from different sources of rumen fluid used on *in vitro* incubations

Rumen fluid	1	2	3	4	5	6	7	8	9	10	11	12	SEM	P-v
Gas production (ml/g substrate)	175.0	190.7	189.8	195.1	178.4	183.7	191.6	185.4	190.6	167.6	172.4	194.1	1.76	<0.001
CH_4 production (ml/g substrate)	38.4	48.5	38.9	38.9	37.8	38.1	38.7	38.3	38.6	37.2	37.5	38.9	0.27	<0.001
Total VFA (mmol/g substrate)	1.72	1.77	1.35	2.08	1.92	2.15	2.04	2.37	2.52	1.76	1.83	1.86	0.06	<0.001
VFA (mmol/mol)														
Acetate	567.9	584.7	512.5	562.1	506.5	550.3	569.0	593.7	641.2	516.3	515.2	574.1	4.87	<0.001
Propionate	306.6	246.0	390.3	312.4	366.2	327.1	256.6	254.5	216.5	318.1	364.5	266.5	5.15	<0.001
Butyrate	103.3	130.4	83.6	93.5	102.3	94.2	138.2	122.9	114.4	157.6	95.3	138.9	2.96	<0.001
NH_3-N (mmol/g substrate)	0.99	1.38	1.20	1.26	0.75	0.93	1.34	1.62	1.45	1.55	0.78	1.29	0.058	<0.001

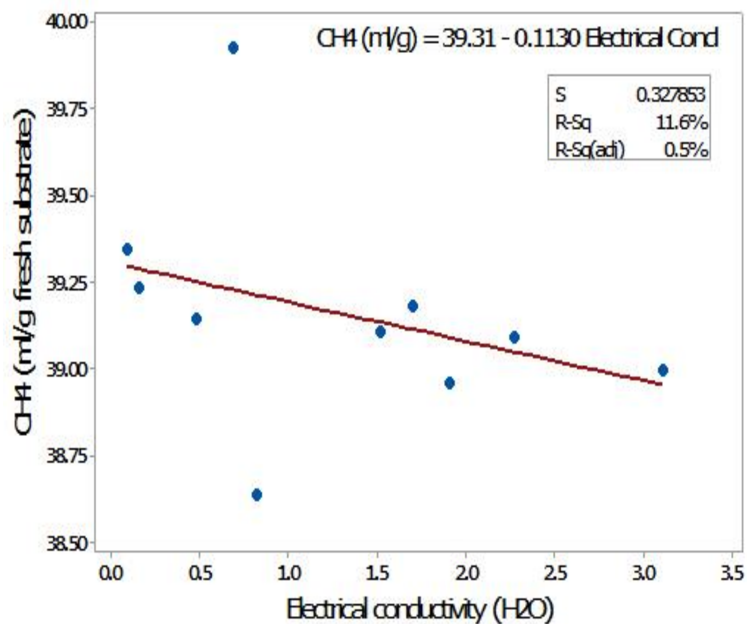
2.3.2 Overall effects of biochar compounds

Overall, addition of biochar reduced CH_4 production by 3.75±1.53% and total gas production by 2.11±1.6% of that in control (no added biochar). Addition of biochar to incubations did not change total amounts of VFA or acetic acid produced during *in vitro* fermentation; however, the amounts of propionate (0.97; SEM 0.006, $P<0.001$) and NH_3-N concentrations were lower ($P<0.001$) when biochar was included in incubations (Table 2.5). There was a negative correlation between electrical conductivity (dS/m) of biochar compounds with CH_4 production (ml/g substrate)($r=-0.648$, $p=0.04$) and total VFA (mmol/g substrate) ($r=-0.673$, $P=0.03$). The scatterplot summarize the results (Figure 2.3). The scatterplot suggests a linear relationship between electrical conductivity and CH_4 and VFA produced during *in vitro* incubations, with larger values of electrical conductivity tending to be associated with lower values of CH_4 and VFA . No other significant correlations were found between physicochemical properties of biochar compounds and parameters studied.

Table 2.5 Parameters expressed as proportion of control samples and results from T-tests (SEM and P values) with the hypothesis that values from samples which included biochar were different from 1 (control)

	Mean	SEM	P-value
Gas production (ml/g substrate)	0.98	0.003	<0.001
CH_4 production (ml/g substrate)	0.96	0.002	<0.001
Total VFA (mmol/g substrate)	1.00	0.018	0.797
VFA (mmol/mol)			
Acetate	1.01	0.002	0.334
Propionate	0.97	0.006	<0.001
Butyrate	0.98	0.009	0.105
NH_3 (mmol/g substrate)	0.78	0.014	<0.001

2.3 a)



2.3 b)

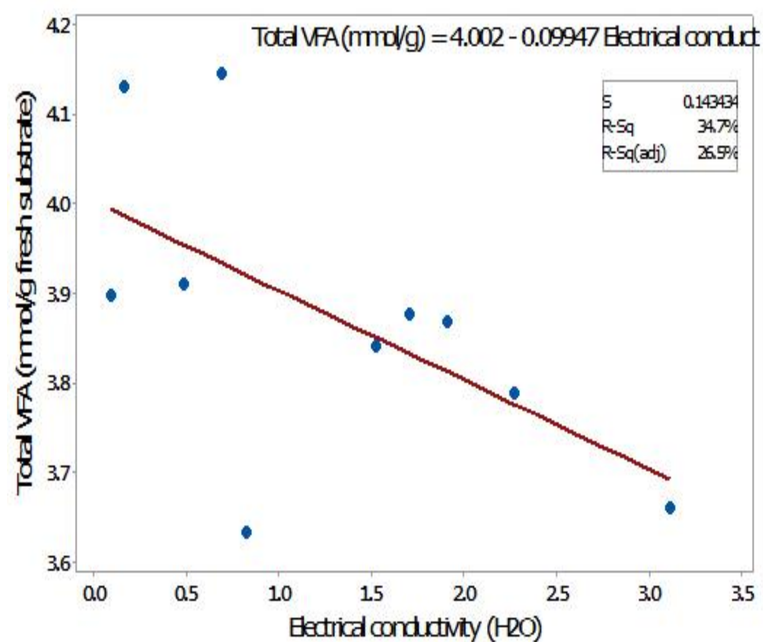


Figure 2.3 Linear regression between electrical conductivity (dS/m) of biochar compounds versus CH_4 produced (ml/g substrate) and total VFA produced (mmol/g substrate) from *in vitro* incubations

2.3.3 Effect of inclusion level of biochar on rumen fermentation

There were no differences ($P>0.05$) between adding biochar at 10 or 100g/kg on gas and CH_4 production, nor for fermentation parameters (Table 2.6)

Table 2.6 Effects of different levels of biochar inclusion on *in vitro* gas production and fermentation parameters after 24h incubation

	10 g/kg	100 g/kg	SEM	P-Value
Gas production (ml/g substrate)	185.0	184.1	1.00	0.35
CH_4 production (ml/g substrate)	39.2	39.1	0.16	0.65
Total VFA (mmol/g substrate)	2.11	2.16	0.030	0.11
VFA (mmol/mol)				
Acetate	557.5	558.0	1.91	0.77
Propionate	304.2	300.2	2.02	0.05
Butyrate	114.0	114.9	1.19	0.42
NH_3-N (mmol/g substrate)	1.21	1.21	0.028	0.90

2.3.4 Effects of preparing biochar at different temperatures on rumen gas production and fermentation

Total gas ($P=0.05$) production was slightly greater when biochar was produced at 700 rather 550 ^{0}C. Process temperature had no effect on CH_4 production or VFA proportions. In contrast, NH_3-N and total VFA concentration were lower when pyrolysis temperature was 550 ºC than 700 ºC ($P<0.05$) (Table 2.7).

Table 2.7 Effects of inclusion of biochar compounds prepared at different temperature in gas production and fermentation parameters *in vitro* after 24 h incubation

	550ºC	700ºC	SEM	P-Value
Gas production (ml/g substrate)	183.6	185.5	1.00	0.05
CH_4 production (ml/g substrate)	39.1	39.2	0.16	0.33
Total VFA (mmol/g substrate)	1.90	1.96	0.045	0.03
VFA (mmol/mol)				
Acetate	557.2	558.3	1.91	0.58
Propionate	304.2	300.2	2.02	0.81
Butyrate	113.7	115.2	1.19	0.21
NH_3-N(mmol/g substrate)	1.17	1.25	0.03	0.01

2.3.5 Effect of preparing biochar from different feedstocks on rumen gas production and fermentation

Biochar feedstocks influenced CH_4 production (P=0.05), but had no effect on total gas production (P=0.09). Methane production was lowest with biochar produced from MSP, with RH and SWP highest, and OSR and WSP intermediate (Table 2.8). Total VFA concentrations were influenced by biochar feedstock with extent of production ranked (lowest to highest): oilseed rape straw; miscanthus straw; wheat straw; soft wood pellets and rice husks for total VFA, acetate and butyrate. There were no significant differences in VFA molar proportions between different biochar feedstocks. Ammonia concentrations for feedstocks were lowest for miscanthus straw and greatest for rice husk with oil seed rape straw, soft wood pellets and wheat straw intermediate (in order of ascending concentration).

Table 2.8 Effects of inclusion of biochar compounds from 5 different feedstocks in gas production and fermentation parameters *in vitro* after 24 h incubation compared to control vaues

	Control	MSP	OSR	RH	SWP	WSP	SEM	P-value
Gas production (ml/g substrate)	188.9	182.5	183.6	186.5	185.6	184.5	1.59	0.09
CH_4 production (ml/g substrate)	39.5[a]	38.8[b]	39.1[ab]	39.5[a]	39.3[a]	39.1[ab]	0.25	0.05
Total VFA (mmol/ g substrate)	2.72[a]	2.09[b]	2.05[b]	2.21[c]	2.20[c]	2.14[bc]	0.01	0.01
VFA (mmol/mol)								
Acetate	558.8	563.2	557.1	556.9	555.6	556.0	3.03	0.08
Propionate	303.1	300.5	303.6	299.9	300.7	306.2	3.20	0.27
Butyrate	115.3	112.4	114.2	116.0	116.0	113.7	1.88	0.25
NH^3-N (mmol/g substrate)	1.89[a]	0.93[b]	1.22[c]	1.34[d]	1.28[cd]	1.29[cd]	0.04	<0.001

SEM: standard error of the mean

[a,b,c] Means values within a row with unlike superscript letters were significantly different: P<0.05

2.4 DISCUSSION

Including biochar in *in vitro* rumen fluid incubations reduced total gas, VFA and CH_4 production to a limited and NH_3-N concentrations to a greater extent.

2.4.1 *In vitro* gas fermentation technique

In vitro techniques are used to screen for compounds in laboratorial conditions, allowing a less invasive methodology and lowering costs (Castro-Montoya et al., 2010; Mitsumori et al., 2002). *In vitro* techniques are used to screen for compounds in laboratorial conditions, allowing a less invasive methodology and lower costs compared with *in vivo* studies. Depending on the research question, *in vitro* studies can be valuable for initial screening of antimethanogenic compounds and informing on the suitability for further evaluation *in vivo*. For the first experiment of this thesis the *in vitro* batch culture technique was used to test biochar. The method was considered the most appropriate according to the state of biochar research for ruminant diets. The knowledge of biochar compounds as antimethanogenic additive for ruminants is scarce and little is known about the implications for animal production and health. It is especially useful to estimate the influences on rumen fermentation, total gas and CH_4 production (Getachew et al., 1998). The most important factor regarding the inoculum, is the presence of sufficient microbial activity to support fermentation, which can be determined by measuring absorbance of the inoculum (following a 50-fold dilution) at 600 nm (Nagadi et al., 2000). Rumen fluid taken following an overnight fasting is less active than that taken 2 h after feeding, but microbiota population is more consistent in its composition and activity with less variation between animals and sampling days (Williams, 2000). Therefore, in the current experiment samples were taken before the morning fed and it was assumed that inocula had enough microbial activity. Most *in vitro* studies use an inoculum obtained by bulking rumen fluid from several animals for each assay (Bodas et al., 2008; Patra et al., 2006; Patra and Yu, 2014). When using the *in vitro* gas production technique, it is usually recommended that the most

consistent results are obtained by taking samples combining rumen fluid from a minimum of three animals (Williams et al., 1990). However, such an approach does not allow the assessment of animal to animal variation in response. In the current experiment, animal to animal variation was specifically addressed by using 12 different sources of rumen fluid (in most cases from individual animals) incubations. Despite the fact that there was substantial animal to animal variation, estimated to be four (gas produced / g substrate DM) to ten (CH_4 produced / g substrate DM) times greater than the variation associated with the biochar treatments imposed, the overall effect of and differences between biochar types were successfully detected. It should be noted that at least some of the animal to animal variation will be related to feed consumption and quality, as although fresh feed was last offered 24h before rumen samples were obtained, individual patterns of feed intake would have differed from animal to animal and the nutritional quality of feed from replicate to replicate.

Other considerations for the *in vitro* test were carefully taken to optimise the results of the screening (Yanez-Ruiz et al., 2016); 4 independent incubation runs were carried out, same diet composition was fed to donor animals than the substrate incubated *in vitro* (Mould et al., 2005), and fresh rumen fluid was maintained under anaerobic conditions at 39◦C (Rymer et al., 2005) and inoculated into *in vitro* vessels within 1 h post collection.

Still, the *in vitro* technique has some limitations. The model is static and cannot include some animal factors such as differences in ingestive behaviour, rumen size, fill and digesta passage kinetics (Patra et al., 2010). Batch cultures techniques may not emulate what occurs in animals, as the rumen resembles a continuous rather than culture system. Therefore, a positive outcome *in vitro* does not guarantee that the same treatment will have a similar effect *in vivo* (Hristov et al., 2012). Most of the comparison between *in vivo* and *in vitro* results about CH_4 reduction of compounds have shown that inhibition potential is often overestimated *in vitro* (Martinez-Fernandez et al., 2014; Martinez-Fernandez et al., 2013a). The lack of adaptation in short-term *in vitro* incubations may lead to larger CH_4 mitigation *in vitro* compared to the *in vivo* situation (van Zijderveld, 2011). .

2.4.2 Effects of biochar on gas production

These preliminary results demonstrate that biochar decreased CH_4 production from *in vitro* rumen fluid incubations. Biochar reduced the overall extent of fermentation (gas production) to 0.96 and CH_4 emissions to 0.95 of control values. More importantly the ratio of CH_4 to total gas in samples to which biochar had been added was 0.98 of control values. Therefore biochar caused only a small reduction in CH_4 production. The level of addition was selected based on previous research. The extent of reduction with biochar incubated compared with controls was significantly greater in Leng et al. (2012a) experiments (14%) when compared with the reduction observed in the current study (5%). Hansen et al. (2012) reported a 20% reduction in CH_4 production (although not significant) *in vitro* when three different biochars were added at 90 g/ kg DM. However, in both studies no biological replication of the rumen fluid was possible as only a single source of rumen fluid was used (Hansen et al., 2012; Leng et al., 2012a). Calvelo Pereira et al. (2014) investigated feeding ruminants biochar as a vehicle for delivery of biochar in faeces to pastoral soils. Different types of biochar were either incorporated into grass silage at harvest or added directly to hay and included in *in vitro* assays. In this experiment, biochar did not reduced CH_4 production or change the rumen fermentation (Calvelo Pereira et al., 2014). The differences in the rate of CH_4 reduction between studies may be a consequence of the different biochar compounds tested and different substrates used for incubations, then the range of results is not surprising and difficult to analyse. The level of biochar inclusion were based on previous *in vitro* studies (Hansen et al., 2012; Leng et al., 2012a). Addition of 100 g biochar/kg substrate rather than 10g/ kg did not increase the effect of biochar and therefore suggests that 10 g/kg addition had achieved the maximum response. The apparent maximum effective inclusion (<= 10 g/kg) for reduction of CH_4 production agrees with Leng et al. (2012a).

2.4.3 Effects of biochar compounds on rumen fermentation

Total VFA concentrations were not significantly reduced with the inclusion of biochar, but there were differences in fermentation inhibition effect between biochars prepared from different feedstocks. Total VFA (mmol/g substrate) produced from

incubations with MSP biochar compounds was lower in comparison to incubations from RH and SWP compounds, which is in line with the difference in CH_4 produced between biochar feedstocks. Molar proportion of the main VFA (acetate, propionate and butyrate) was not different between biochar feedstock.

Ammonia concentrations were unexpectedly reduced when biochar was included in incubations compared to substrate controls. The reduction was most marked in miscanthus-derived biochar (0.58 of control) and biochar prepared at 550^{0}C had a greater effect than preparation at 700 ^{0}C. Since the *in vitro* incubation is a sealed system, there are two possible reasons for this difference. First, the differences in NH_3-N concentrations could be due to a reduction in proteolysis and deamination of nitrogenous constituents of the feed substrate or increased incorporation of NH_3-N into MP (Patra and Saxena, 2009; Patra and Yu, 2014) or a combination of these two processes (Wallace et al., 1994). It is likely that the observed reduction in NH_3-N in this study would not impair MPS as rumen fermentation was not overall affected by biochar inclusion then a reduction in proteolysis seems more probable (Cardozo et al., 2005). Only Calvelo Pereira et al. (2014) have also reported NH_3-N concentrations *in vitro* with biochar addition but these authors found no differences between treatments.

Biochar has shown to enhance nitrification on soils and increase the number of NH_3-N-oxidizing bacteria (Ball et al., 2010; Lehmann et al., 2011), and in some studies, biochar in soils reduced leaching of NH_3-N (Ding et al. 2010). This has been attributed to the cation exchange capacity of the negatively charged biochar and indeed in laboratory studies, NH_3-N is adsorbed from solution by biochar (Gai et al., 2014; Winning et al., 2014). In these laboratory studies, the efficacy of biochar in adsorbing NH_3-N was inversely related to the temperature at which was produced (increased pyrolysis temperature reduces cation exchange capacity) and to the influence of feedstock on cation exchange capacity. Thus, an alternative explanation for the effect of biochar on NH_3-N concentrations is that NH_3-N was adsorbed by biochar. In the *in vivo* situation, binding of NH_3-N by biochar may be beneficial as any NH_3-N bound when NH_3-N concentrations are high immediately after feeding would be released when NH_3-N concentrations declined and therefore would

improve synchrony between the supply of NH_3-N and energy (from degraded CHOs) for rumen MPS.

The increase or decrease in NH_3-N concentration may be considered of interest depending on the diet being fed and the extent of the effect. If the diet is high in rumen degradable protein a decrease in NH_3-N production could be a desirable effect, decreasing the losses of N in the urine, but if NH_3-N concentration is low, such decrease in NH_3-N concentration, below a certain threshold, could impair an optimal MP growth.

2.4.4 Properties of biochar compounds and mechanism of action

A significant and negative correlation was found between electrical conductivity of biochar compounds and total VFA production and CH_4 production. No significant correlations were found between the composition and particle size distribution of biochar compounds and parameters studied. Biochar compounds were not fermentable as the observed reductions in CH4 produced were not associated by a change in VFA production.

As described in the introduction section, the effects of biochar compounds in soils have been largely studied but the understanding of the mechanism of action of biochar compounds on rumen fermentation and gas production is a greater challenge. One hypothesis could be that biochar effect on CH_4 production is linked to their physical properties, modifying the environment for CH_4 producing microbial population. In the soil and composting environments, the balance between methanogenic archaea and methanotrophic organisms was altered favourably towards methanotrophic rather than methanogenesis with biochar application (Feng et al., 2012). In a different trial by Leng et al. (2012a), biochar was used either untreated or after washing to remove water soluble material. The amount of CH_4 produced was lower with washed biochar, which suggested that the insoluble component of biochar was more important for reducing CH_4 production than the soluble component. Leng et al. (2012b) suggested that biochar reduced CH_4 production from steers by stimulating an increased biomass of microbial consortia

that oxidise CH_4. However, the evidence for CH_4 oxidation in ruminants is limited (Kajikawa et al., 2003). Other possibilities suggested have been creation of micro-environments by the large surface area of biochar promoting microbial growth and facilitating both primary (hydrogenotrophic) and secondary (acetoclastic) fermentations (Leng et al., 2014). Recently, research has purposed that electrical conductivity of biochar surfaces may facilitate direct electron transfer among syntrophic organisms (Liu et al., 2011), facilitating methanogenesis by electrical conductance between organisms (Leng et al., 2013).The negative correlation found between electrical conductivity and CH_4 production in this study may support this theory.

2.5 CONCLUSIONS AND IMPLICATIONS

In this study, it was shown *in vitro* that biochar compounds decrease CH_4 production by 5% with 10 g/kg substrate level of inclusion. As biochar is an inert material and not fermentable, it is likely that biochar included in the diet, would reduce the intake of digestible feed and therefore may reduce energy supply to the animal if it cannot increase feed intake to compensate. The direct consequence will be a reduction in performance. On the other hand, the reductions in rumen NH_3-N concentration observed with biochar may change the balance of nitrogenous constituents in animal excreta as less NH_3-N may be absorbed from the digestive tract and excreted as urea in urine, thus decreasing the soluble nitrogenous constituents of manure or slurry. If NH_3-N is excreted bound to biochar then it may contribute to improved N retention in soils.

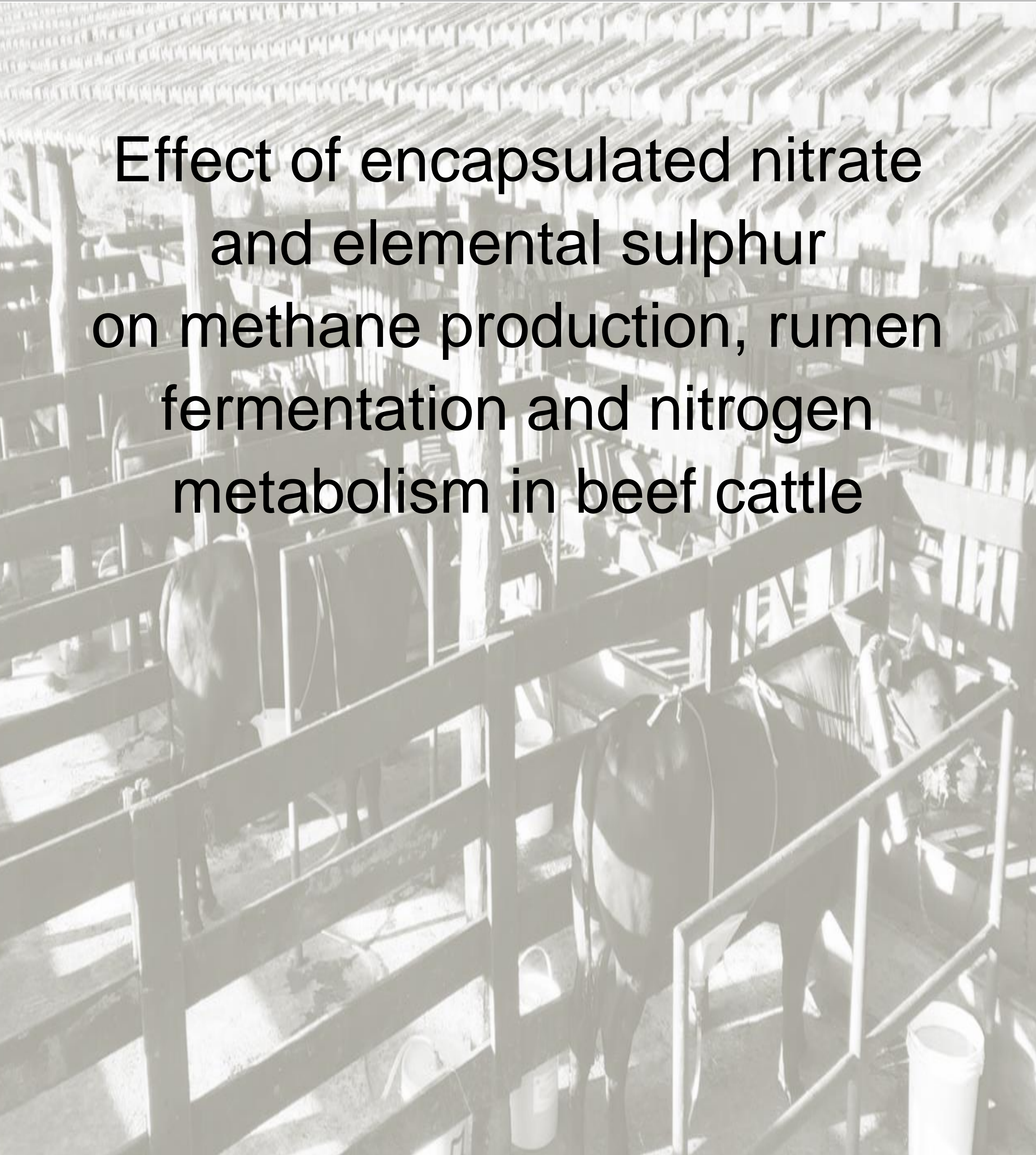
Including biochar in animal feed as a means of applying biochar to soil and pasture has been suggested (Calvelo Pereira et al. 2014; Joseph et al. 2015). Incorporating biochar into ensiled grass had no adverse effects on the resulting silage (Calvelo Pereira et al., 2014) and Joseph et al. (2015) reported little change in the properties of biochar as it passed through the gut of cattle. Adding biochar up to 100g /kg feed substrate in the current experiment did not adversely effect rumen fermentation.

By any mean, there are many questions that should be addressed before practical use of biochar as ruminant additive such as availability, dose needed, and effect on performance, safety, and method of addition. From previous *in vitro* studies, the amount of additive needed to achieve significant reduction in CH_4 exceed practical feeding limits for high producing animals, as cows might not be able to consume sufficient feed energy if too much additive replaces a part of the daily feed ration (Hansen et al., 2012). The effect of biochar compounds on eating behaviour and palatability would need to be evaluated. The resources needed to test *in vivo* biochar compounds were beyond the resources available for this study.

2.6 FUTURE WORK

Further *in vitro* experiments would be needed in order to confirm these findings and to gain insight into the mechanism of action of biochar compounds. Future *in vitro* experimental design should include the use of different biochar compounds with larger surface area, a wider range of biochar level of inclusion, incubations with different substrates, study of substrate degradability and/or profile of gas produced in the *in vitro* tests with longer incubation time (24-48 h).

CHAPTER 3



Effect of encapsulated nitrate and elemental sulphur on methane production, rumen fermentation and nitrogen metabolism in beef cattle

Chapter 3.

3.1 Introduction

Nitrate supplements have been extensively investigated and recognised as promising additives for reducing rumen CH_4 production, and also providing a valuable source of non-protein nitrogen (NPN) for ruminant diets (Lee and Beauchemin, 2014; Leng, 2008). Nitrate has been tested showing similar results in feed intake and production in ruminants compared with urea *in vivo* (Lee and Beauchemin, 2014; Helmer and Bartley, 1971; van Zijderveld et al., 2011a; Velazco et al., 2014; Guo et al., 2009; Nguyen et al., 2015; Nolan et al., 2010; Sophal et al., 2013) and *in vitro* studies have reported that MPS was superior in the presence of nitrate compared to urea (Guo et al., 2009; Tillman et al., 1965).

Nevertheless, a toxic effect of nitrate-containing feeds has been observed for many years (Lewis, 1951; Wright and Davison, 1964), constraining its use as a feed additive (Alaboudi and Jones, 1985; Allison and Reddy, 1984; van Zijderveld et al., 2010). To attenuate the negative effects of nitrate supplementation, several strategies have been proposed, such as gradual acclimatisation of animals to nitrate consumption (Li et al., 2012; Nolan et al., 2010; van Zijderveld et al., 2011a; Velazco et al., 2014), and the use of sulphur compounds in combination with nitrate (Takahashi et al., 1998; van Zijderveld et al., 2010). Nevertheless, the combination of strategies for lowering CH_4 emissions and reducing nitrate toxicity have scarcely been investigated.

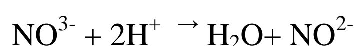
Urea is generally considered a low cost product, readily available and easy to use, and is a valuable source of NPN for ruminants (Helmer and Bartley, 1971). Non protein nitrogen sources could be an economical replacement for part of the protein in feedlot rations, since ruminant microbiota are able to convert NPN into protein (Allison, 1969; de Almeida Rufino et al., 2016). Most studies have used urea as a NPN source in control diets to test the effect of nitrate on CH_4 emissions and N metabolism (Lee et al., 2015b; Lee and Beauchemin, 2014). However there is little

information about nitrate containing diets, where nitrate replaces true protein rather than urea. The independent effect, mode of action and application of each additive will be explained in detail below.

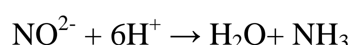
3.1.1 Nitrate

Nitrate is a natural component of crude proteins in forage consumed by ruminants, but is rarely present in cereal grains and protein concentrates. Growth stage influences the quantity of nitrate present in forages, *i.e*. higher in young plants compared with mature ones. Non-natural sources of nitrate are commonly available as commercial fertilizers in the form of nitrate salts. Research has been carried out using nitrate salts as a source of nitrogen, alternative to urea, in ruminant diets and the feeding of nitrate has been associated with inhibition of methanogenesis (Leng, 2008).

Different metabolic pathways for nitrate assimilation in ruminant are known. Dissimilatory and assimilatory nitrate reduction are the predominant nitrate reduction pathways in the rumen with NH_3-N as the end product. Dissimilatory reduction of nitrate is unaffected by the presence of NH_3-N in the culture, and rapid conversion of nitrate to NH_3-N occurs even at high concentrations of NH_3-N (Qingxiang et al., 2011). Dissimilatory nitrate reduction is suggested to be the dominant pathway of nitrate metabolism in the rumen (Leng, 2008). The primary function of nitrate appears to be to re-oxidize reduced pyridine nucleotides (e.g. nicotinamide adenine dinucleotide (NADH) which limit the growth rate of microorganisms (Leng, 2008). Nitrate reduction to NH_3-N is a more favourable pathway than the reduction of CO_2 to CH_4 (Lee and Beauchemin, 2014). The two steps in dissimilatory nitrate reduction are:



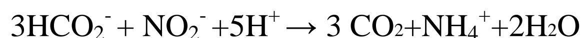
$$NO^{3-} + 2H^{+} \rightarrow H_2O + NO^{2-}$$



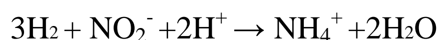
$$NO^{2-} + 6H^{+} \rightarrow H_2O + NH_3$$

Assimilatory nitrate reductase involves enzymes that catalyse the reduction of nitrate to nitrite then to NH_3-N. For this pathway nitrate is reduced to nitrite by NADH reduction and nitrite is reduced to NH_3-N by assimilatory nitrite

ammonification producing ATP. High NH_3-N concentrations have an inhibitory effect on the assimilatory process. Formate and H_2 are the common electron donors in assimilatory nitrite ammonification. These substrates are oxidized according to the following equations:

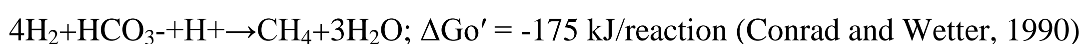


$$3HCO_2^- + NO_2^- + 5H^+ \rightarrow 3\ CO_2 + NH_4^+ + 2H_2O$$

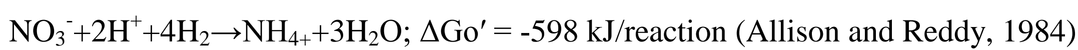


$$3H_2 + NO_2^- + 2H^+ \rightarrow NH_4^+ + 2H_2O$$

The two processes, methanogenesis and nitrate reduction to NH_3-N, depend on electron sources such as hydrogen, but the reduction of nitrate to NH_3-N yields more energy than the reduction of CO_2 to CH_4 (van Zijderveld et al., 2010a) as demonstrated below. G is defined as the free energy change between reactants and products. A given population of cells, metabolising a substrate under conditions with a defined ΔG, will produce more biomass than another population metabolizing the same substrate via a pathway with a less negative G (Janssen, 2010):



$4H_2$+HCO_3-+H+→CH_4+$3H_2O$; $\Delta Go' = -175$ kJ/reaction (Conrad and Wetter, 1990)



NO_3^-+$2H^+$+$4H_2$→NH_{4+}+$3H_2O$; $\Delta Go' = -598$ kJ/reaction (Allison and Reddy, 1984)

Reduction of CH_4 production from domestic ruminants with the addition of nitrate to diets has been shown both *in vitro* (Allison and Reddy, 1984; Iwamoto et al., 2001; Jones, 1972; Lin et al., 2011; Patra and Yu, 2013) and *in vivo* (Allison et al., 1981; El-Zaiat et al., 2014; Hulshof et al., 2012; Newbold et al., 2014; Patra and Yu, 2013; Soissan et al., 2014; Sophal et al., 2013; Troy et al., 2015; van Zijderveld et al., 2011a; Velazco et al., 2014; Veneman et al., 2015). The hydrogen sink is considered the main mechanism whereby nitrate reduces rumen CH_4 production. Nitrate lowers CH_4 production (yield) increasing bacteria in the rumen, that use nitrate as a terminal electron acceptor capturing H_2, with less H_2 available for methanogenesis. Other mechanisms for lowering CH_4 production have been proposed (Yang et al., 2016): *e.g*, nitrite toxicity could affect the number of methanogens and/ or alter the metabolism of hydrogen producers (Marais et al., 1988). The CH_4 mitigating effect of nitrate seems to be consistent and persistent over time (El-Zaiat et al., 2014;van Zijderveld et al., 2011a;Guyader et al.,

2016).Theoretically, each mole of nitrate reduced could reduce CH_4 production by one mole (Ungerfeld and Kohn, 2006), 16g or 22.4 L of CH_4 (van Zijderveld et al., 2010). Stoichiometrically, 100 g of dietary nitrate reduced to NH_3-N in the rumen should lower CH_4 emissions by 25.8g.

However, in most studies, supplementary nitrate did not achieve the theoretical maximum CH_4 mitigation effect (Table 3.1). The efficacy of CH_4 mitigation in comparison to the control ranged from 95% (El-Zaiat et al., 2014) to 49% (Newbold et al., 2014). The reasons for the apparent inefficiency of nitrate as an anthimethanogneic agent are the excretion of nitrate and nitrite in the urine, changes in ruminal ecology related to nitrate addition that stimulates additional H_2 production and nitrate stimulates formate production by methanogens that is converted to H_2 (Leng, 2014).

Moreover, the efficiency of CH_4 mitigation decreases as nitrate ingestion increases (Lee and Beauchemin, 2014; Leng, 2014). The relationship between nitrate dose and CH_4 reduction have been shown to be curvilinear, but as the rate of CH_4 production is not known, the most effective nitrate dose could not be estimated and would strongly depend on diet (Leng, 2014). There is a balance between the amount of N from nitrate that could be utilised by rumen microbiota and the potential reduction in CH_4 production that can be achieved. In addition, different nitrate salts have been shown to differ in nitrate availability thus affecting the CH_4 reduction potential and toxicity. Calcium nitrate seems to be less effective compared to sodium and potassium nitrate, probably due to lower solubility of calcium (Latham et al., 2016).

The reduction in CH_4 emissions would predict an overall benefit in energy metabolism by the animal, if the energy lost with CH_4 production is conserved in microbial biomass. However, an improvement in performance or production with dietary nitrate inclusion has not been detected (van Zijderveld et al., 2011a; Hegarty et al., 2016), which implies that using nitrate to lower CH_4 emissions may not re-direct additional metabolizable energy towards animal production (Lee and Beauchemin,2014).

Table 3.1 Effect of nitrate addition on *in vivo* CH_4 emission (summary of studies)

Author	Species	NO_3 intake (g/kg DM)	Control diet (Forage:Concentrate)	Control Nitrogen	CH_4[1] (% control)	CH_4[2] (% control)
(Ascensao, 2010)	Steers	28	16:84	Urea	42	
(Alaboudi and Jones, 1985)	Sheep		50:44	?		
(El-Zaiat et al., 2014)	Sheep	27	40:60	Urea		95
(Hulshof et al., 2012)	Steers	22	60:40	Urea	27	87
(Newbold et al., 2014)	Steers	30	65:35	Urea	28	49
(Nolan et al., 2010)	Sheep	40	Hay	Urea	23	78
(Lee et al., 2015b)	Heifers	25	55:45	Encapsulated Urea	21	82
(Li et al., 2012)	Sheep	30		Urea	35	97
(Lund et al., 2014)	Dairy cattle	20	55:45	?	31	
(Soissan et al., 2014)	Sheep	20	Oaten chaff	Urea	15	72
(Sophal et al., 2013)	Cattle	50		Urea	43	
(Troy et al., 2015)	Steers	21	50:50	Rapeseed meal	17	80
	Steers	21	8:92	Rapeseed meal	0	0
(Velazco et al., 2014)	Steers	26	20:80	Urea	17	78
(van Zijderveld et al., 2010)	Sheep	26	90:10	Urea	32	89
(van Zijderveld et al., 2011a)	Dairy cattle	21	66:34	Urea	16	59

[1] % Decrease in CH_4 yield (g/kg DMI) with nitrate inclusion compared to control

[2] % CH_4 reduction observed from theoretically expected (1 g of nitrate reduced could reduce CH4 production by 0.26 g)

However, nitrate can have negative effects on animal productivity. A potential negative effect of nitrate supplementation to ruminants is nitrite toxicity. In the rumen, nitrate is quickly converted to nitrite which, can be absorbed across the rumen wall and converts blood haemoglobin (Diaz et al., 2009) to methaemoglobin (MetHb). MetHb is unable of carrying O_2 and therefore toxic for the animal (Lee and Beauchemin, 2014). In ruminants the normal physiological concentration of MetHb is less than 1% total Hb (Godwin et al., 2014). A level of MetHb above 20 % total Hb (for both sheep and cattle) is considered to be a risk for toxicity (Cockrum et al., 2008). Methaemoglobinemia could reduce productivity and in severe cases lead to the death of the animal. Other negative effects of nitrate consumption are that nitrite acts as a vasodilator which could cause a fatal reduction in blood pressure, and that nitrite reduces the conversion of carotenoids to vitamin A, thereby causing a vitamin A deficiency (Marais et al., 1988; Mehmet Ozedmyr, 2014).

The addition of nitrate to ruminant diets has been shown to decrease DMI in some studies (Hulshof et al., 2012; Newbold et al., 2014; Hegarty et al., 2016). The negative effects on DMI can be an indicator of nitrate poisoning or reduction of organoleptic properties of diets caused by nitrate supplementation (Bruning-Fann and Kaneene, 1993). The effects in DMI from previous studies are reported in Table 3.2. The extent of reduction in DMI varied from 5% in some studies (Sophal et al., 2013), to 16% (Velazco et al., 2014). In most studies DMI was not affected by nitrate inclusion levels above 20 g/kg DM (Ascensao et al., 2010; El-Zaiat et al., 2014; Lee et al., 2015a; Nolan et al., 2010; Soissan et al., 2014; Troy et al., 2015; van Zijderveld et al., 2011b), while there where significant reductions in other studies (Hulshof et al., 2012; Li et al., 2012; Lund et al., 2014; Newbold et al., 2014; Sophal et al., 2013). The discrepancy between studies about the effects of nitrate on DMI and feed pattern could be attributed to different dietary composition, form and level of nitrate, animal species, and feeding method. Sheep have been shown to have the highest tolerance to nitrate poisoning across all ruminants (Qingxiang et al., 2011), because they have the greatest capability of all ruminants to convert methemoglobin back to hemoglobin. Furthermore, feed consumption rate has been reported as one of the factors that affects nitrate poisoning when supplementary nitrate is fed to cattle (Lee and Beauchemin, 2014), and restricted compared with *ad libitum* feeding is

associated with higher nitrate toxicity (Lee et al., 2015b; Soissan et al., 2014). Yet, the effects of nitrate on feed consumption rate and eating behaviour are scarcely reported.

To reduce the possible toxicity of nitrate supplementation, several strategies are proposed. Gradual adaptation to nitrate has been extensively used to decrease nitrite accumulation in the rumen, and therefore lower the risk of methaemoglobinemia. However, adaptation times and doses implemented have been variable, and different degrees of success have been observed between studies. The length of adaptation time to nitrate supplementation varied from 28 days (El-Zaiat et al., 2014; van Zijderveld et al., 2010; van Zijderveld et al., 2011a; Velazco et al., 2014) to 7 days (Li et al., 2012) (Table 3.2). Most of the studies have not recorded toxic MetHb levels after a period of adaptation (Alaboudi and Jones, 1985; El-Zaiat et al., 2014; Li et al., 2013; Nolan et al., 2010; van Zijderveld et al., 2011a; Velazco et al., 2014) (Table 3.2). The rate of reduction of nitrate and nitrite to NH_3-N increase with exposure to nitrate and this is associated with increases in populations of nitrate reducing bacteria (NRB). Allison and Ready et al. (1984) reported that between 3 and 6 days are required for microbial adaptation to nitrate. Therefore, the accumulation of nitrite in the rumen depends to some extent on how long the animal has been accustomed to nitrate in the feed (Qingxiang et al., 2011). Conditions which promote slow release of nitrate in the rumen may reduce the toxic effects of nitrate. Following this idea, slow release forms of nitrate are being developed as one strategy to reduce the potential toxicity of nitrate (Lee et al., 2015b; de Raphaelis-Soissan et al., 2017). The experimental ENP use in this study is protected by an international patent and was manufactured by GRASP Ind. & Com. LTDA (Curitiba, PR, Brazil). The product was manufactured using the double salt of calcium and ammonium nitrate (CAN) decahydrate coated with a controlled-release matrix. The product was designed to release 50, 80, and 100% of nitrate within 4, 12, and 30 h, respectively. The product was composed as follows (% of DM): 86.17% DM in as-fed basis, 14.98% N, 60.83% NO3–, and 17.84% Ca) (El-Zaiat et al., 2014).

Different compounds in combination with nitrate might lower its toxicity and be more effective and practical in mitigating CH_4 emissions from ruminants without

impairing feed digestion (Iwamoto and Asanuma, 1999; Patra and Yu, 2013). For example, the suppressing effects of some sulphur (S) compounds on ruminal nitrate reduction have been reported *in vitro* (Takahashi, 1989) and *in vivo* (Takahashi et al., 1998; van Zijderveld et al., 2010). Sulphur compounds combined with nitrate can act as electron donors in the reduction of nitrite to NH_3-N, reducing nitrite accumulation in the rumen and therefore toxicity of nitrate (Latham et al., 2016; Leng, 2008). This effect is due to the sulphate–nitrate interrelationships in anaerobic environments. Some sulphur-reducing bacteria (SRB) can use nitrate as an electron donor for the oxidation of S and some nitrate reducing bacteria uses nitrite as the electron acceptor to reoxidize the excreted sulphide to sulphate. Some NRB strains are commercially available to avoid nitrite toxicity in cows fed high nitrate forage (Jeyanathan et al., 2013).

The effect of nitrate on rumen fermentation varies between studies (Table 3.3) Some studies have reported no effect in rumen fermentation with nitrate addition (Li et al., 2012; van Zijderveld et al., 2010). Most studies have shown a shift in the VFA profile from propionate to acetate (Hulshof et al., 2012; Nolan et al., 2010; Soissan et al., 2014; Troy et al., 2015). The effects in NH_3-N are variable, as no effects on VFA profile were reported in some studies (Nolan et al., 2010; Soissan et al., 2014; Sophal et al., 2013) whereas a decrease (El-Zaiat et al., 2014; Lee et al., 2015b) or an increase were found in others (Hulshof et al., 2012)(Table 3.3).

Van Zijderveld et al, (2011) showed a persistent reduction in CH_4 production in dairy cattle with nitrate addition to a forage based diet (66:34) over 4 consecutives periods: 24 days plus 17 days of sampling. The persistency of the effect in CH_4 reduction is an essential requirement for any promising antimethanogenic additive and that was the first evidence *in vivo*. However, the reduction in CH_4 production was only 59% of the theoretical potential. The persistency of reduction in CH_4 production over long term studies have been reported by others (Troy et al., 2015; Guyader et al., 2016; Duttie et al., 2017). However, a decrease in fat and protein corrected milk yield was recorded when both nitrate and linseed were added to the diet (Guyader et al., 2016) and a reduction in FCR was observed in nitrate fed animals (Duttie et al., 2017).

Table 3.2 Toxicity effects of nitrate addition (MetHb and DMI) (summary of studies)

Author	Species	NO_3 (g/kg DM)	Adaptation time (days)	Control Nitrogen	DMI (% of control)	% Met Hb (Max)
(Ascensao, 2010)	Steers	28	18	Urea	117	
(Alaboudi and Jones, 1985)	Sheep		14	?		2
(El-Zaiat et al., 2014)	Sheep	27	28	Urea	100	1
(Hulshof et al., 2012)	Steers	22	16	Urea	93	
(Newbold et al., 2014)	Steers	30	25	Urea	93	20
(Nolan et al., 2010)	Sheep	40	18	Urea	100	0.6
(Lee et al., 2015b)	Heifers	25	14	Encapsulated Urea	97	
(Li et al., 2012)	Sheep	30	7	Urea	87	
(Lund et al., 2014)	Dairy cattle	20	1	?	89	
(Soissan et al., 2014)	Sheep	20	14	Urea	99	45
(Sophal et al., 2013)	Cattle	50	14	Urea	95	
(Troy et al., 2015)	Steers	21	28	Rapeseed	100	4
	Steers	21	28	Meal	100	4
(Velazco et al., 2014)	Steers	26	28	Urea	84	1
(van Zijderveld et al., 2010)	Sheep	26	28	Urea	98	7
(van Zijderveld et al., 2011a)	Dairy cattle	21	28	Urea	100	19

Table 3.3 . Effects of nitrate on rumen fermentation (summary of studies)

Author	Species	NO_3 (g/kg DM)	Adaptation days	Days nitrate	Control Nitrogen	RF Sampling[1]	$N\text{-}NH_3$ concentration	TotalVFA. A:P ratio
(Ascensao, 2010)	Steers	28	18	6	Urea			
(Alaboudi and Jones, 1985)	Sheep		14	21	?	2 h		T =, >A
(El-Zaiat et al., 2014)	Sheep	27	28	64	Urea	3 h	<	>T, =A:P
(Hulshof et al., 2012)	Steers	22	16	8	Urea	2h	>	>A:P
(Newbold et al., 2014)	Steers	30	25	8	Urea			
(Nolan et al., 2010)	Sheep	40	18	3	Urea	Day mean	NE	> T, >A:P
(Lee et al., 2015b)	Heifers	25	14	14	Urea Encaps	Day pattern	<	
(Li et al., 2012)	Sheep	30	7	28	Urea	1h before		NE
(Lund et al., 2014)	Dairy cattle	20	1	1	?			
(Soissan et al., 2014)	Sheep	20	14	48	Urea	2,5h	NE	>A:P
(Sophal et al., 2013)	Cattle	50	14	14	Urea	2h	NE	< T
(Troy et al., 2015)	Steers	21	28	84	Rapeseed meal	2h		>A:P
(Velazco et al., 2014)	Steers	26	28	14	Urea			
(van Zijderveld et al., 2010)	Sheep	26	28	7	Urea	24h		NE
(van Zijderveld et al., 2011a)	Dairy cattle	21	28	17	Urea			

[1]Rumen fluid sampling time-hours after feeding
NE=No effect

3.1.2 Urea

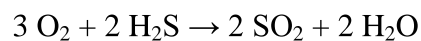
Urea is a cheap source of NPN used to feed livestock. Its use in rations has fluctuated with protein and urea prices, and with mixed to negative experiences in experiments and field situations (Kertz, 2010). Urea can be sprayed onto straw directly, or be available in a mixture with other feeds. The mixture can be given in a liquid or solid form (Schiere et al., 1989). Once ingested, urea is soluble and is hydrolysed in the rumen in 20-60 mins by microbial enzymes to produce NH_3-N, which is converted into MP, thus providing additional protein to the host animal. Ruminal ureolytic bacteria are abundant and urease activity in rumen fluid is consistently high, converting urea to NH_3-N quickly. Using urea as a source of nitrogen for MPS may be more economical than using natural protein sources (Shain et al., 1998). However, microbial populations need readily available energy to use urea efficiently and more often than not, imbalances with the availability of energy from the degradation of CHOs are often observed (Thompson et al., 1972). This fact could impair the use of urea as a feed additive (Alves et al., 2014). Urea is not recommended to exceed 1% of DM in the concentrate, approximately 135 g/cow daily, and not more than 20% of total dietary CP (Kertz, 2010). An excess of urea provided as a source of N for protein synthesis, has been reported to decrease DMI and MPS (Broderick and Reynal, 2009; Holder et al., 2015) and could decrease the reduction of nitrite to NH_3-N, thereby allowing nitrite to accumulate in the rumen (Eryavuz et al., 2003).

In conclusion, reductions in feed consumption when a high level of urea is included in diets and a loss of dietary N resulting from rapid hydrolysis in the rumen have encouraged researchers to seek new ways of improving NPN utilization in ruminant rations (Alves et al., 2014; Guo et al., 2009; Thompson et al., 1972).

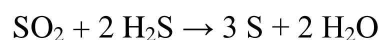
3.1.3 Sulphur compounds

Sulphur(S) is required by all animals due to the important biochemical roles that S-containing compounds have in metabolism such us the production of amino acids (Ammerman and Goodrich, 1983). Ruminants S requirements can be met by supplying either organic (S-containing amino acids in protein) (Malik et al., 2015) or inorganic S (from forages and mineralized salts) (Fron et al., 1990). Rumen microorganisms are able to use inorganic S to synthesize the S-containing amino acids (*e.g*, methionine, cysteine, homocysteine, and taurine) (Sokolowski et al., 1969). Most natural rations fed to ruminants contain adequate S to meet the animal requirements. Water is another source of S that should be taken into account.

Sulphur compounds are mostly metabolised in the rumen (Bray and Till, 1975) by dissimilatory reduction, with the generation of ATP and hydrogen sulphide (H_2S) as an electron sink or by the assimilatory process where S compounds are reduced for incorporation into other organic compounds necessary for cell survival (Ungerfeld and Kohn, 2006). The absorbed sulphide is oxidized to sulphate in blood and liver and is distributed to extracellular fluid. Then, sulphate can be recycled within the rumen, via saliva or go directly to the large intestine. Sulphide and S form a recycling system (Gould, 1998):



$$3\ O_2 + 2\ H_2S \rightarrow 2\ SO_2 + 2\ H_2O$$



$$SO_2 + 2\ H_2S \rightarrow 3\ S + 2\ H_2O$$

Sulphur is a poor electron acceptor for H_2 and the strongest electron acceptor that may compete with methanogenesis is the oxidised form of sulphate. However, oxidation of elemental S under anaerobic conditions has still not been explained. Nevertheless, in the presence of S, some methanogenic archaea produce H_2S by dissimilatory S reduction, while methanogenesis is reduced (Stetter and Gaag, 1983). In these sulphur-reducing heterotrophs, the reduction of S to HS^- is proposed to be a mechanism for the disposal of excess H_2 (Hedderich et al., 1998). However, S is not the best substrate for S respiration because of its low solubility in water

$$H_2 + S^{2-} \rightarrow HS^- + S_{n-1}^{2-} + H^+ \quad \Delta Go' = -31\ kJ/reaction$$

Several bacteria such as *Wollinella succinogenes* can use S for growth using different electron donors including H_2 or formate. In anaerobic environments, the reduction of elemental S to H_2S is stoichiometrically linked to the oxidation of acetate to CO_2 (Oae and Okuyama, 1992).

The potential toxicity of S compounds is the major limiting factor for its diet addition. Absorbed sulphate could result in a metabolic acidosis and may have a detrimental effect on average daily gain, feed intake, and net energy value of the diet (Drewnoski et al., 2014; Zinn et al., 1997). Excess sulphide while required for MPS, could be toxic. The low solubility of sulphide facilitates its transfer into the rumen gas phase. Hydrogen sulphide gas can be eructed, inhaled or absorbed through the lungs. Sulphur-induced polioencephalomalacia (S-PEM) is attributed to the production of H_2S gas in the rumen (Barton et al., 2006; Gould, 1998). The toxicity of S is influenced by the method of administration and roughage level in the diet (Drewnoski et al., 2012). Cattle on high-roughage diets compared to high-concentrate diets were more tolerant to dietary S and therefore, presented less risk of S-PEM (NRC, 2005). This could be explained by an increase in H_2S with low rumen pH in presence of concentrate diets. NRC (2005) define the maximum tolerable concentration of dietary S at 3g/kg DM for diets containing at least 85% concentrate, and a concentration of 5 g/kg DM, for diets containing more than 40% forage. At higher levels, reductions in performance may be expected. A strong interaction between nitrate and S utilization in the rumen has been observed. As microorganisms need S to utilize nitrogen for protein synthesis, the NRC (1984) recommends that dietary S allowances are based on a knowledge of N:S ratio in MP (Zinn et al., 1997). The optimal ratio between N and S is between 9:1 and 16:1 (Silva et al., 2014). A greater ratio between N and S available for the animal, must result in a waste of dietary N by ruminants (Bird, 1972), as microorganisms may not be able to fix all the N provided. In addition, the source of S used has an effect on the availability of S for MPS (Fron et al., 1990; Johnson et al., 1971; Kahlon et al., 1975; McSweeney and Denman, 2007). Supplementation of the diets of ruminants with S compounds increased the use of N by rumen bacteria, decreasing the accumulation of nitrite (Takahashi, 1989).

As explained before, previous research has focused only on comparisons between nitrate and urea, with few comparisons between nitrate replacing sources of true protein. To date, no study has compared treatments with true protein, urea, and nitrate and the combination of each NPN with added sulphur.

3.1.4 b Aim of the study

The primary objective of this study was to examine the effects of encapsulated nitrate in steer diet on enteric CH_4 production and nitrate toxicity. The secondary objective was the study of the effect of encapsulated nitrate on nutrient digestibility, N utilization and MPS. In addition, the additive effect of inorganic S with NPN sources on enteric CH_4 emission, and MPS was assessed.

The hypotheses of the present study were:

- Partial replacement of true protein, or urea by encapsulated nitrate could reduce CH_4 emissions without impairing N utilisation.
- Elemental S above requirements could reduce nitrate toxicity, improve N utilisation and further decrease CH_4 production.

3.2 MATERIAL AND METHODS

The protocol used in this experiment was in accordance with guidelines of the Brazilian College of Animal Experimentation (COBEA – Colégio Brasileiro de Experimentacão Animal) and approved by the Ethics, Bioethics, and Animal Welfare Committee (CEBEA – Comissão de Ética e Bem Estar Animal) of the School of Veterinary (Faculdade de Ciências Agrárias e Veterinárias (FCAV)), UNESP, Jaboticabal campus. All experimental procedures were made available by SRUC's Animal Welfare and Ethical Review Body, the Animal Experiments Committee.

3.2.1 Experimental design, Animals and Diets

This experiment was conducted at Setor de Avaliação de Alimentos e Digestibilidade da FCAV, UNESP, Campus Jaboticabal, São Paulo, Brazil, from September the 4th, 2015, to December the 22nd, 2015.

3.2.1.1 Experimental design

The experiment lasted 106 days and was divided into five 21 day periods (P). Each period consisted of 14 days for adaptation to diets and 7 days for measurements.

3.2.1.2 Animals

Five crossbred Angus x Nelore steers each fitted with a permanent rumen cannula were used in a 5x5 Latin square design. Animals were weighed individually before the start and at the end of the experiment. At the beginning of the experiment, animals were approximately 15 months old. Mean initial and final body weights were 327 ± 20.1 and 423 ± 41.3, respectively (mean±SD) and a mean live weight gain of 0.9± 0.2 kg/day was recorded. Body weights were used to determine the feed allocation. The animals were fed the assigned dietary treatments at 07:00 h each day. The animals were housed individually in outdoor pens of 9 m^2 and clean water was provided *ad libitum* (Figure 3.1).

Figure 3.1 Angus x Nelore steer in its corresponding experimental unit

3.2.1.3 Diets

The chemical composition of the ingredients and details of formulation (g/kg DM) are presented in Tables 3.5 and 3.6, respectively. Chemical analysis of the ingredients was performed before the start of the experimental period. Diets were formulated according to the Brazilian Tables of Nutrient Requirements for mixed breed beef cattle—BR CORTE system (Valadares Filho et al., 2010) to achieve an average daily gain (ADG) of 1.2 kg. Diet formulations were performed using theoretical chemical composition of ingredients and were formulated to be isonitrogenous (CP~150g/kg DM). Crude protein content of diets was above the level recommended for finishing beef cattle (National, 1996). Throughout the adaptation periods (days 1 to 14), the quantities of food offered were adjusted to allow approximately 100g/kg surplus in relation to the total consumed the previous day. During the measurements period (days 15 to 21) diets were adjusted to provide 90% of voluntary feed intake (VFI, restricted feeding) on a DM basis, to promote complete consumption of the offered rations.

The feed ingredients were obtained from local suppliers (Agromix, Jaboticabal-SP, Brazil). The basal diet consisted of Tifton 85 hay (hybrid of *Cynodon dactylon*) as roughage (Figure 3.2 a) and a concentrate mixture in a 500:500 ratio

(g/kg, DM basis). After collection, the Tifton 85 hay was chopped into lengths of about 20-30 mm on site with a forage chopper and stored in a dry, covered and ventilated place before daily use. The concentrate was composed of soya-bean meal and ground maize. The ingredients for the concentrates were ground in a hammer mill fitted with a 2 mm screen. The concentrates were mixed and a mineral-vitamin supplement (BELLNUTRI 100g/kg, Multi Tec, Jaboticabal,SP, Brazil) was added to the mixture (Figure 3.2.b). Steers were randomly allocated to five different dietary treatments (Table 3.4).

Table 3.4 Experimental treatments

Treatment	Treatment specifications
1) Control	Control, True protein (soya-bean meal)
2) Urea	Urea (8 g/kg DM)
3) Urea +S	Urea (8g/kg DM) +Elemental Sulphur (2.4 g/kg DM)
4) Nitrate	Encapsulated Nitrate (20 g/kg DM, 14.3 g nitrate /kg DM)
5)Nitrate +S	Encapsulated Nitrate (20g/kg DM) + Elemental Sulphur (2.4 g/kg DM)

Three concentrate premixes were prepared according to the diets formulated: mixture 1, for the control diet treatment, mixture 2, for urea and urea +S diets, and mixture 3, for the nitrate and nitrate + S diets. Encapsulated nitrate (EN) and/or elemental S were weighed and incorporated manually daily into the appropriate concentrate mixture according to treatments. For the control treatment the main source of protein was soya-bean meal. The urea and EN added for treatments 2 and 3, and 4 and 5, respectively, replaced part of the soya-bean meal in the rations. The inclusion level of urea on treatments 2 and 3 was 8g/kg of DM. The inclusion level of EN on treatment 4 and 5 was 20 g/kg DM, equivalent to 14.3 g nitrate/kg DM. The level was increased by 25 % of EN final dose (20 g/ kg DM) every 4 days during the adaptation period, to avoid possible nitrate toxicity (Table 3.7). The source of nitrate used was EN (Lee et al., 2015b). Elemental S (2.4 g/kg DM) was added to

urea (treatment 3) and EN (treatment 5). Encapsulated nitrate was supplied by GRASP Ind. & Com. LTDA, (Curitiba, Parana, Brazil) (Figure 3.2 c). Elemental S was supplied by Brasil Quimica Ind. (Batatais, SP, Brazil). For diet formulation, as NPN sources replaced part of the true protein of the diets, the net energy content decreased and the amount of fermentable CHO provided by soya bean meal became lower. To maintain diets energy content, the amount of maize, which is high in energy and low in proteins, provided was increased.

The S additive selected for this experiment was elemental S (sulphur flowers), which has been shown to be suitable as a S supplement for cattle (Silva et al., 2014), also increasing N retention (Sokolowski et al., 1969), and was readily available. The S content of the control (soya bean meal diet), urea and nitrate diets was approximately 3 g/kg DM, and S added diets provided a total of 5.1 g S/kg DM (Table 3.6). Therefore, the S level in the control diet was within the range recommended by NRC 1996 for finishing cattle (1-4 g/kg DM) (National, 1996). The S containing diets had a N:S ratio of 5:1 (40 g/S day) and the diets without additional S 9:1(22 g/S day). All diets met requirements for RDP (734 g/d).

Table 3.5 Chemical composition of diet ingredients (g/kg DM)

Ingredients	Dry Matter	Organic Matter	Ash	Crude Protein	NDF	EE
Tifton 8 hay	928	930	70	125	705	15
Soya-bean meal	939	933	67	508	264	17
Maize	920	987	13	95	203	40
Urea	990			2890		
EN[1]	847			907		
Elemental sulphur	100					

[1]Encapsulated nitrate was manufactured by GRASP Ind. & Com. LTDA and EW|Nutrition GmbH; 17.6% N, 19.6% Ca, and 71.4% NO3− on a DM basis. The source of nitrate was the double salt of calcium ammonium nitrate decahydrate [$5Ca(NO_3)2 \cdot NH_4NO_3 \cdot 10H_2O$]. Chemical Analysis gave different value for N content 14.6%
NDF: Neutral Detergent Fibre

Table 3.6 Experimental diets formulation and chemical composition (g/kg DM)

	Treatments (g/kg)				
	Control	Urea	Urea +S	Nitrate	Nitrate +S
Tifton 85 Hay	500	500	500	500	500
Soya-bean meal	100	40	40	40	40
Maize	370	427	425	415	413
Urea		8	8		
EN				20	20
Elemental Sulphur			2.4		2.4
Mineral-Vitamin[1]	25	25	25	25	25
Chemical composition					
DM	928	927	925	925	923
Organic matter	928	923	921	912	909
CP	151	146	146	140	140
UDP[2]	90	92	92	86	86
DUP[2]	61	54	54	54	54
NDF	446	441	440	438	438
NO_3				14.6	14.6
S	2.9	2.7	5.1	2.7	5.1
NPN:N total	0.0:24.1	3.7:23.3	3.7:23.3	2.9:22.4	2.9:22.4
N:S	9:1	9:1	5:1	9:1	5:1

[1]Composition of the mineral mixture(Ca - 160 g; P - 40 g; Mg - 5 g; S - 40 g; N- 160 g;Cu' 945mg Mn - 730 mg; Zn - 3,500 mg; I - 70 mg; Co - 56 mg; Se - 18 mg; F (max.) - 400 mg).
[2] Calculated from ingredient values from Valadares Filho et al., 2010.

Figure 3.2 Feed ingredients of diets

a) Tifton 85 hay b) Concentrate mixture c) Encapsulated nitrate

3.2.2 Sampling and Measurements procedures

Sampling days and adaptation to nitrate diets is detailed on Figure 3.3 and Table 3.7.

3.2.2.1 Feed sampling and intakes

Feed offered and refusals were recorded daily for the entirety of the trial. Dry matter contents of ingredients were used to adjust the daily feed offered from previous consumption (the day before). Samples of Tifton hay and concentrate mixture offered were collected, stored and subsamples were taken for chemical analysis.

Fresh feed intake in the first 3 h after the morning feed was measured on days 3, 6, 9, and 12 of adaptation to evaluate if the treatments had an effect on palatability and eating behaviour; feed remaining in feed bunks at 3 h after feeding was collected, weighed and then placed back in the feeders.

During the measurement period, samples from the refusals were collected and composited. The samples were dried for 72h at 55° C in a forced air oven, and ground in a Wiley mill (1 mm screen). Dried and ground samples were stored at room temperature until chemical analysis.

3.2.2.2 Blood sampling

Blood samples (5 ml) were taken 3 hours after feeding on days 1, 4, 7, 10 and 14 from the jugular vein, using BD Preset^{TM} safety blood gas syringes. Animals were conducted to the handling crush and blood sampling from all experimental animals was done within 30 mins. Each sampling day coincided with the day of increase in EN dose to test the possible toxicity of nitrate

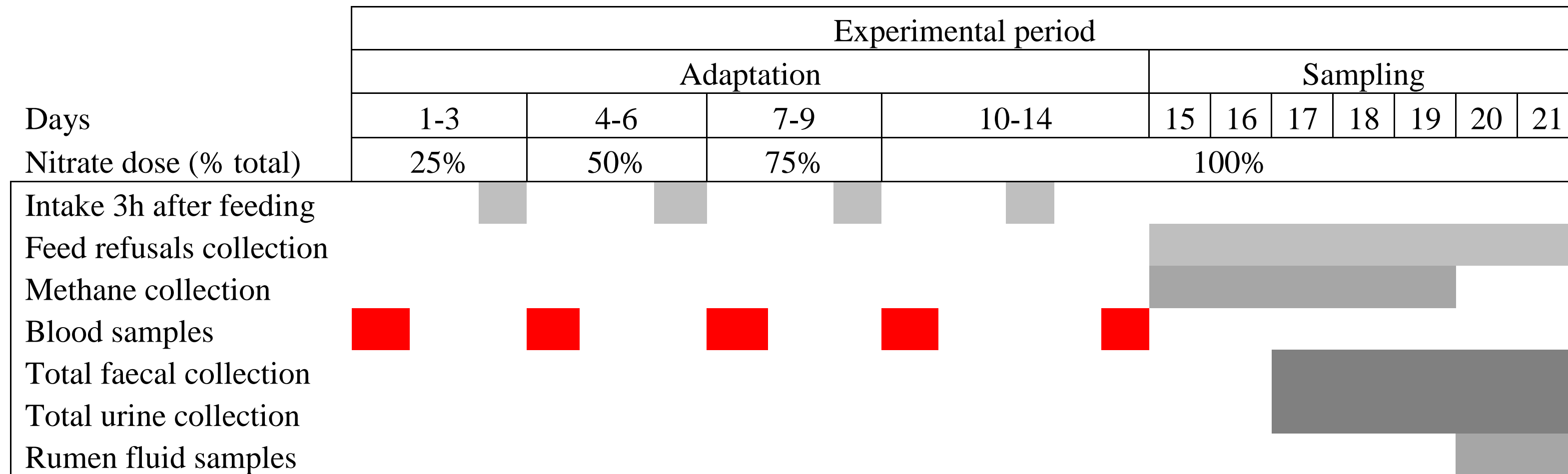


Figure 3.3 Experimental timetable

Table 3.7 Experimental timeline used to gradually increase dietary encapsulated nitrate (EN) fed to steers with ad libitum intake

Day	EN(% of DM)	Total NO_3^-(% DM)	NO_3 (g/kg DM)
1 to 3	0.5	0.4	3.7
4 to 6	1.0	0.8	7.3
7 to 9	1.5	1.2	11.0
10 to 14	2.0	1.5	14.6

3.2.2.3 Methane gas collection

Methane production was measured from individual steers for five consecutive days during the measurement period (days 15 to 20). Methane concentration was determined using the sulphur hexafluoride tracer gas technique (SF6).

Permeation tubes: the source of SF6

The permeation tubes were filled with SF_6 gas and calibrated to determine the release rate. The number of tubes filled exceeded the number required for the experiment to allow further selection of those with standardised emission rates. SF_6 release rate was determined through serial weighing every 5 days over 6 weeks, at a controlled temperature of 39°C. The permeation tubes selected had release rates from 0.35 to 1.22 mg SF_6/day. The pre-calibrated permeation tubes filled with SF_6 were placed into the rumen through the cannula 7 days before the beginning of the first measurement period, to ensure that SF_6 concentration had reached equilibrium in the rumen.

Sampling systems

Air filters, flow restrictor capillary tubes and quick connect stem assemblies were used to connect the sampling point (just above the animal nostrils) to the collection vessel (PVC canisters). The steel capillary tubes were tested before sampling to assure constant flow rates. The capillary tubes were connected to canisters and the pressure was measured every 24 h for three days. The air flow rates desired were 0.45 to 0.55 ml/min. When the capillary tube air flow was slightly different from desired, the capillary tube was adjusted by crimping, changing the diameter and thus the air flow. New restrictor capillary tubes were created simply by cutting additional pieces to the set length. Capillary tubes were mechanically protected from bending, with plastic tubes. PVC canisters (both V- or U-shaped) were used for air collection. The canisters were tested before sampling period, to avoid unexpected leaks, by evacuating them, recording the initial vacuum (>90 kPA) using a digital meter, and measuring the remaining vacuum 48 h later. If the vacuum

has fallen below 87 kPA, the canister was rejected because assumption of leaks. Where possible canisters were sealed and reused.

Sampling procedure

Prior to sample collection, the canisters were purged, with air, to ensure that the evacuated vessels used were free of residual CH_4 and SF_6 from their previous use; three repeated evacuation and flushing of canisters were done with mechanical vacuum pump (Berndt et al., 2014) (Figure 3.4 a). The initial vacuum of the canisters was maximised (93.8 kPA). Animals were trained to wear halter and canisters for three days prior to experimental period. Halters and polyvinyl yokes were fitted on the animal´s head on the first day of measurement. A pre-evacuated PVC canister was placed above the animal's neck daily immediately before feeding and connected to the halter (Figure 3.4 b). The initial pressure of the canisters was recorded prior to connection to the halter. Every CH_4 collection day, two extra canisters were placed in the area nearby the animals to correct for daily background (blanks). Expired gases were collected continuously for 24 hours into evacuated PVC canisters. After 24 hours the canisters were disconnected and pressure measured. Starting and final time points were recorded. Any leaking, broken or blocked apparatus detected during collection were replaced. Three days (of total of five) of measurements per animal was the minimum accepted as successful sampling. Accordingly, when the collection failed more than two days in one animal, the collection was repeated for an extra day. Common causes of sampling failure were blockage of the air inlet by particles of feed, soil or drinking water, and breakage of canisters.

Figure 3.4 a) Steer wearing halter and canisters **b) PVC Canister and connections, attached to a mechanical vacuum pump**

Analysis of breath samples

After gas samples had been collected two sub-samples were analysed to determine CH_4 and SF_6 concentrations by gas chromatography (Vlaming et al., 2007). The method followed for sub-sampling was over-pressurising each collection canister by diluting with high-purity N, recording the pressure so that successive sub-samples could be pushed out by the pressure. Three certified gas mixtures were used as standards to interpret the results from the gas chromatography (Tang et al., 2013). Sample analysis immediately followed analysis of the three standards. After mounting a canister, the sample loop opened and a diaphragm pump connected to the GC exhaust was turned on, drawing the sample into the GC at ~30 ml/min for the 30 seconds the sampling loop was open. The pump was turned off after the sampling loop has closed. Run time per sample was three minutes. After each sampling period, canisters were analysed sequentially until all samples were analysed. Duplicate samples from each canister were analysed. Mean of duplicates were used for calculations. The variation between CH_4 areas from canister sub-samples should be less than 5%. If samples were > 5%, another subsample from that canister was analysed. Output from the gas chromatographs was processed by the computerized data acquisition system associated with each chromatograph according to Johnson et al., (2007). This provided an integrated area under each individual peak in the chromatogram. Peak identities were assigned by comparison to a chromatogram obtained from standard runs with CH_4 and SF6 (Johnson et al., 2007).

3.2.2.4 Faeces and urine collection

Total daily faecal and urine collections were carried out for five consecutive days from individual animals (days 16 to 21) during the measurement week of each period. Faeces excreted were collected into plastic containers for 24 hours. At the end of each sampling day, faeces collected were weighed, mixed and sub-sampled. The samples were then dried for 72 h at 56ºC in a forced air oven. Samples were composited proportionally to daily DM output by animal and period. Samples were ground in a Wiley mill to pass through a 1 mm screen, and stored at room temperature until chemical analysis.

Total daily urine was collected by urine funnel collectors attached to the body of the animal. The funnel collectors were connected to a flexible polyethylene tube, which directed the urine to lidded plastic containers containing 200 ml 20% sulphuric acid (H_2SO_4) to ensure pH remained acid and avoid loss of nitrogenous compounds. At the end of each 24-hour collection period, the total weight and volume of urine excreted was determined. Aliquots of approximately 50 ml undiluted urine were collected and composited by period and steer and stored at -20ºC for subsequent analysis (total N, DM, creatinine and uric acid). Another aliquot of 10 ml was collected daily, diluted 1:5 (v/v) with 1.36 M H_2SO_4 for allantoin analysis. Samples were stored at -20ºC.

3.2.2.5 Rumen content sampling

On day 20 of each period, rumen contents samples were collected from each steer manually via the rumen cannula at 0, 2, 4, 6, 12, and 24 h after feeding. The samples were filtered through four layers of gauze. Three aliquots were collected (around 20 ml) and stored at -20ºC, two for VFA and NH_3-N analysis, and one for any additional analysis needed. The pH of the rumen fluid was measured immediately after samples were obtained using an electric portable pH meter (Nova Técnica, PHM, Piracicaba, SP).

3.2.3 Laboratory analysis

Diet, faecal and refusal samples were analysed for DM by drying at 105º C for 24 h and ash was determined by combustion at 525 º C for 6 h according to AOAC (1990). Neutral detergent fibre (NDF) content was determined following Van Soest et al, (1991) and adapted for the ANKOM 200 Fibre Analyser (Ankom Technology, Fairport, NY, USA). Heat-stable α-amylase was included in the NDF solution (Mertens, 2002) and results were corrected for residual ash. Nitrogen concentration in each sample was determined by rapid combustion (850°C). Conversion of all N combustion products to N_2 was performed, and subsequently measurement by thermoconductivity cell (Leco®, model FP-528 N analyser; LECO Corp., St. Joseph, MI, USA) and multiplied by 6.25 to record total CP.

For VFA analysis, rumen fluid samples were thawed and centrifuged for 15 mins at 13,000 rpm at 4º C and 0.5 ml of supernatants were transferred to a vial with addition of 0.1 ml of formic acid as internal standard and quantified by gas chromatography (SHIMADZU® model 20-10, automatic injection) using a capillary column (SP-2560, 100 m × 0.25 mm in diameter and 0.02 mm in thickness, Supelco, Bellefonte, PA) according to the methodology of Johnson et al. (1995). The VFA determined were acetic, propionic, isobutyric, butyric, isovaleric and valeric acids. The total concentration of VFA was calculated as the sum of these VFA.

For determination of NH_3-N concentration, the rumen fluid was made alkaline with KOH and NH_3-N was determined by titration after distillation according the methodology of Fenner et al., (1965) adapted for use in Kjeldahl distillation (TECNAL®, Distiller Nitrogen TE-036/1).

The concentration of allantoin in the urine was determined using colorimetric methods based on those described by Young and Conway (1942) using a UV-spectrophotometer reading at 522 nm (SHIMADZU® UV-1800 Spectrophotometer). The method adopted to measure the uric acid was described by Fujihara et al., (1987) Creatinine concentrations were determined by semi automatic biochemical analyzer (LABTEST® Diagnóstica, Lagoa Santa, MG, Brazil). The yield of total microbial N

from the rumen was calculated using equations of Chen et al. (1990) modified by Orskow, (2004). Whole blood MetHb concentration was determined within 30 min of sampling using a blood gas analyser (ROCHE® Cobas b 123 POC system).

3.2.4 Calculations and statistical analysis

Nutrients consumed (OM, CP, NDF) were calculated according to diet offered and refusals analysed, and total DM consumption. The total DM intake during adaptation was averaged for each steer and period and the percentage of refusals as a proportion of the total ration offered was calculated. Feed consumed in first 3 h after feed offered was expressed as total DMI and as a % of total daily DMI. The percentage and composition of refusals during the measurement week was calculated and analysed. Apparent total-tract digestibility was calculated from nutrients consumed and excreted in faeces during the total collection period (5 days): ([nutrient intake-nutrient output in faeces] /nutrient intake).

Daily CH_4 emissions were calculated from the specific SF_6 permeation rates and CH_4/SF_6 ratio of concentrations in breath samples, after adjustment for background gas concentrations according to the equation shown below. Average DMI for the corresponding period was used to calculate CH_4 yield (g/kg DMI).

$$CH_4\ (g/h) = SF_6\ \text{release rate}\ (g/h) \times [CH_4\ (g/m^3)]\ /\ [SF_6\ (g/m^3)]$$

Total N consumed and excreted by faeces and urine was used to calculate N balance. N retained in the body was calculated by the difference between N intake and N outputs (faeces, urine).

Allantoin and uric acid urinary excretion were expressed as total mmol per day using total daily urine volume. The total purine derivatives excreted were estimated as the sum of allantoin and uric acid. Purine derivatives in urine (mmol/day) were used to estimated total rumen MPS using the equation of Chen for *Bos indicus* (Orskov and Miller, 1988). Efficiency of Microbial N synthesis was calculated as the ratio of Mic N and kg digestible organic matter in the rumen (DOMR):

$$Mic\ N^{1}(g/d) = PD\ absorption\ (mmol/d)*0.726$$

$$PD\ absorption\ (mmol/d) = PD\ excretion\ (mmol/d)-(0.147*MBW^{2})/0.85$$

[1] Mic N=microbial N flow
[2] MBW=Metabolic body weight

Creatinine is an indicator of body weight and varies between animals (XB Chen, 2004) Creatinine concentration [C] is relatively constant from day to day for an animal. Thus the ratio of [PD]/[C] should be independent of urine volume, where creatinine is used here effectively as urine volume marker. The direct [PD]/[C] ratio is linearly correlated with daily PD excretion. PDC index was calculated as follows:

$$PDC\ index= [PD]/[Creatinine]*\ W^{0.75}$$

Total VFA were expressed as the total concentration (mmol/l), and the main VFA both as a concentration (mmol/l) and as a proportion of the total (mol/100 mol).

3.2.4.1 Statistical analysis

The intake, apparent digestibility, N balance, MPS and CH_4 production were analysed as a 5*5 Latin Square using the General Analysis of Variance procedure of GenStat (version 11.1 for Windows; VSN International Limited). The model included the fixed effects of treatments and the random effects of animal and period. In addition, orthogonal comparison contrasts were calculated using the contrast option from the Latin Square analysis. Comparisons were made between the following treatments:

- Control vs. NPN treatments
- Urea treatments vs nitrate treatments
- Presence or absence of S effects
- Interactions between NPN treatments and S addition.

Ruminal pH, VFA and NH_3-N data at time 0 (pre-feed) were analysed as a 5*5 Latin Square. In addition, data for rumen fermentation were analysed with time point as a repeated measure with time point 0 included as a covariate. The model included fixed effects for treatment, time and the interaction between these two variables. Interactions between linear and quadratic effects of time and treatment contrasts defined above were examined to understand the effects of treatment on changes in concentrations of VFA and NH_3-N after feeding. The results are reported as least square means and standard error of mean (SEM) for each treatment. Statistical differences were declared at P values <0.05.

3.3 RESULTS

3.3.1 Adaptation period

3.3.1.1 Blood methaemoglobin

Methaemoglobin values (mean, max and min) for steers receiving nitrate containing diets from day 7 of adaptation are presented in Table 3.8. During the adaptation period, MetHb blood content (% total Hb) was negligible for all steers receiving control and urea treatments diets (0.7 to 1.2 %).

Overall, nitrate supplementation did not affect blood MetHb. Three animals presented slightly increased MetHb values (>6 %) on day 10 of the adaptation period. However MetHb values of two of them decreased to baseline level at the end of the adaptation period (day 14), when only one animal presented an slightly elevated MetHb level (Klug and Reddy, 1984) (Figure 3.5).

Table 3.8 Blood MtHb values (% total Hb) on days 7, 10 and 14 of adaptation. Animals were fed 15 g/kg of EN on day 7 and 20 g/kg of EN on days 10 and 14

	Day 7-15g/kg EN		Day 10-20g/kg EN		Day 14-20g/kg EN	
MetHb (%)	Nitrate	Nitrate+S	Nitrate	Nitrate+S	Nitrate	Nitrate+S
Mean	1	1.3	4.6	2.5	4.0	1.5
Minimum	0.8	0.7	0.9	0.9	1.0	1.1
Maximum	1.4	3.1	13.7	7.2	9.9	2.2

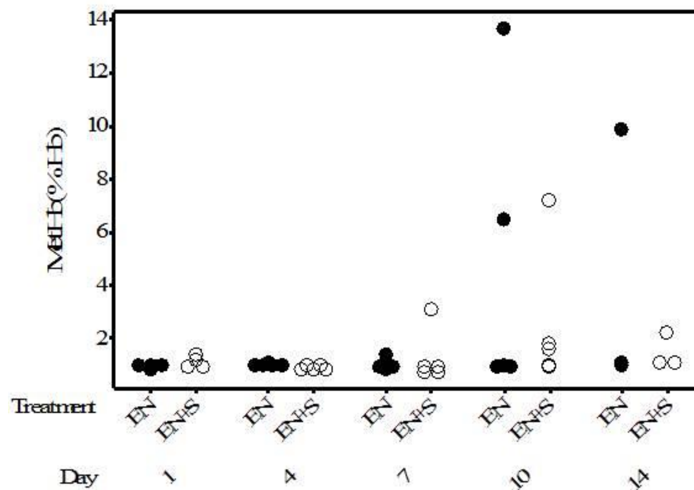


Figure 3.5 Blood MetHb values (% total Hb) for individual steers receiving the nitrate and nitrate +S diets on days 1, 4, 7 and 10 of adaptation period

3.3.1.2 Nutrient intake, digestibility and feed consumption rates

Dry matter intake during the adaptation period was not affected by treatments (P>0.05; Table 3.9). Crude protein intake was lower (P=0.01) with NPN sources than with the control diet and nitrate containing diets had lower CP intakes than the urea containing diets (P=0.02). The quantity of refusals was 111±6 g/kg total daily DM offered and was unaffected by treatments (P>0.05). There was no effect of S addition on intake.

Feed consumption rates for steers during the first 3 h after feeding were obtained from different days during adaptation periods, corresponding to different dietary nitrate levels (Table 3.10). The actual feed consumed and the proportion of total daily feed consumption from 0 to 3 h after feeding were not affected by treatments on day 3 (when 3.7 g/kg of nitrate was provided) (P>0.05). When the level of nitrate inclusion was increased to 7.3 g/kg of DM on day 6 (50% of final dose) a decrease in feed consumption (g /kg DM/d consumed), was observed for nitrate containing diets compared with urea containing diets (P=0.02). However no difference between treatments was observed for feed consumption rate at the end of adaptation when the final dose of nitrate was achieved (14.6/kg; day 12; P>0.05).

Table 3.9 Nutrient intakes and refusals (DM, expressed as a proportion of daily feed offered) from steers fed the different diets: Control (True protein), Urea, Urea + S, Nitrate or Nitrate + S, during adaptation to experimental diets

	Treatments						P- values for Contrasts			
	Control	Urea	Urea+S	Nitrate	Nitrate+S	SEM	Control v NPN	Nitrate v Urea	Added S	NPN * S Interaction
Intake, kg/d										
Dry matter	8.9	8.7	8.4	8.2	8.2	0.27	0.13	0.18	0.63	0.46
Organic matter	8.3	8.1	7.8	7.5	7.5	0.25	0.06	0.09	0.56	0.47
CP	1.3	1.3	1.2	1.1	1.2	0.04	0.01	0.02	0.56	0.48
NDFc[1]	4.4	4.2	4.0	4.0	4.0	0.13	0.04	0.32	0.36	0.32
refusals (g/kg total DM offered)	120	111	104	112	107	1.0	0.64	0.16	0.81	0.39

SEM- Standard error of the mean
[1] Neutral detergent fibre corrected for ash

Table 3.10 Intake the first 3 h after fresh feeding was offered as total intake and as a proportion of the total daily intake by steers fed the different diets: Control (True protein), Urea, Urea + S, Nitrate or Nitrate + S, during adaptation to experimental diets

	Treatments						P- values for Contrasts			
3h after feeding*	Control	Urea	Urea+S	Nitrate	Nitrate+S	SEM	Control v NPN	Nitrate V Urea	Added S	NPN * S Interaction
Actual feed consumption, kg DM										
Day 3-25% Nitrate	4.7	5.1	4.8	4.5	4.3	0.42	0.90	0.21	0.65	0.93
Day 6-50% Nitrate	3.4	4.2	3.9	3.3	3.3	0.27	0.39	0.02	0.62	0.48
Day 9-75% Nitrate	4.8	3.6	4.4	3.4	3.0	0.38	0.03	0.07	0.56	0.14
Day 12-100% Nitrate	3.5	3.9	3.4	2.9	3.4	0.44	0.75	0.28	0.94	0.28
Proportion of feed consumption, % total consumed										
Day 3-25% Nitrate	53.7	50.2	51.1	49.1	50.8	4.06	0.47	0.88	0.76	0.92
Day 6-50% Nitrate	42.6	48.5	45.7	35.3	41.5	3.27	0.96	0.02	0.61	0.20
Day 9-75% Nitrate	44.4	45.6	48.4	38.6	43.8	4.74	0.95	0.26	0.42	0.80
Day 12-100% Nitrate	36.4	46.6	34.7	32.5	41.4	4.73	0.65	0.45	0.75	0.05

SEM- Standard error of the mean
*Measurement days corresponded with 2 days after nitrate inclusion level was increased by 25% of final dose

3.3.2 Sampling period

3.3.2.1 Feed intake and refusal composition

During the sampling period, the feed offered was restricted to provide 90% of voluntary feed intake. Consequently, the amount of refusals was negligible (39±17.5 g/kg feed offered). The composition of refusals was compared with diets formulated within each treatment (Table 3.11). Refusals DM, OM, NDFc were less or tended to be less than feed offered in all treatments, except in urea +S diet, where no difference were found. No differences were found in CP content between refusals and feed offered in any of the diets.

3.3.2.2 Enteric methane emissions

Daily enteric CH_4 production (g/day) and yield (g/kg DMI) are presented in Table 3.12. There were no differences in CH_4 emissions (g/kg) between treatments. However, a numerical decrease in CH_4 production was observed with nitrate treatments compared with urea treatments.

Table 3.11 Diets vs refusals composition. Diet composition analysed at the beginning of the experiment. Samples of refusals obtained from day 15 to day 21, composited for animal and period

Treatments		Dry matter	Organic matter	CP	NDFc
Control	Diet	92.8	92.8	15.1	47.2
	Refusal	81.6	88.8	15.8	31.3
	SEM	1.65	0.86	0.58	0.14
	P-value	0.01	0.03	0.46	0.02
Urea	Diet	92.7	92.3	14.7	46.8
	Refusal	82.5	90.3	14.8	31.9
	SEM	1.55	0.49	0.73	3.75
	P-value	0.01	0.05	0.93	0.05
Urea+S	Diet	92.5	92.1	14.6	46.7
	Refusal	78.7	90.4	14.6	40.2
	SEM	1.99	0.61	0.79	5.40
	P-value	0.01	0.13	0.99	0.44
Nitrate	Diet	92.5	91.2	14.0	46.5
	Refusal	79.5	88.7	13.7	27.0
	SEM	1.34	0.67	0.41	5.45
	P-value	0.00	0.06	0.64	0.06
Nitrate+S	Diet	92.3	90.9	14.0	46.4
	Refusal	80.2	88.6	13.9	27.5
	SEM	1.37	0.66	0.35	3.65
	P-value	0.00	0.07	0.76	0.02

Table 3.12 Methane produced by steers fed the different diets: Control (True protein), Urea, Urea + S, Nitrate or Nitrate + S (g/day and g/kg DMI)

	Treatments						P-value for contrasts			
CH_4	Control	Urea	Urea+S	Nitrate	Nitrate+S	SEM	Control v NPN	Nitrate V Urea	Added S	NPN * S Interaction
g/day	121	139	85	73	71	30.1	0.40	0.21	0.36	0.41
g/kg DMI[1]	13.6	15.9	10.4	9.6	9.4	2.72	0.47	0.21	0.32	0.35

SEM- Standard error of the mean
[1] DMI measured from sampling period

3.3.2.3 Nitrogen metabolism

Total N intake (Table 3.13) was lower with NPN treatments (P=0.01) compared with control. Nitrogen intake and digestibility were lower with nitrate containing diets than with urea containing diets (P=0.03 and P=0.05). Daily urinary N excretion (g/d and % of N intake) was lower from steers fed NPN diets compared to the control diet (P<0.05). N retention tended to be greater with NPN treatments (Table 3.13) than control diets (P=0.08). There were no statistical significance differences in PD excretion between treatments and nor therefore in MPS (Table 3.14). The PDC index was not different between treatments (P>0.05).

Table 3.13 Nitrogen utilization and excretion by steers fed the different diets: True protein (control), Urea, Urea + S, Nitrate or Nitrate + S

	Treatments						P-value for Contrasts			
	Control	Urea	Urea+S	Nitrate	Nitrate+S	SEM	Control v NPN	Nitrate V Urea	Added S	NPN * S Interaction
N intake, g/d[1]	209	198	191	179	179	9.0	0.01	0.03	0.62	0.65
Rumen NH_3-N, mg/dl	21.5	14.4	14.0	17.9	19.6	1.78	0.03	0.02	0.70	0.55
N ATTD, g/kg[2]	75.2	73.8	76.7	73.9	72.9	1.06	0.80	0.05	0.37	0.12
Faecal N										
g/d	50.0	47.9	43.2	44.2	45.0	3.93	0.13	0.74	0.49	0.35
% of intake N	23.8	24.3	22.1	24.7	25.3	0.80	0.80	0.05	0.37	0.12
Urinary N										
g/d	90.2	63.9	69.1	59.6	70.1	7.38	0.01	0.75	0.16	0.62
% of intake N	43.9	33.4	36.8	33.9	39.4	2.92	0.03	0.59	0.15	0.73
Retained N[3]										
% of N intake	31.4	42.0	41.7	39.9	42.7	6.80	0.08	0.91	0.81	0.75
% of N absorbed	40.8	55.3	53.7	52.8	56.8	9.34	0.08	0.97	0.86	0.68

SEM- Standard error of the mean [1]N intake measured during the total collection of faeces and urine (d 16 to 20)
[2]Nitrogen apparent total-tract digestibility
[3]Intake-faeces-urine

Table 3.14 Urinary excretion of purine derivatives and Microbial Protein Synthesis (MPS) by steers fed the different diets: Control (True Protein), Urea, Urea + S, Nitrate or Nitrate + S

	Treatments						P-value for Contrasts			
ITEM	Control	Urea	Urea+S	Nitrate	Nitrate+S	SEM	Control v NPN	Nitrate v Urea	Added S	NPN * S Interaction
Purine derivatives, mmol/d										
Allantoin	84.7	75.3	78.4	65.8	79.5	7.32	0.25	0.58	0.27	0.48
Uric Acid	8.1	6.3	7.7	6.6	6.7	0.68	0.12	0.62	0.26	0.36
Creatinin (mmol/l)	8.5	9.0	11.4	9.3	9.7	1.26	0.10	0.25	0.47	0.92
PD excretion	92.8	81.6	86.1	72.4	86.2	7.72	0.22	0.57	0.26	0.56
Microbial N[1]										
g/d	68.6	59.1	62.9	51.2	63.0	6.61	0.22	0.57	0.26	0.56
g /kg DOMR[2]	22.9	22.1	22.7	19.5	24.4	2.43	0.81	0.86	0.28	0.40
PDC index[3]	116.5	122.1	91.4	97.8	105.3	12.7	0.22	0.14	0.98	0.36

SEM- Standard error of the mean

[1] Values calculated according to (XB Chen, 2004)for Bos indicus

[2] digestible organic matter in the rumen

[3] [PD]/[Creatinine]* $W^{0.75}$; W body weight, PD and creatinine concentrations in mmol/l

3.3.2.4 Rumen fermentation

Treatment did not affect total VFA concentrations in the rumen fluid (Table 3.15). None of the main VFA concentrations (mmol/l), molar proportions or pH differed between diets (Table 3.15). The NH_3-N rumen concentrations before feeding were higher for the animals receiving the control diet than those fed the NPN sources (P=0.03). The NH_3-N concentration was lower in urea-containing diets when compared with nitrate-containing diets (P=0.02). Time after feeding had an effect on all rumen fermentation parameters analysed (P<0.01; data not shown). The effect was quadratic for all parameters (P<0.01). E.g., NH_3-N concentration increased after feeding up to 2 h, decreasing after with all treatments (Figure 3.6).

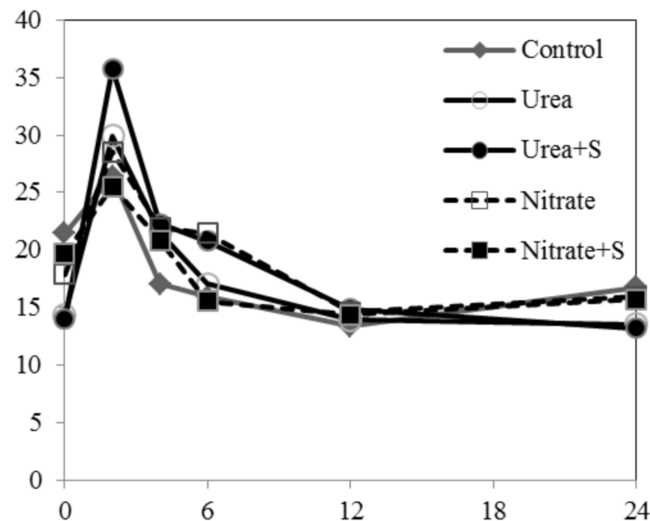


Figure 3.6 Daily rumen NH_3-N concentration pattern (mg/dl) (hours after feeding) of steers fed the different diets

Table 3.15 Rumen fermentation parameters before feeding of steers fed the different diets: Control (True protein), Urea, Urea + S, Nitrate or Nitrate + S

	Treatments						P-value for Contrasts			
ITEM	Control	Urea	Urea+S	Nitrate	Nitrate+S	SEM	Control v NPN	Nitrate v Urea	Added S	NPN * S Interaction
Total VFA, mmol/l	121.2	128.2	95.2	87.3	81.7	18.86	0.30	0.18	0.33	0.48
Acetate, mmol/l	82.5	88.2	69.8	63.3	56.9	13.02	0.39	0.17	0.36	0.65
Propionate, mmol/l	21.1	21.6	14.2	13.0	13.2	3.05	0.13	0.14	0.27	0.24
Butyrate, mmol/l	11.9	13.3	8.6	8.4	7.5	2.18	0.34	0.20	0.23	0.41
VFA, mol/100 mol										
Acetate	69.4	68.9	73.4	72.4	68.8	2.19	0.57	0.80	0.84	0.09
Propionate	16.8	17.5	15.3	15.0	16.7	1.28	0.62	0.67	0.85	0.16
Butyrate	9.5	10.1	8.7	9.6	9.2	0.50	0.93	1.00	0.10	0.36
NH_3-N, mg/dl	21.5	14.4	14.0	17.9	19.6	1.78	0.03	0.02	0.70	0.55
pH	6.82	6.78	6.79	6.88	6.83	0.06	0.99	0.23	0.70	0.58

SEM- Standard error of the mean

The interactions between treatment and time for rumen parameters are presented in Table 3.16. There were significant interactions for total VFA concentration, acetate and propionate concentrations and molar proportions, and NH_3-N concentration between urea and nitrate diets and time ($P<0.05$). In addition, the change in molar proportions of propionate during the day differed when S was added to NPN diets.

Table 3.16 Interaction between treatments and time points for rumen parameters measurements for the different contrasts studied

	P-value for contrasts							
	Control v NPN		Nitrate vUrea		Added S		NPN * S Interaction	
	Lin	Quad	Lin	Quad	Lin	Quad	Lin	Quad
Total VFA,				0.01				0.07
Acetate, mmol/l				0.03				0.06
Propionate,		0.02		0.01				0.04
Butyrate, mmol/l			0.02					
VFA, mol/100								
Acetate			0.01			0.09		
Propionate		0.06		0.01	0.01			
Butyrate			0.08		0.05			
NH_3-N, mg/dl	0.06		0.05					
pH								

Lin=linear effect
Quad=quadratic effect

Total VFA (mmol/l) concentration with the nitrate diets increased up to 12 hours after feeding, when the maximum values where achieved, and decreased afterwards, whereas with urea diets VFA concentration remained constant during the day, remaining below the values obtained with nitrate diets from 6 to 18 h after feeding (Figure 3.7 a). VFA concentrations were similar between urea and control treatments. Acetate molar proportion of total VFA tended to be greater in nitrate diets than in urea diets, and the opposite was observed with propionate molar proportion (Figures 3.7 b and 3.7 c). Propionate molar proportion presented interaction between time and inclusion or not of sulphur to NPN diets, with sulphur addition to NPN promoting lower propionate molar proportions after feeding than with no sulphur added (P=0.01; Figure 3.8). Ammonia concentration tended to be greater in urea than in nitrate-containing diets 2 h after feeding (Figure 3.9).

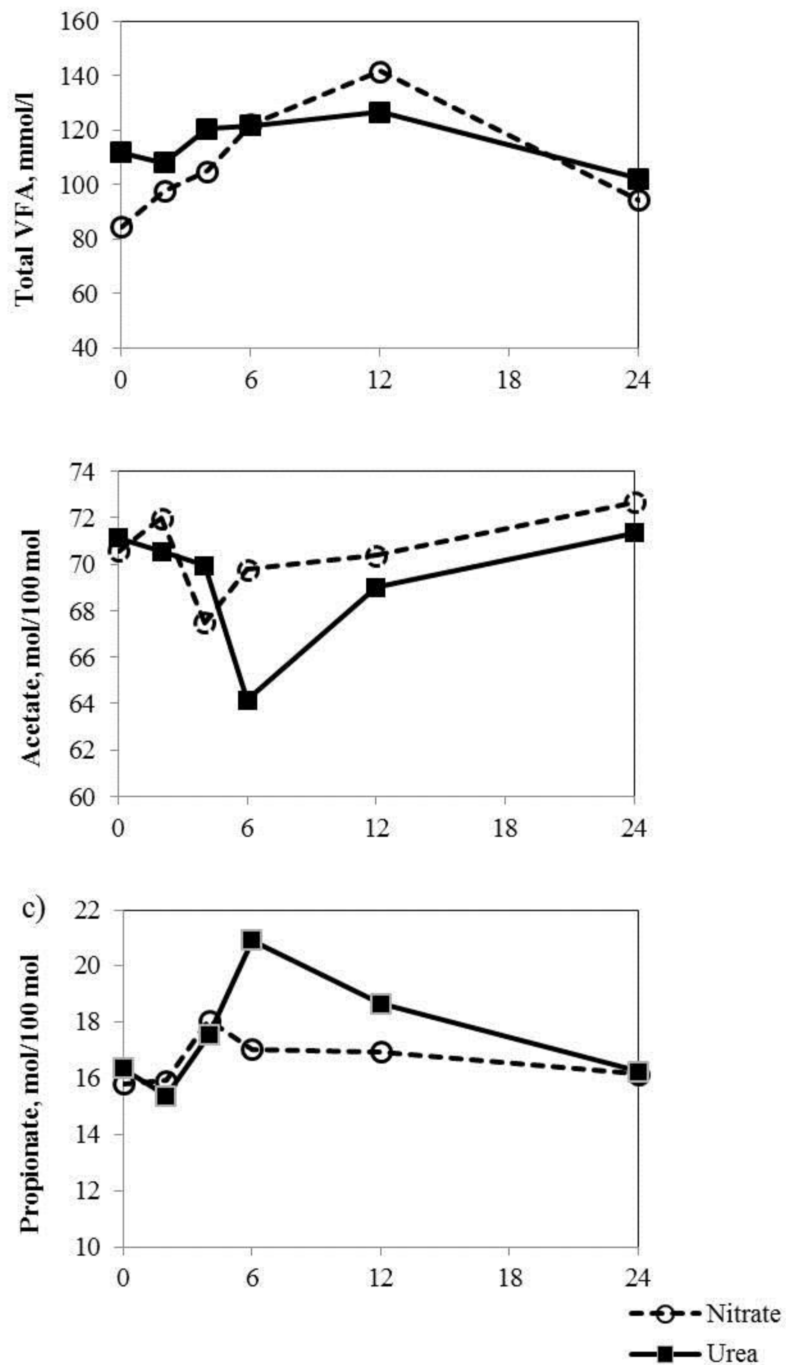


Figure 3.7 Daily pattern (hours after feeding) of rumen VFA production from steers receiving nitrate containing diets vs urea diets

a) Total VFA (mmol/l)

b) Acetate molar proportion

c) Propionate molar proportion

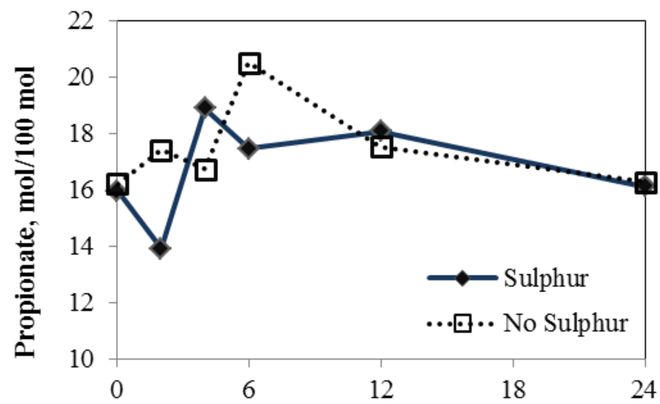


Figure 3.8 Propionate molar proportions during the day (hours after feeding) from steers receiving sulphur treatments vs no sulphur containing ones

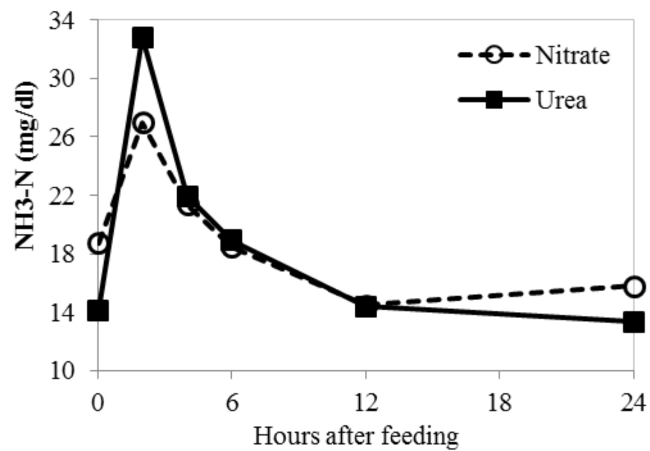


Figure 3.9 Daily pattern of NH_3-N rumen concentration (hours after feeding) from steers receiving nitrate diets vs urea diets

3.4 DISCUSSION

The hypotheses of the present study were that partial replacement of true protein or urea by encapsulated nitrate would reduce CH_4 emissions, without impair N utilisation and that elemental S above requirements could reduce nitrate toxicity, improve N utilisation and further decrease CH_4 production.

3.4.1 Effect of dietary nitrate and sulphur on methane production

Nitrate addition to diets produced a numerical decrease in enteric CH_4 production compared with the urea and control treatments. The absence of a statistically significant difference between treatments could be due to the small sample size. To further explore the significance of CH_4 reduction effect, CH_4 data was analysed together with CH_4 data from another set of five animals to increase the sample size. The other data were obtained from 5 Nellore breed steers that were used together with the animals from the present experiment in an identical design experiment and at the same time. Similar numerical differences in CH_4 produced between diets were observed from the 10 animals, compared with the results obtained in this experiment (Table 3.17).

Consistent and persistent reductions in CH_4 production as a result of dietary nitrate have been extensively documented (Lee and Beauchemin, 2014). In this experiment, steers consumed 14.3 g nitrate/kg DM, with a potential to decrease CH_4 by 3.7 g CH_4/kg DMI (Hulshof et al., 2012). The CH_4 mitigation achieved was 2.8 g CH_4/kg DMI (29%), resulting in a 76% efficiency.

Table 3.17 Methane production from steers fed different dietary treatments (combining studies)

	Treatments						P-value for contrasts			
CH_4 (g/kg DMI)	Control	Urea	Urea+S	Nitrate	Nitrate+S	SEM	Control v NPN	Nitrate v Urea	Added S	NPN * S Interaction
Angusx[1]	13.6	15.9	10.4	9.6	9.4	2.72	0.47	0.21	0.32	0.35
Nellore	14.1	12.4	11.2	12.75	11.7	1.71	0.15	0.73	0.39	0.95
Angus+ Nellore[2]	13.2	13.4	10.4	10.4	9.9	2.03	0.18	0.23	0.22	0.38

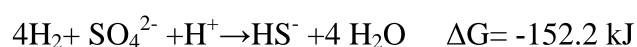
[1]values from this study (presented in results section)

[2] values combined 10 experimental animals

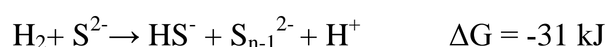
One mole of nitrate (Soissan et al., 2014) incorporates 4 moles of H in the reduction to NH_3-N having the potential to reduce CH_4 formation by 1 mol (16 g)(Lee and Beauchemin, 2014). However, the different levels of addition of nitrate, with different basal diets and species used across studies have differed in CH_4 mitigation effect. The studies with steers and forage diets have been limited: Hulshof et al. (2012) reported a 27% reduction in CH_4 yield (87% of the theoretical potential) in steers fed sugar-cane based diets supplemented with calcium nitrate (22 g/kg DM). Similarly, Troy et al. (2015) supplemented steers with calcium nitrate (21.5 g/kg DM), and reported a reduction of CH_4 production of 80% from theoretically expected with forage diets. The effect of feeding encapsulated nitrate (25 g/kg DM) to steers was studied by Lee et al. (2015b). These authors reported that nitrate decreased CH_4 emissions from steers by 83% of the theoretical expected. Several factors such as variable levels of absorption of nitrite and nitrate affected by retention time in the rumen and diets, the presence of different bacteria populations, the concentration of H_2 in the rumen and/or interaction between them may interfere with the efficiency of nitrate to reduce CH_4. A possible increase in H_2 concentration with nitrate inclusion has been reported (van Zijderveld et al., 2011a; Veneman et al., 2015) affected the efficiency of CH_4 reduction. As enteric H_2 production is another loss of energy, it would be desirable to measure H_2 concentration, when nitrate inclusion is studied. High doses of nitrate above certain threshold have been reported to have lower efficiency to lower CH_4 emissions (Lee and Beauchemin, 2014). Once a day feeding used in the present study could also have reduced the magnitude of CH_4 mitigation, as CH_4 reduction seems to happened within 12 h after feeding (Lee et al., 2015b). Therefore, daily ration provided in more than one meal over a day may increase the CH_4 reduction potential.

Elemental S (2.4g /kg of DM) did not reduce CH_4 emissions from nitrate fed animals. The numerical reduction of CH_4 production from urea treatments with sulphur inclusion is difficult to interpret. A decrease in total VFA production was also observed in the urea +S treatment, which could suggest a reduction in fermentation and therefore in CH_4 produced with S addition to urea diets. In addition,

the molar proportion of propionate and butyrate were decreased with S addition to urea diets, and acetate proportion was increased. This shift in VFA profile has been generally associated with reduction in CH_4 production. It could be that S additive acted as an alternative H sink, but with no significant effect with nitrate. To the best of my knowledge no study has used elemental S to reduce enteric CH_4 production. Van Zijderveld et al.(2010) studied the effect of adding nitrate and/or sulphate (26 g/kg DM) to sheep diets, and achieved a reduction in CH_4 independent and additive with nitrate inclusion. The source of S was the oxidized form (sulphate) and the inclusion rate (26g /kg of DM) was well above the amount require for protein synthesis. It could be that sulphate promotes interactions between SRB and NRB bacteria in the rumen. In addition, based in free energy calculations, elemental S has lower affinity for H_2 than sulphate, therefore, having lower CH_4 mitigation effect.



$$4H_2 + SO_4^{2-} + H^+ \rightarrow HS^- + 4\ H_2O \quad \Delta G = -152.2\ kJ$$



$$H_2 + S^{2-} \rightarrow HS^- + S_{n-1}^{2-} + H^+ \quad \Delta G = -31\ kJ$$

In the current study, daily CH_4 production (g/day) was on average lower than expected from forage based diets according to Intergovernmental Panel on Climate Change (IPCC). The IPCC estimates a CH_4 production of 49 kg/animal/year for young cattle in Latin America (CHANGE, 2006). Methane productions were 4.6% of GE (13.6 g CH_4/kg DMI or 121 g/day) in the control diet, which is less than most studies with beef cattle using SF6 technique (Ricci et al., 2013). The IPCC 2006 tier II model estimates enteric CH_4 production to be 6.5% of gross energy (GE) for forage based fed cattle. Therefore, the CH_4 values from this study were more than 30% lower than expected from IPCC calculations. However, the molar proportions of propionate (16 mmol/ mol) and acetate (70 mmol/mol) in this study were consistent with the forage-mixed based diet fed. The low values reported were similar to studies where forage based diets (barley silage, sugar cane, respectively) were fed and CH_4 productions were estimated with the SF6 technique (Boadi et al., 2004; Hulshof et al., 2012).

This study used the SF6 technique because it is the standard for CH_4 measurements in Brazil and was the method available, although the technique has more variability between animals and within animals than Calorimetric methods

(Pinares-Patiño and Clark, 2008) and disadvantages caused by cannulation have been reported (Beauchemin et al., 2012). Cannulation has the risk of leakage of gas from the rumen, and therefore large number of animals is needed to overcome the additional variability (Beauchemin et al., 2012; Boadi et al., 2002). In addition, it was reported that SF6 underestimate CH_4 emissions from animals in pens (McGinn et al., 2006). Taking into accounts all the constraints mentioned for CH_4 measurements, the CH_4 values in this study are used to compare effect between treatments, and not for evaluating total enteric CH_4 production from steers fed forage diets.

3.4.2 Nitrate toxicity

When looking at nitrate supplementation in ruminant diets, most studies have implemented an acclimatisation strategy to reduce the risk of toxicity (Leng, 2008). The present study implemented a period of adaptation (14 days) to nitrate with an increase of 25% of the final dose every 3 days and animals did not show any apparent signs of toxicity. Several *in vivo* studies have demonstrated that the stepwise introduction of nitrate into the diets prevent nitrate toxicity (Hulshof et al., 2012; Li et al., 2012; Li et al., 2013; Nolan et al., 2010; van Zijderveld et al., 2010).

In this study, DMI was not affected by treatments during adaptation period with *ad libitum* feeding. Similar results have been reported elsewhere (Lee et al., 2015b; Soissan et al., 2014; Troy et al., 2015; van Zijderveld et al., 2011a). A decrease in DMI and ADG with nitrate addition without adaptation was reported in beef cattle (Hegarty et al., 2013). The results of this study and others suggest that the stepwise adaptation to nitrate may avoid a decrease in DMI. In addition, the effective level of nitrate in this study was lower compared with other studies as the encapsulation process was not considered when the level of nitrate was selected (14.3 vs 20 g/kg DM).

Feed consumption and MetHb levels were measured 3 h after feeding *ad libitum* on 4 different days, corresponding to different levels of nitrate inclusion (alternate days for each measure). It was assumed that blood MetHb concentration

achieved maximums 3 h after feeding when the majority of the ration has been consumed (38 to 51%) (Crawford et al., 1966). In the present study, animals receiving nitrate containing diets presented MetHb values below the threshold for toxicity (Leng, 2008), and did not present signs of toxicity. Differences in MetHb concentrations in animals receiving nitrate have been reported from previous studies. Most of studies are in agreement with the current one, where a period of adaptation to nitrate seems to prevent methaemoglobinemia (El-Zaiat et al., 2014; Nolan et al., 2010; Troy et al., 2015; Velazco et al., 2014). Contrarily, high MetHb levels in blood (up to 45%) were recorded (Soissan et al., 2014) when 20 g of nitrate/kg DM was added in sheep diets with 14 days of adaptation. The suggested reason for the increase in toxicity in the study by Soissan et al. (2014) compared with the current one was that diets were fed at a restricted level and contained only low levels of readily fermented CHO, which could increase pH and reduce nitrite reduction. The *ad libitum* feeding used in this study contributed to prevent toxicity effect.

Although a slight decrease on the feed consumption rate on day 9 of adaptation period with nitrate containing diets was observed, feed pattern during adaptation period seems not to be affected by treatments: there was no significant lower DMI because treatments and feed consumption rates at the end of adaptation were not different between treatments. Animals consumed nearly half of the daily ration within first 3 hours after feeding (51% on day 3, 43% on day 6, 44% on day 9 and 38% on day 12). Daily feed intake was changed in the study by Lund et al. (2014), where nitrate addition without an adaptation period significantly reduce cows feed intake in the first hours after feeding. Refusals composition was used as an indirect way of measuring the effect of additives on feeding behaviour and palatability. Ash and NDF content were lower in refusals compared with diets what may suggest a selection against concentrate. However, the lack of differences between CP contents of feed offered and refusals suggested that animals did not select against the feed additives. To the best of my knowledge, no previous study has recorded the refusals composition from animals receiving nitrate treatments. In agreement with the current study, no effect in feed intake or feed consumption was observed with *ad libitum* feeding and 20 g/kg DM of EN (Lee et al., 2015a). Few studies have reported the effects of feeding nitrate on total apparent digestibility. The

current study did not show any difference in digestibility in agreement with the results of Nolan et al. (2010). Contrarily, Lee et al. (2015a), reported a linear increase in total DM digestibility with encapsulated nitrate.

Co-existence with cellulolytic bacteria promotes the growth of *Selemonas ruminantium* which reduce nitrite in the rumen, whereas co/existence with amilolytic bacteria has been reported to increase nitrite accumulation (Lin et al., 2013). Therefore, it has been suggested that enhance of fibre fermentation may reduce nitrite accumulation in the rumen through the increase in cellulolytic bacteria (Yoshii et al., 2003). On the contrary, an inhibition of cellulolytic bacteria may explain the decrease in DMI sometimes observed in animals fed nitrate, because a decrease in NDF digestion, affect rumen fill and thus decrease DMI (Latham et al., 2016.). The amount of fermentable CHO and high content of hay (50%) was adequate to maintain healthy conditions of the rumen. In conclusion, the level of addition, feeding method, time of adaptation, and the provision of fermentable CHO in the form of starch and fibre may have protected the steers in this study from nitrate toxicity.

3.4.3 Effect of nitrate and sulphur on nitrogen utilization and microbial protein synthesis of steers

In the current experiment, the chemical analysis of additives was carried out after diets were formulated. The N content of EN was lower than the formulation given by the provider. Therefore, although diets were formulated to be iso-nitrogenous, NPN treatments had lower N contents compared with control. The increase in N retained and lower NH_3-N concentration from steers with NPN diets compared with control diet might indicate N content of diets balance with energy supply. Opposingly, higher N content in control diet may promote greater N losses via urine, as N was over the requirements for microbial growth and/or not synchronise with the supply of energy. There were no differences in N excreted and retained between the urea and nitrate diets. Purine derivatives were used as an indirect way to measure MPS in the rumen (Chen et al., 1990). The similarity in MPS among the diets may reflect that N content was high and not limiting microbial

growth in any of the diets. This fact was supported by NH_3-N levels adequate for fermentation (>8.5 mg/dl), ADG, and no significant changes in total VFA production (Mota et al., 2015).

This study does not support previous results where the addition of S to diets increased utilization of nitrate and nitrite and N retention (Sokolowski et al., 1969) and S altered fermentation pattern (N:S, 8:1)(Fron et al., 1990). The most important issue regarding sulphur-containing compounds for ruminants is that the ratio with N should be optimum in the diets (Napasirth et al., 2013). Therefore, it was unlikely that the extra S in the current study would improve MP synthesis as diets with no added S had adequate N:S (9:1) ratios and the addition of elemental S was not accompanied by an increase in N content in diets. In previous studies, where the addition of S increased N retention, S contents of control diets were lower and probably below the requirements for optimal MP synthesis (N:S,18:2) (McSweeney and Denman, 2007), and (N:S, 18:1) (Sokolowski et al., 1969) . The source of S tested in the present study should also be considered. Comparing inorganic vs organic S sources, it has been reported that inorganic S is mainly dissimilated to sulphide before utilization for MPS, whereas organic S can be incorporated directly into MP without entering the ruminal sulphide pool (McSweeney et al., 2009). In this study, elemental S needed to be reduced to sulphide before entering the rumen pool. However, limited *in vivo* studies have compared the relative benefit when ruminants are fed organic or inorganic sources of S. Silva et al. (2014) found that some microorganisms were able to degrade inorganic S sources into sulphide, incorporating this compound to produce amino acids while other microorganisms preferred organic S. During a trial with lambs, Johnson et al. (1971) determined that approximately three times more inorganic S was required in comparison with organic S to meet the maintenance requirement for S. Moreover, elemental S has been reported to have a lower apparent absorption than other inorganic source of S as sulphate, or methionine (Fron et al., 1990; Hedderich et al., 1998) and to be 35% as available for ruminal growth and 50% as digestible as sulphate salts (Zinn et al., 1997). Therefore, elemental S used in this study seems less convenient as an additive to improve MPS than sulphate or organic source of S.

3.4.4 Effect of nitrate and sulphur on rumen fermentation

Overall, the source of N did not affect rumen fermentation. Consistently, no effect on total VFA concentrations or the proportions of the main VFA were observed in others studies in response to nitrate when sampling was done before feeding (Li et al., 2012; van Zijderveld et al., 2010). When average daily NH_3-N values were compared between treatments, no differences were observed in this study. The same was reported by Nolan et al, (2010) from sheep fed urea or nitrate. Ammonia concentration were greater with urea than with nitrate-containing diets 2 h after feeding, Similarly, lower NH_3-N concentrations 3 h after feeding with encapsulated nitrate compared to urea has been reported (El-Zaiat et al., 2014) and similar daily pattern was reported by Lee et al. (2015b) when encapsulated nitrate was fed to steers. Similarly, NH_3-N concentrations were lower *in vitro* by using a nitrate-based compared with urea-based media after 24 h of incubation (Guo et al., 2009; Lin et al., 2011). When rumen fluid taken from steers 5 h after feeding adapted or not to nitrate and from urea fed animals was incubated, NH_3-N concentration of *in vitro* incubations where higher with urea compared with nitrate at the beginning of incubation and were lower at 3 and 6 h with urea respect to nitrate(Lin et al., 2013). If it is considered that rumen fluid samples were taken 5 h after feeding the pattern of NH_3-N is comparable with the present study. In contradiction, an increase in NH_3-N concentration 2 h after feeding nitrate compared with urea based diet has been observed in sheep (Hulshof et al., 2012).

The reduced NH_3-N concentration after feeding with nitrate can possibly be attributed to the longer time needed for nitrate to be reduced to NH_3-N compared with urea that is highly soluble and immediately converted to NH_3-N. An alternative interpretation could be that nitrate was not quantitatively converted to NH_3-N, as may be expected if nitrate reduction was by the dissimilatory pathway.

A significant interaction was observed between treatments and time. The results of this study are comparable with previous publications where an increase in acetate proportions and decrease in propionate proportions were reported 2 h after feeding with nitrate compared to urea (Farra and Satter, 1971, Hulshof et al., 2012,

Soissan et al., 2014, Troy et al., 2015). However, in the present study the greatest differences in molar proportions of acetate and propionate between studies were observed 6 h after feeding. It is possible that the later change in fermentation pattern in response to nitrate in this study compared with the previous ones could be due to the source of nitrate used that will be slower release to the rumen. The results from the current and previous studies seem to imply that change in fermentation parameters with nitrate inclusion and therefore CH_4 production would be produced in the first hours after feeding (up to 6 hours) and a consistent tendency of increasing acetate and decreasing propionate with nitrate addition has been verified.

Recently, it has been discovered an alternative pathway for nitrate ammonification with elemental S as an electron donor in deep sea (Slobodkina et al., 2017). If the previous is confirmed in the rumen environment, elemental S could be an alternative electron donor to sulphide for the S dependent dissimilatory nitrate reduction to NH_3-N. From this study, this pathway is not confirmed as S added to nitrate during the day did not increase NH_3-N production, although NH_3-N concentration was higher with S addition to nitrate before feeding. Nitrate and/or S may promote NRB and SRB populations that utilise organic compounds and may be responsible of the conversion of propionate and butyrate to acetate with a concomitant increase of H_2 in the rumen. The threshold level of H_2 for nitrate reduction is extremely low, and therefore nitrate will create the favourable condition for syntrophic metabolism of butyrate or propionate to acetate (Leng, 2014). The stimulation of these populations and increase in H_2 may also explain a lower apparent efficiency of nitrate in CH_4 mitigation. It appears that any change in microbial ecology will increase the amount of H_2.

The daily increase tendency in total concentration of VFA with nitrate diets versus urea might indicate an increase in production rate when nitrate rather than urea is a major source of N for the rumen microbial population. Animals in all treatments consumed similar amounts of feed in first 3 h after feed supply, which indicates that any difference in rumen fermentation are unlikely to be caused by feed intake (van Zijderveld et al., 2011a).

3.5 CONCLUSIONS AND IMPLICATIONS

The reduction of CH_4 production with the addition of nitrate to forage diets from steers was confirmed. As hypothesized, no effects on N metabolism and MPS were observed. The level of addition of encapsulated nitrate (20 g/kg of DM, equivalent to 14.3 g nitrate /kg DM), *ad libitum* feeding, and the time of adaptation (14 days) chosen for this study were adequate to avoid nitrate toxicity. The addition of elemental S to NPN sources did not significantly affect CH_4 production, N balance, digestibility or rumen fermentation.

3.5.1 Implications

Nitrate as a NPN source could be recommended for use wherever ruminants are supported on low protein forages or on good quality diets to reduce the amount of true protein needed and/or urea, and reduce CH_4 emissions. It is essential to stimulate maximum conversion of nitrate to NH_3-N so nitrate can replace dietary protein or urea sources (Yang et al., 2016). Taking into account the literature results, the incorporation of nitrate as a major source of N for ruminants needs to consider the method of feeding. For example, nitrate supplementation in grazing animals may need to use slow release nitrate preparations to ensure that animals access nitrate at intervals over a day so that synchrony of feed intake (fermentation rate) and availability of N is achieved in the rumen (Leng, 2008). Nitrate should not be added to diets already adequate in RDP supply as excretion of excess N can lead to increased nitrous oxide (N_2O) production from soil after manure application (Yang et al., 2016). Moreover, excessive RDP from NPN sources in diets could have negative effects on feed intake and animal production. Therefore, feeding nitrate as a substitute for urea, at levels greater than 25g/kg of DM is not suggested as urea level is recommended to be less than 10 g/kg of DM (Kertz, 2010). As most organisms that reduce sulphate are also capable of reducing nitrate or nitrite, nitrate uptake may be stimulated by a source of S. However, the risks of feeding excessive sulphur should be considered depending on the diet.

It was hypothesised that the absence of effect of S in the present study could be due to the source utilised (elemental S) and the dose. Sulphur sources for ruminants can be added as elemental S, sulphate or organic sulphur compounds, and the chemical forms of S provided have different availabilities. This study brings insight about the necessity to develop more *in vivo* studies about the benefit of different S sources for ruminants, and in combination with N compounds.

The use of nitrate as a CH_4 mitigation strategy needs to consider the excretion of N_2O. The relative efficacy of CH_4 mitigation by nitrate may be lower by a rise in N_2O production from nitrate fed animals (Soissan et al., 2014). A small increase in N_2O because nitrate addition to diets could have large effects in GHG emissions because N_2O high global warming potential (Latham et al., 2016). Nitrous oxide excretion when nitrate was included *in vitro* was studied observing a low production (Kaspar and Tiedje, 1981) and appeared to be a by product of dissimilatory nitrate reduction, as denitrification has not been observed in the rumen (Leng and Preston, 2010). Recently, a study has shown that nitrate included in the diet of dairy cows at level of 14 and 21 g kg DM increased the emission of N_2O suggesting that denitrification may be ocurring in the rumen or in the mouth (Petersen et al., 2015) as N_2O was realased directly from the animals. Furthermore, the study demonstrated that N_20 was exhaled from the animal and did not arise from excreta or feed products (Petersen et al., 2015). A different study has also shown a small increase in N_2O emission with incluison of different nitrate sources to sheep diets compared with urea (de Raphaelis-Soissan et al., 2017).

Recently a study has been published comparing nitrate coated with paraffin and oil with nitrate not coated and demonstrated that coating nitrate with paraffin can improve the safety of nitrate supplementation, as shown by the lower blood MetHb concentrations (de Raphaelis-Soissan et al., 2017). In addition, the nitrate coated with paraffin and oil lead to lower N_2O released compared with nitrate unprotected, suggesting that the differences in nitrate and nitrite concentrations in rumen and blood significantly change the portioning of ruminal nitrate into N_2O when feeding nitrate protected compared with unprotected nitrate.

To conclude, as also shown by others (Lee and Beauchemin, 2014) nitrate can safely be used as a CH_4 mitigation additive and replace urea as a source of NPN for ruminants. To the best of my knowledge, the current study demonstrated for the first time that nitrate can replace part of protein of the ration without adverse effects on N metabolism and rumen fermentation. Encapsulated nitrate provide a promising additive to lower CH_4 production and maybe safer than conventional nitrate salts.

3.6 FUTURE WORK

Additional long-term studies with large numbers of animals are needed to explore the effects of EN on CH_4 production and growth performance of beef cattle. The last results reported by Soissan et al. (2017) indicate that encapsulated could be promising to lower nitrate toxicity and may produce lower amounts of N_2O compared with nitrate salts. Therefore, direct comparisons between nitrate and encapsulated nitrate are recommended to validate the effectiveness of EN vs nitrate in CH_4 mitigation effect and safety. It would be of interest to measure the concentration of nitrate, nitrite, and NH_3-N in the rumen and plasma during the day after feeding both additives, together with daily pattern of CH_4 production and Blood MetHb. The measurement of the different forms of N could add information about the absorption and metabolism of nitrate compounds.

To demonstrate if nitrate could improve MPS compared with urea, experimental diets should be formulated to be limiting in protein and include a diet with a high proportion of protein N as a positive control. Further research is needed to validate the use of encapsulated nitrate as a substitute for part of the protein in the ration.

It would be worthy to study different sources of S, such as sulphate salts or organic compounds with greater availability and CH_4 reduction potential than elemental sulphur. The adequacy of using cannulated animals for CH_4 measurements with SF6 should be also considered. Future work should contemplate different nitrate

addition levels, the method of addition to diets and times of feeding. Acclimation procedures to nitrate have not been clearly stated. Therefore, more *in vivo* studies are needed to establish minimum conditions for successful adaptation to nitrate-containing diets.

Meat and milk characteristics could be analysed and determine if nitrate and nitrite residues in animal products increase when nitrate is fed. Nevertheless, from previous research it is likely that the potential health risk to human from eating animal products containing nitrite, is very low (Cockburn et al., 2013). A recent study measured nitrate and nitrosamines content in beef meat from animals fed nitrate and did not detect nitrosamines levels to account for concern for human safety (Hegarty et al., 2013) and similar results were reported when analysing residues in milk (Guyader et al., 2016). As a conclusion, the necessity for this analysis is questionable.

CHAPTER 4

Assessment of the effects on the rumen microbial community of adding nitrate to diets containing different proportions of concentrate

Chapter 4.

4.1 INTRODUCTION

Methane is a potent greenhouse gas that contributes widely to global warming and climate change. Ruminants are the main anthropogenic source of CH_4 entering the atmosphere. The rumen is the main foregut compartment in ruminants where most fermentation occurs. This process is mediated by the rumen microbial population involved in the primary breakdown of plant polymers, followed by fermentation of monomers to produce short chain fatty acids and other nutrients needed for biomass synthesis. During this breakdown, a large amount of H_2 is produced. The H_2 needs to be removed for the correct functioning of the system and this is done mainly by methanogenic archaea. Methanogens gain energy by reducing CO_2 with electrons from H_2 oxidation, producing CH_4, which has no nutritional value for the animal.

Methanogens are the only members of the rumen microbiota able to produce CH_4 (Kittelmann et al., 2015). Several factors can affect this archaeal population including the availability of growth factors, number of bacterial H_2 producers and non producers and interactions between H_2-producing and -consuming synergistic populations (Firkins and Yu, 2015). Therefore, the interactions of archaea with other rumen microbes could play an important role in CH_4 production pathways (Janssen and Kirs, 2008; Leahy et al., 2013; Zhou and Hernandez-Sanabria, 2009). Knowledge about rumen microbiology has increased in recent years due to new molecular techniques, such as next-generation sequencing, but the correlation between rumen microbial population and CH_4 production is still not clear.

Many compounds have been evaluated for their ability to reduce enteric CH_4 production by ruminal microorganisms (reviewed in chapter 1). Understanding the mechanisms of methanogenesis and the microorganisms involved is important in

developing effective mitigation strategies for enteric CH_4 production (Morgavi et al., 2010a, Zhou et al., 2012, Leahy et al., 2013; Ross et al., 2013;). Among the feed additive options for lowering enteric CH_4 emissions from ruminants, nitrate has been identified to be promising (Newbold et al., 2014, Lee et al., 2015, Nguyen et al., 2015; Veneman et al., 2015) but it suffers from variability in efficacy for reasons that are not well understood (Yang et al., 2016).

Consequently, understanding the microbial ecology and how rumen bacteria and methanogens interact and contribute to rumen function could provide new insight into additives causing long term reductions in CH_4 (Attwood et al., 2011; Leahy et al., 2013).

4.1.1 Relationships between the rumen microbiome and CH4 production

As mentioned previously, archaea are responsible for CH_4 production in the rumen. However, other microbes also affect CH_4 production either by competing for H_2, bacteria H_2 producers or by their effect on the numbers of archaea or other members of the microbiota (Morgavi et al., 2010a).

Total number of archaea have been correlated with CH_4 production in some studies (Wallace et al., 2014; Wallace et al., 2015), but no correlation was found in others (Zhou et al., 2011, Danielsson et al., 2012, Kittelmann et al., 2014; Shi et al., 2014, Danielsson et al., 2017). The relationship between methanogenesis and the abundance of archaea population was not clear in the recent study of Tapio et al. (2017a), which suggested a closer relation with methanogenic structure than abundance. Other studies highlighted that the expression of genes involved in methanogenesis pathways is more relevant for total CH_4 production than methanogens abundance (Shi et al., 2014).

The interactions between bacteria and protozoa are important and could play an important role in CH_4 production pathways. Holotrich ciliate protozoa are highly active in the rumen and produce H_2 that could be used by methanogens to produce

CH_4. In addition, protozoa harbour methanogen populations, an example of interspecies H_2 transfer (Krumholz et al., 1983). The removal of protozoa from the rumen is associated with decreased CH_4 emission (Belanche et al., 2014; Newbold et al., 2015; Tapio et al., 2017).

Rumen microbial bacteria produce substrates needed for methanogenesis, thereby contributing indirectly to CH_4 production. For example, interspecies H_2 transfer has been described between cellulolytic bacteria and methanogens (Wolin et al., 1997). Animals that are considered high CH_4 emitters have been correlated with larger communities of H_2-producing bacteria (*Ruminoccocus*, *Prevotella, Clostridiales*) as shown by others (Kittelmann et al., 2014; Tapio et al., 2017). Nevertheless, some non H_2-producing cellulolytic bacteria exist in the rumen (*e.g. Fibrobacter*), that could replace H_2 producers without impairing fibre digestibility and reducing CH_4 production (Morgavi et al., 2010a). In agreement, a high abundance of family *Succiniovibrionaceae* (non H_2 producer) within Proteobacteria in wallabies has been correlated with low emissions of CH_4 from these animals (Pope et al., 2011) and Proteobacteria abundance, predominantly *Succinivibrionaceae*, has been negatively correlated with CH_4 emissions (Wallace et al., 2015, Tapio et al., 2017). This negative correlation between *Succiniovibrionaceae* abundance and CH_4 production has also been reported in beef cattle (Wallace et al., 2015) and dairy cows (Danielsson et al., 2017).

Prevotella has been observed to be positively correlated with CH_4 production (Kittelmann et al., 2014). However, *Prevotella* has a great variation in the ability to utilise different substrates among OTUs (Schloss and Westcott, 2011), with some *Prevotella* OTUs correlated with a high CH_4 phenotype, while others were associated with low emissions suggesting the existence of functional differences within the genus *Prevotella* genus (Danielsson et al., 2017; Tapio et al., 2017).

4.1.2 Microbial populations involved in nitrate-nitrite metabolism

Nitrate reduction to NH_3-N in the rumen is achieved by both bacteria and protozoa (Lin et al., 2011). Nitrate can change the rumen bacterial community through the toxicity of nitrite or competition for H_2 produced in fermentation and there is a shift in the VFA profile from propionate to acetate (Guo et al., 2009; Lee and Beauchemin, 2014; Lin et al., 2013). Nitrate lowers rumen CH_4 production due to the presence of NRB in the rumen, that use nitrate as a terminal electron acceptor. Therefore, CH_4 emissions can be significantly decreased with nitrate supplementation with only minor, but consistent, effects on the rumen microbial population and its function (Veneman et al., 2015).

Some studies reported the effect of nitrate on rumen populations during *in vitro* incubations (Lin et al., 2011; Marais et al., 1988; Yoshii et al., 2003). Few studies have addressed the microbial populations involved in nitrate metabolism *in vivo* (Asanuma et al., 2015; Lin et al., 2013; Popova et al., 2017; Veneman et al., 2015; Zhao et al., 2015). The effect of nitrate addition is more dramatic in the liquid phase, which is most likely related to the fact that nitrate is readily soluble in water (Lin et al., 2013). Free-living bacteria within the liquid phase seems more vulnerable to nitrate inclusion compared the attached bacteria, consistent with observations that the solid-associated bacterial community is more stable to perturbation (Welkie et al., 2010). Nitrate addition seems to stimulate nitrate- and nitrite-reducing bacterial members (Leng, 2014). Therefore, an increase in nitrate reducers would be expected in animals adapted to nitrate (Iwamoto et al., 2001). Traditionally, *Wolinella succinogenes, Veillonella parvula and Selenomonas ruminantium* were identified as important for nitrite-nitrate metabolism in the rumen (Asanuma et al., 2002; Iwamoto et al., 2002; Yoshii et al., 2003) and *Selemonas* species have been confirmed to play an important role in nitrate and nitrite reduction in the rumen (Asanuma et al., 2015). *Selenomonas ruminantum* and *Streptoccocus bovis* increased with nitrate addition to diets (Asanuma et al., 2015; Zhao et al., 2015) and the increase in *S. ruminantum* was confirmed over a long period of nitrate addition (Yoshii et al., 2003). *S. ruminantum and S. bovis* have been identified as important proteolytic bacteria in the rumen,

suggesting an increase in protein degradation in the rumen with nitrate addition (Asanuma et al., 2015). More recently, *Mannheimia succiniciproducens* and *Campylobacter fetus* were discovered to be, somehow, involved in nitrate metabolism (Lin et al., 2013). *Campylobacter fetus* and *Mannheimia succiniciproducens* abundance in steers increased linearly with nitrate addition level (Zhao et al., 2015). A number of other nitrate reducing bacteria, such as *Desulfivibrio* can inhabit the rumen at low abundance, and could be increased during long exposure to nitrate (Latham et al., 2016). The effect of nitrate addition on relative abundance of cellulolytic bacteria as *F.succinogenes, R.flavefaciens* and *R.albus* differed between studies, with a decrease in some studies (Asanuma et al., 2015; Iwamoto et al., 2002) and an increase in another (Zhao et al., 2015). It was reported that *F. succinogenes* is inhibited by nitrate in the nitrate unadapted rumen during *in vitro* culture (Hulshof et al., 2012; Zhou et al., 2012). *Prevotella* population was increased by nitrate addition *in vitro* (Patra and Yu, 2013) but nitrate treatment significantly decreased the relative abundance of *Prevotella in vivo* in dairy cows (Veneman et al., 2015). The decrease in propionate with nitrate addition could be linked to the decrease in *Prevotella* abundance in the last study.

Some studies have reported a decrease in the relative abundance of total number of archaea with nitrate addition (Patra and Yu, 2013; van Zijderveld et al., 2010; Veneman et al., 2015) while no effect was observed in others studies (Patra and Yu, 2014; Popova et al., 2017). Protozoa population forms hydrogen and contains electron transport carriers that might transfer electrons during nitrate reduction (Yang et al., 2015). Furthermore, protozoa have been suggested to increase nitrate reduction by bacteria but little is known about the specific role of protozoa fraction in nitrate metabolism (Lin et al., 2011). Some studies suggest a negative effect of nitrate in protozoa population (Asanuma et al., 2015), while others reported no effect (Guyader et al., 2016; van Zijderveld et al., 2010). The role of other members of the rumen microbiota involved in nitrate-nitrite metabolism is not clear hindering the application of nitrate as a dietary additive. Community profiling of rumen microbiota from animals fed different diets with nitrate addition could highlight the microbial species involved in nitrate reduction and help to understand the mechanism of action on CH_4 reduction and on the potential toxicity of nitrate in ruminants.

4.1.3 Molecular biology techniques to study the rumen microbiome

Next-generation sequencing (NGS) targeting DNA sequences have been used as powerful tools to study the gut microbiota. In this context, whole genome sequencing (WGS) is an effective way of gaining information on how rumen microbiota interact and contribute to rumen function and CH_4 production. At present, WGS information is publicly available for a small number of rumen bacteria (Leahy et al., 2013). However, the main focus of rumen bacterial community analysis has been the study of rumen bacteria involved in substrates degradation and therefore involved directly in growth and productivity of ruminants. More recently, in 2014 The Hungate 1000 project (http://www.rmgnetwork.org/hungate1000.html).was established to produce a reference set of rumen microbial genome sequences by sequencing the genomes of available cultivated rumen bacteria and methanogenic archaea, together with representative cultures of rumen anaerobic fungi and ciliate protozoa.

One experimental approach is amplicon sequencing, where a particular gene or fragment is amplified using polymerase chain reaction (PCR) method and the sequence determined (Di Bella et al., 2013). The purpose is to identify the organisms in a sample. The rRNA amplicon sequencing provides an accurate DNA sequencing method to determine in greater detail different members of the rumen community when compared with the PCR-based methods (e.g. Denaturing Gradient Gel Electrophoresis (DGGE)) or traditional cultured-dependent methods (Wallace et al., 2014). Amplifying and sequencing variable regions of the 16S rRNA gene, which is ubiquitous in bacteria and archaea is used to determine the taxonomic composition of the microbiome by comparing them with existing databases. Most molecular techniques for identification and classification of bacteria and archaea have been based on the nucleotide sequences of the 16S ribosomal RNA gene (Ozutsumi et al., 2005). However, information about methanogens is still scarcely. There are currently 21 rumen methanogen sequences available on NCBI (Sayers et al., 2011). PCR amplification is the first essential step to increase the DNA yield of microbial cells that are present in low numbers (Zhou et al., 2011). For amplicon sequencing, a gene

fragment will be amplified and the sequence determined. Accordingly, segments of the 16S rRNA gene will be amplified for the preparation of clone libraries in order to determine prokaryotic taxonomic composition in samples (Di Bella et al., 2013). However, the length of the gene (on average 1550 bp) means that it is not possible to sequence the entire gene. Usually one or more of the nine variable (V) regions of the 16S rRNA gene are amplified and sequenced, using particular sets of primers. The selection of the region depends on the target species the study is interested on and the sequencing method applied. For example, the Earth Microbiome Project (Gilbert et al., 2010) is standardized to amplify the V4 region, since that region can detect most bacteria and archaea populations (Caporaso et al., 2012). Therefore, for this study the V4 region of the 16S rRNA gene was targeted.

The MiSeq Illumina platform (Illumina, San Diego, CA) allows community amplicon sequencing at a lower cost per sequence than other platforms. Each MiSeq run produces one paired-end lane read for a pool of samples. The MiSeq generates 1.5 Gb per day from 5 million 150-base paired-end reads (Caporaso et al., 2012).

4.1.4 Experiment setup and aim of the study

Rumen contents and CH_4 data from 74 steers were used in this study (Table 4.1). After rumen fluid was collected from steers, the DNA was extracted. The V4 hypervariable region of the bacterial and archaea 16S rRNA gene was amplified and sequenced using an Illumina MiSeq instrument.

Hypothesis: Nitrate addition changes the rumen microbial population profile in a different way depending on the basal diet. The aims of the study were:

- To assess the differences in microbiota between different basal diets (high concentrate diets vs forage diets).
- To identify changes in rumen microbiota associated with addition of nitrate to the different basal diets.
- To identify microbial population affected by nitrate and correlated with CH_4 production from these animals (biomarkers development).

4.2 MATERIAL AND METHODS

4.2.1 Animal study

Rumen fluid samples and CH_4 production data from two studies with steers conducted in 2013 and 2014 were used for this study (Troy et al., 2015) and (Duthie et al., 2016). Finishing beef steers were fed a range of dietary treatments. Throughout both experiments all steers were offered one of the experimental dietary treatments *ad libitum* once daily at approximately 1.05 times of actual daily intake. In both experiments animal daily CH_4 outputs from the animals were measured using respiration chambers. Rumen fluid samples used for sequencing were obtained from each animal within 2 h of the animals leaving the respiration chambers by inserting a stomach tube nasally and aspirating manually (Troy et al., 2015). Animals selected and dietary treatments from each year experiment are explained below. In summary, the rumen content samples used for sequencing were: 40 rumen samples from the chamber period of Experiment 1 (2013); 34 rumen samples from chamber from Experiment 2 (2014) (Table 4.1).

Table 4.1 Animal and samples selected for the experiment

Basal diet	Concentrate		Mixed	
Treatment	Control	Nitrate	Control	Nitrate
Experiment 1	10	10	10	10
Experiment 2			17	17

4.2.1.1 Experiment 1. Experimental setup

Forty steers were selected based on the availability of rumen samples for sequencing. The animals were introduced into the chambers according to body weight (mean BW 696 ± 43 kg). The steers were fed one of two basal diets using a diet mixing wagon, consisting of (g/kg DM) forage to concentrate ratios of either (i) 520:480 (Mixed) or (ii) 84:916 (Concentrate) (Table 4.2). Within each basal diet the steers were offered one of two treatments: (i) Control containing rapeseed meal as the main protein source which was replaced with (ii) Nitrate in the form of calcium nitrate (Calcinit, Yara, Oslo, Norway; 21.5 g nitrate/kg diet DM), (Troy et al., 2015).

Table 4.2 Ingredients composition of Mixed and Concentrate diets (g/kg DM)

Diet	Mixed		Concentrate	
Ingredient	Control	Nitrate	Control	Nitrate
Grass Silage	189	193		
WCBS	316	316		
Barley Straw			84	82
Bruised barley grain	340	392	739	803
Rapeseed meal	128	43	146	57
Calcinit		28		28
Molasses	20	21	21	21
Minerals*	10	9	10	10

WCBS, whole crop barley silage; Calcinit, calcium nitrate.
*Contained (mg/kg): Fe, 6036; Mn, 2200; Zn, 2600; Iodine, 200; Co, 90; Cu, 2500; Se 30; (µg/kg): vitamin E, 2000; vitamin B12, 1000; vitamin A, 151515; vitamin D, 2500.

Steers were adapted to the experimental diets in two stages. During stage one (day -56 to day -28) the animals were adapted to the basal diets. During stage two (day –28 to day 0), steers were adapted to the treatments over a second four week period with an increase of 25% of the final dose of nitrate every 7 days. Following the completion of the 56 day periods steers were successively moved to respiration chamber measure their CH_4 emissions. After leaving the respiration chamber unit rumen fluid samples were taken (Troy et al., 2015).

4.2.1.2 Experiment 2. Experimental setup

Thirty four animals were selected. Rumen fluid samples were taken when the animals left the respiration chambers. The steers were fed one basal diet prepared using a diet mixing wagon and consisting of (g/kg DM) forage to concentrate ratios of 557:443 (Mixed) (Table 4.3). Within the Mixed basal diet the steers were offered one of two treatments: (i) Control containing rapeseed meal as the main protein source which was replaced with (ii) Nitrate in the form of calcium nitrate (Calcinit, Yara, Oslo, Norway; 21.5 g nitrate/kg diet DM).

The experimental protocol followed the same procedures as for the 2013 experiment.

Table 4.3 Ingredients composition of experimental diets (dry matter basis; g/kg)

Ingredient	Control	Nitrate
Grass Silage	210	211
WCBS	347	347
Bruised barley grain	336	388
Rapeseed meal	79	0
Calcinit	0	28
MDG	0	0
Molasses	19	20
Minerals*	9	9

WCBS, whole crop barley silage, MDG, maize dark grains, Calcinit, calcium nitrate.
*Contained (mg/kg): Fe, 6036; Mn, 2200; Zn, 2600; Iodine, 200; Co, 90; Cu, 2500; Se 30; (μg/kg): vitamin E, 2000; vitamin B12, 1000; vitamin A, 151515; vitamin D, 2500

4.2.2 DNA extraction from rumen fluid

DNA extraction was performed using the QIAmp DNA Stool Mini kit (Qiagen, Manchester) with a method based on the technique described by Yu and Morrison, (2004). The method is based on repeated bead beating plus column filtration. Ruminal fluid samples were centrifuged, most of the supernatant removed and 0.25 g of pellet content was transferred to bead tubes containing zirconia beads (0.3 g of 0.1 mm and 0.1 g of 0.5 mm). Then samples were homogenised for 3 min in total (30sec on, 5 minutes off) at speed 6.5 on FastPrep FP120 cell disrupter (Qbiogene, Inc., France). From this point onward, the Yu and Morrison (2004) technique was followed except for the final elution step. The final samples were diluted in AE buffer, either in 50 or 100 µl depending on the DNA concentration, and stored at -20º C in duplicate. DNA concentrations were determined with NanoDrop ND 1000 Spectrophotometer (Thermo Fisher Scientific). Sample quality was determined using 260/280 and 260/230 ratios. The ratio 260/280 expresses the ratio between nucleic acids (absorbance maxima at 260nm) and proteins (absorbance maxima at 280 nm). A ratio of ~1.8 is generally accepted for pure DNA. The samples used in this study had values between 1.8 and 1.9. A low value may be the result of a contaminant. The absorbance at 230 nm is accepted as being the result of other contamination. The expected ratio 260/230 is ~2.2. In this experiment if any value differed significantly from these reference values, DNA extraction from that sample was repeated. The samples used in this study had ratios 260/230 between 2.1 and 2.3. The DNA concentration was recorded and if values were higher than 400 ng/µl diluted in 50 ul of buffer, the sample was diluted again (added 50 µl of buffer). If the concentration was lower than 60 ng/µl, the DNA yield was considered insufficient and DNA extraction was repeated.

4.2.3 PCR amplification of 16S rDNA and amplicon library preparation

Total DNA extracted from individual rumen fluid samples were diluted to a concentration of 10 ng/µl with nuclease-free water. The amplicon libraries were generated by PCR amplification of the hypervariable V4 region of bacterial and archaeal 16S rRNA gene. The V4 region was amplified with region-specific primers (515F/806R) that included the Illumina flowcell adapter sequences. All primers contained the Illumina adapters for MiSeq sequencing. The reverse amplification primer also contained a unique twelve base barcode sequence for sample identification within the pool. The amplification primers were adapted from to include nine extra bases in the adapter region of the forward amplification primer (Caporaso et al., 2012) to support paired-end sequencing on the HiSeq/MiSeq (Illumina, San Diego, CA). The amplification and sequencing primers additionally contained a new pad region to avoid primer-dimer formation with the modified adapter (Appendix 4.1). The primer sequences, including the 2,167 valid secondary-structure checked Golay-barcoded reverse primers, are provided in Appendix 4.2. Primers were obtained from Integrated DNA Technologies (Leuven, Belgium).

16S rRNA gene amplicons were generated using 30 PCR cycles for Illumina MiSeq sequencing. For each DNA sample, 50 µl of reaction mix was prepared. The PCR solution (Phuc et al., 2009) contained 2.5 µl 10 µM of each Primer (forward and reverse), 1 µl of 10 µM Deoxynucleotide (dNTP) Solution Set, 100mM, 0.5 µl of Taq Polymerase (NEB Hot Start High-Fidelity DNA Polymerase), 10 µl of PCR Q5 Reaction Buffer, 10 µl of High GC content Enhancer, 21.5 µl of molecular grade water and 2 µl of DNA template. A no-template negative control (nuclease-free water) and a positive control (Deoxyribonucleic acid sodium salt from Escherichia coli strain B Genomic, from SIGMA), was used for each PCR reaction. Q5 Reaction Buffer, dNTPs and Taq Polymerase were distributed from New England Biolabs Ltd (Massachusetts). Amplification was performed as follows: hold at 30ºC, 95ºC for 2 minutes, followed by 30 cycles consisting of 95°C for 15 seconds, 68°C for 20 seconds, 72°C for 1 minute, and hold at 4°C (Figure 4.1).

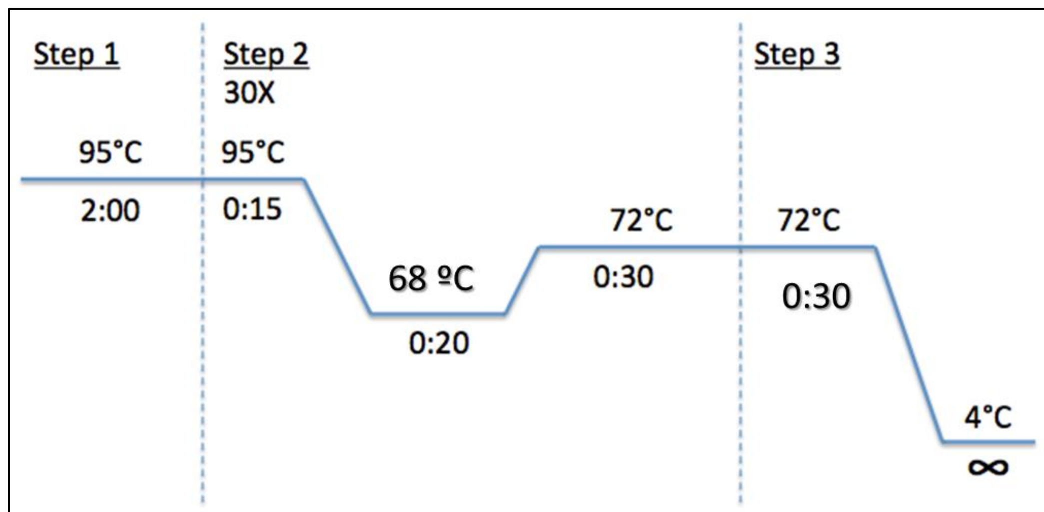


Figure 4.1 PCR reaction (0:00 minutes:seconds)

Libraries were purified using the QIAquick PCR Purification Kit (Qiagen, UK). Samples were eluted in 20 uL buffer EB (supplied in the kit). Samples were then measured on the QuBit (Qubit Fluorimeter 3 from ThermoFisher) and checked for size in a 2% agarose gel with 1x TAE (Figure 4.2). PCR products that showed a band around 390 bp were considered valid. Occasionally, negative template control from some PCR reactions showed a weak band. In those circumstances, tests were done to look for contamination and PCR reactions were repeated with new-clean reagents, tubes and water.

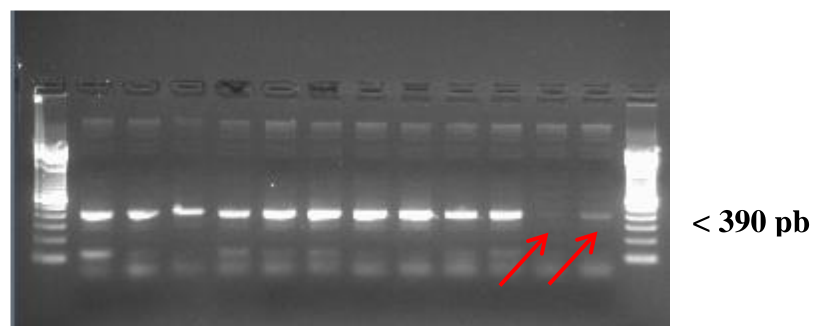


Figure 4.2 PCR products from a single PCR reaction

Penultimate sample is a NTC and the last sample is a Positive control

The barcoded amplicon libraries were then combined into two different groups according to samples classification: Group 1: Experiment 2; n=34, and Group 3: Experiment 1; n=40. The samples were pooled based on QuBit readings thus all libraries were present in the pool in similar concentrations and to obtain a total amount of between 1 and 2 ug DNA. Pools were checked on an agarose gel (2 %). To load the gel, three wells of the gel comb were taped together to result in one large well that allowed 100 ul of pooled library and Green loading Dye (10:7). The pools were run on a 1% agarose gel with 0. 5% TAE for 1.5 h at 110 v. Gels were visualised with a Dark Reader and the band was cut out with a sterile scalpel (Figure 4.3). The band was put in a sterile 25 ml plastic tube. Each gel slice was purified to remove nucleotides, enzymes, salts, agarose, and other impurities from samples, using the QIAquick Gel Extraction Kit (Qiagen, UK) following the manufacturer's specification with the following modifications: isopropanol was not added and the agarose gel band was dissolved by leaving it at room temperature for around 20 mins with hand shaking every five minutes. An extra purification with the QIAquick purification kit was used to remove residual agarose.

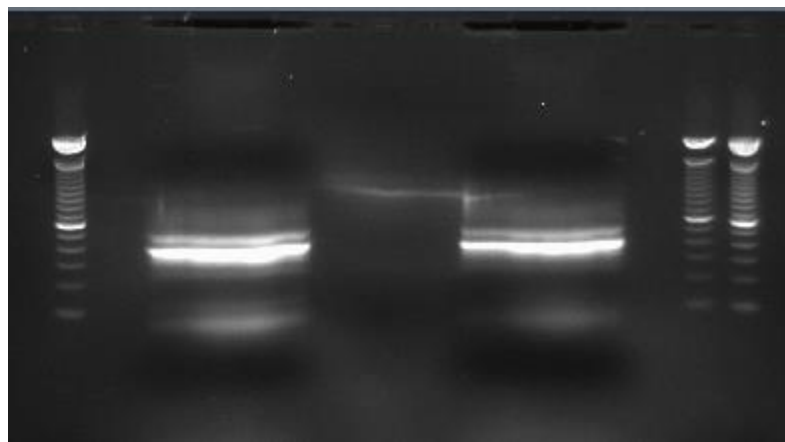


Figure 4.3 1% agarose gel image of the two pools

4.2.3.1 Amplicon library submission and sequencing

The pools of gel-purified libraries were then measured for purity and quantity on the Nanodrop 1000 and further quantified using QuBit and library size was checked on TapeStation (Agilent 2200 Tapestation Instrument). The TapeStation method was recommended by Edinburgh Genomics as the most accurate way to quantify the samples (Figure 4.4). The average size of fragments was 390 bp and the concentration above the minimum recommended from Edinburgh Genomics (>10 nM). The process workflow for rumen fluid samples is shown in Figure 4.6.

The two pools were sent for sequencing to Edinburgh Genomics (Edinburgh Genomics, Ashworth Laboratories, The King's Buildings, University of Edinburgh, Scotland). At the time of library submission, 45 µL of the primers that will be used for sequencing and indexing (15 ul per each pool) were sent (Sequencing Primer Read 1; Sequencing Primer Read 2 and Index sequence primer) in 1.5 mL low bind microfuge tubes (Ambion AM12450). Sequences are provided in Appendix 4.2. Samples passed the Quality Control measurements in Edinburgh Genomics facilities (Figure 4.5).

After cluster formation on the MiSeq instrument, the amplicons were sequenced with custom primers. Samples were sequenced on two paired-end MiSeq runs: The barcode is read using a third sequencing primer in an additional cycle. Once the sample has been brought down to 2nM, the MiSeq Protocol provided by Illumina should be followed for preparation of the library for sequencing.

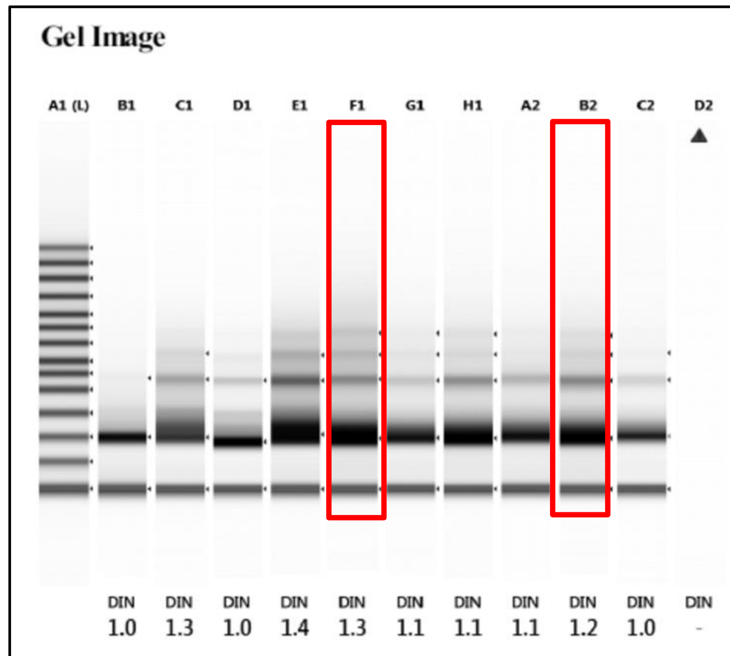


Figure 4.4 Tape Station image. F1Pool 1; B2 Pool 2

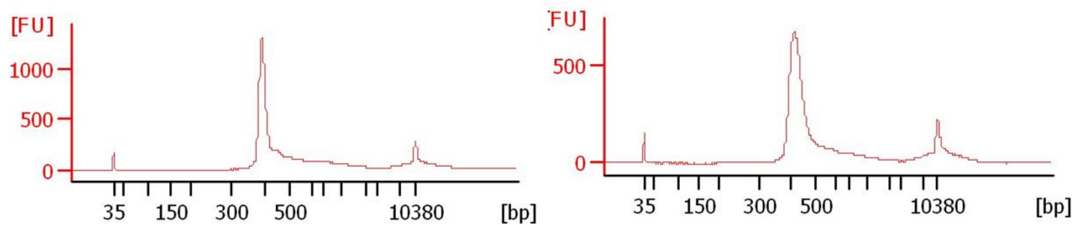
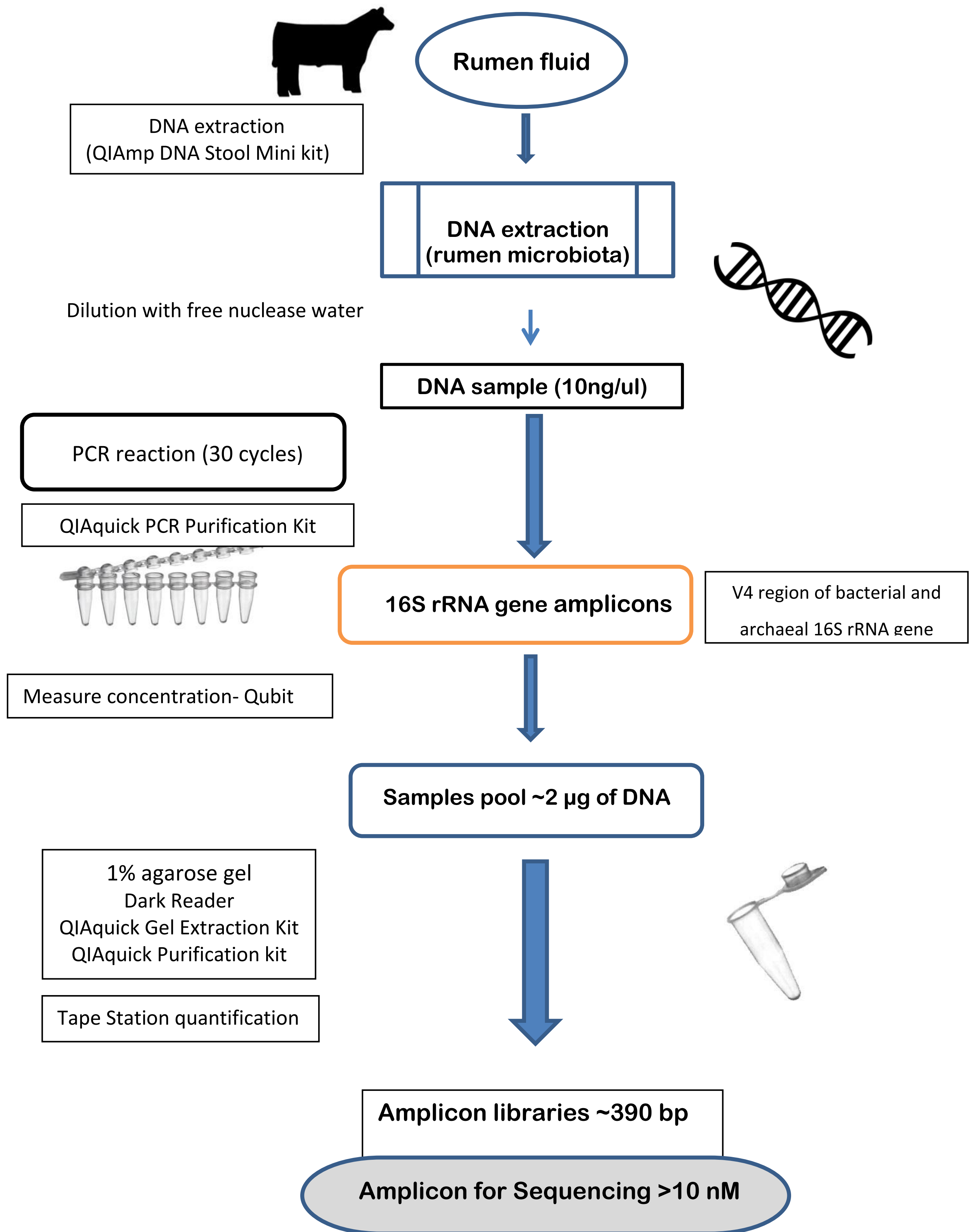


Figure 4.5 Electropherogram of amplicon libraries (Ed. Genomics)

Figure 4.6 Process workflow from rumen fluid collection to Amplicon for sequencing



4.2.4 Bioinformatics and statistical analysis

For CH_4, and H_2 data (g/kg DMI), the General Analysis of Variance procedure of GenStat (version 11.1 for Windows; VSN International Limited) was used with diet as treatment structure. Sequencing analysis was performed by Edinburgh Genomics (Appendix 4.3). In brief, taxonomy assignment of OTUs was performed using QIIME (version 1.9.1) with RDP (Ribosomal Database Project) classification method. Database used for this was GreenGenes 13_8 (latest version, preferred by QIIME). Operational Taxonomic Units (OTU) were assigned with a RDP confidence of >0.8. OTUs with less than 100 reads across samples were removed.

Taxonomies that had zero relative abundance across any individual diet (at genus level) were removed to give a core microbiome across all diets. The number of sequences assigned to individual OTUs was converted to relative abundance by dividing the number of reads aligned to each OTU per sample by the total number of reads present in that sample and multiplying by 100. Alpha diversity was estimated and Shannon diversity indexes were calculated to assess the differences within treatment. Shannon index values are calculated using log base 10. Differences in Shannon diversity indexes between diets were assessed. Shannon diversity index is a quantitative measure that reflects how many different species or OTUs are in a group and is commonly use to present alpha diversity. The Shannon index increases as both the richness and the evenness of the community increase. Filtered relative abundances of OTUs data for each sample were exported to Unscrambler and PCA calculated, using the singular value decomposition (SVD) algorithm, and all sample having equal weighting. The effect of dietary treatment in PC scores was investigated using analysis of variance. Effects of diet and CH_4 yields on PC scores was assessed using REML using diet* CH_4 as fixed model.

General Analysis of variance was used to assess differences between diets in the main phyla, families, genera and OTUs of interest. Where significant different between treatments were detected $P<0.05$, differences between means were identified using least significant differences (GenStat). Correlation between all OTUs and CH_4 data were assessed with Excel correlation test. Spearman correlations were done between relevant OTUs, families and genus and CH_4 yield with Minitab software. Correlations between main microbial taxonomies and H_2 produced were assessed. Values with $P < 0.05$ were classed as significant, with particular interest being taken in taxonomies associated with CH_4 emissions.

4.3 RESULTS

4.3.1 Experiment 1

4.3.1.1 CH_4 yield (g/kg DMI)

Methane production (g/kg of DMI) was greater from steers fed mixed diet compared with steers fed concentrate diet (P<0.01). The average CH_4 production for the mixed and the concentrate diets was 22.9±5.1 and 15.2±3.3 g/kg DMI, respectively. Steers fed the concentrate diet produced less H_2 (0.03 vs 0.09 g/kg DMI; P<0.01) and had smaller acetate to propionate ratio than steers fed the mixed diet (Troy et al., 2015).

When CH_4 production (g/kg DMI) from steers fed mixed diets with (n=10) or without (n=10) the addition of nitrate was studied, CH_4 (g/kg DMI) production tended to be smaller (25 vs 21 g/kg DMI; P=0.07) from steers consuming nitrate containing diets compared with steers not consuming nitrate. There was no reduction in CH_4 production when nitrate was added to the concentrate diet (15.3 vs 15.1 g/kg DMI; P=0.9) (Figure 4.7). Nitrate addition to mixed diet had greater H_2 production (0.05 vs 0.13; P<0.01), where addition of nitrate to the concentrate diet did not change H_2 production (0.02 vs 0.04; P>0.05). Acetate to propionate ratio was greater in both diets with nitrate addition (Troy et al., 2015).

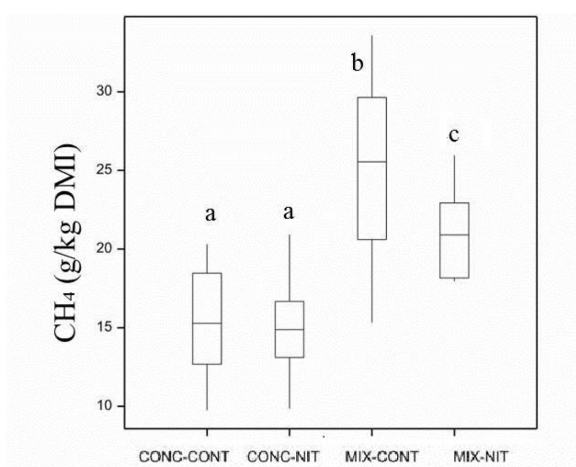


Figure 4.7 Boxplot of CH_4 produced (g/kg DMI) by steers fed the mixed and concentrate diets with and without the addition of nitrate

4.3.1.2 Analysis of Microbial composition (16S rRNA)

After filtering, a total of 8,261,790 reads were obtained for the 40 samples, giving an average of 206,545 ±133,470 reads per sample that were subsequently assigned to an OTU. The number of archaea sequences was 99,117 (1.2% of total OTUs) with an average of 2,478 ±2,066 sequences per sample.

Diversity within group (alpha diversity) was within the expected range (1.2-3.0) for all groups. The mean value across all groups was 2.3. Concentrate diet groups had lower Shannon diversity than mixed diet groups (Table 4.4).

Table 4.4 Shannon index values of samples from steers fed the mixed and concentrate with and without addition of nitrate

Diet	Concentrate Control	Concentrate Nitrate	Mixed Control	Mixed Nitrate	P-value
Shannon Index	1.93[a]	1.96[a]	2.51[b]	2.82[b]	0.001
N of samples	10	10	10	10	

Phylum: Once unclassified taxonomies were removed, 22 taxonomies were recorded at the phylum level. Spirochaetes, Firmicutes, Bacteroidetes, Proteobacteria, Euryarchaeota, Actinobacteria and Fibrobacteres were present at relative abundances >1% across all diets (Table 4.5). Bacteroidetes was the most abundant phylum, representing an average of 42.8±16.9 % (mean ± stdev) of all sequences, followed by Firmicutes (24.3±8.9 %), Proteobacteria (23.9±24.2 %) and Spirochaetes (2.7±6.7 %). Figure 4.8 represents relative abundance at phylum level for all samples corresponding to the 4 different dietary treatments.

At the *family* level, 127 taxonomies were recorded and 210 taxonomies were recorded at the genus level. Once unclassified taxonomies were removed, 97 families remained. OTUs: The number of OTUs identified across all groups was 1957, 11 belonging to archaea and the remaining to bacterial populations.

Table 4.5 Mean relative abundance of main phyla across mixed and concentrate diets with and without nitrate

Taxonomy (Phylum)	Concentrate Control	Concentrate Nitrate	Mixed control	Mixed Nitrate	SED	P-value
Bacteroidetes	43.5[a]	29.8[b]	48.7[a]	49.3[a]	6.95	0.028
Firmicutes	20.6[a]	20.2[a]	26.5[ab]	29.2[b]	3.66	0.032
Proteobacteria	31.0[a]	40.9[a]	14.6[b]	9.1[b]	9.56	0.007
Fibrobacteres	0.2[a]	0.7[a]	2.1[b]	2.1[b]	0.51	0.001
Actinobacteria	0.8[a]	0.5[a]	0.7[a]	1.9[b]	0.49	0.039
Spirochaetes	1.4	5.8	1.6	1.8	3.00	
Euryarchaeota	1.0[a]	0.6[a]	2.3[b]	1.8[ab]	0.61	0.038
Verrucomicrobia	0.3[a]	0.4[a]	1.1[b]	1.5[b]	0.31	0.001

SED: Standard error of differences
Superscript: Different letters indicated that means between treatments were significantly different
Blank space indicated NS differences, P-value >0.05

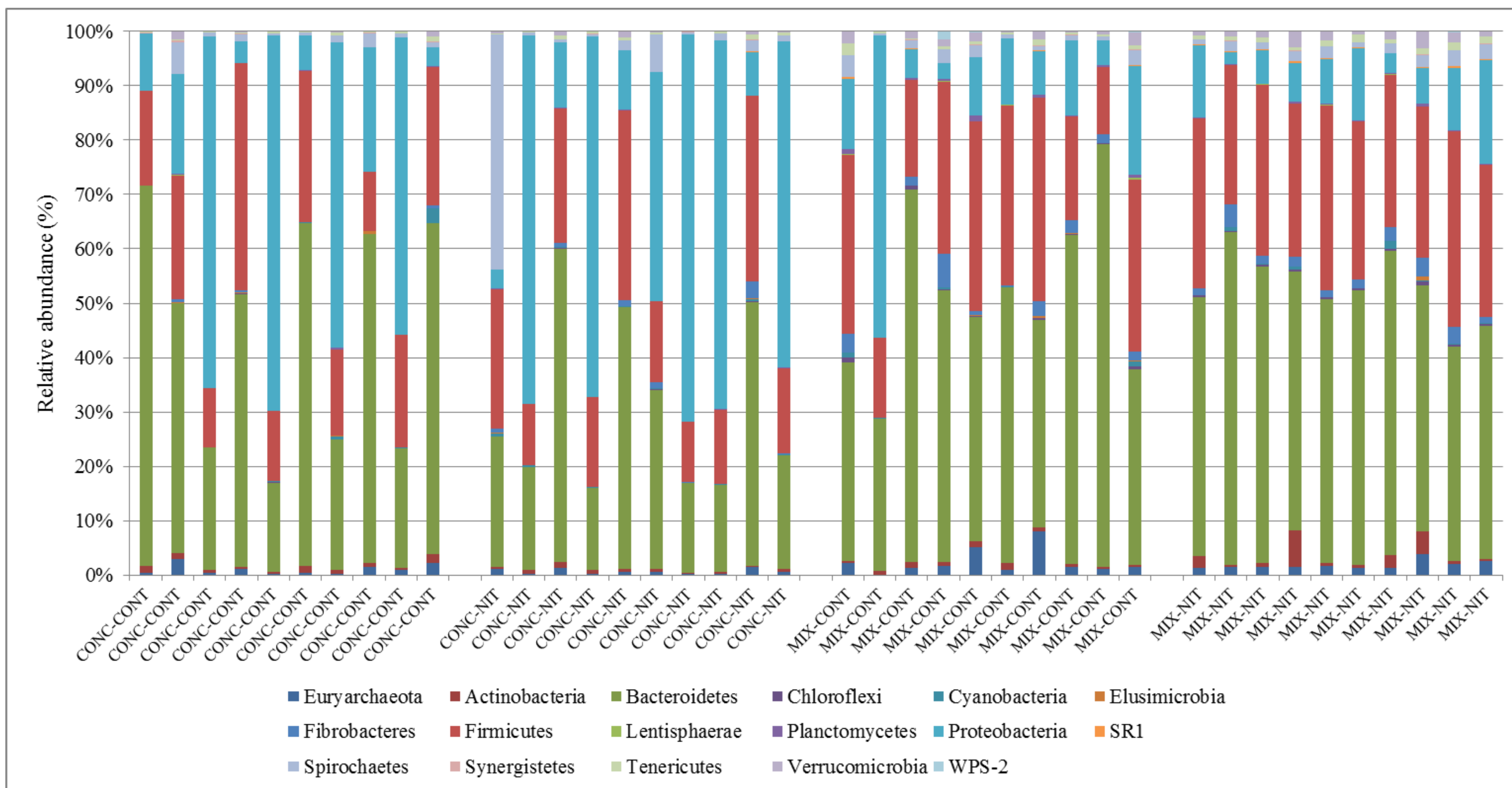


Figure 4.8 Plot of relative abundances at phylum level for individual rumen samples for mixed and concentrate diets with and without nitrate

Bacterial populations

Genus: At the genus level, 113 genera were classified. *Prevotella* dominated, representing on average 35.5±9.8% of all sequences, with 257 OTUs assigned to that genus. Other abundant genera were *Succiniclasticum* (3.3 ±1.9 %), *Ruminococcus* (2.4±1.7 %), *Treponema* (2.2±0.9 %) and *Butyrivibrio* (1.2±0.6 %).

Archaeal populations

The archaeal community was represented by Euryarchaeota phylum. The relative abundance of archaea community across all samples was 1.2±0.6 % of all sequences. *Orders* were represented by Methanobacteriales (class Methanobacteria) (98.0±0.6 %) and E2 (class Thermoplasmata) (1.9±0.03 %). The order Methanobacteriales order included the family *Methanobacteriaceae*, which was dominated by the genus *Methanobrevibacter*, which represented 95.6±4.6 % of all archaea sequences, followed by genus *Methanosphaer*a (2.3±1.3 %), and an unclassified group (0.18±0.19 %). Order Thermoplasmata was represented by the family *Methanomassiliicoccaceae*, identified at the genus level as *vadinCA11*.

OTUs: Eleven OTUs identified at the genus level were assigned to archaea community, including 5 OTUs identified as genus *vadinCA11*, 4 OTUs as *Methanobrevibacter* and 1 OTU as *Methanosphaera*.

4.3.1.3 Effect of basal diet on microbial community.

Phylum: The mean abundance of Bacteroidetes, Firmicutes, Fibrobacteres, Verrumicrobia and Tenericutes, Euryarchaeota phyla and Archaea: Bacteria (A:B) ratio were significantly higher for the mixed diet. Proteobacteria was significantly lower in the mixed diet compared with the concentrate diet (Table 4.6).

OTUs: Comparison of the relative abundance of OTUs detected in the mixed diet and concentrate diet groups showed that 46% of the OTUs were significantly different ($P<0.05$) between these two groups (Appendix 4.4).

Table 4.6 Phyla significantly different (P<0.05) between mixed and concentrate diets (Relative abundance %)

Phylum	Concentrate	Mixed	SED	P<0.05
Bacteroidetes	36.7	49.0	5.04	0.019
Firmicutes	20.4	28.2	2.55	0.004
Proteobacteria	36.0	11.8	6.71	0.001
Fibrobacteres	0.5	2.1	0.36	0.001
Euryarchaeota	0.8	2.1	0.43	0.006
Verrucomicrobia	0.3	1.3	0.22	0.001
Tenericutes	0.3	0.8	0.12	0.001
Archea:Bacteria	0.01	0.02	0.005	0.008

SED: Standard error of differences

Principal components (PC) scores plots between mixed and concentrate diet is presented in figure 4.10. PC-1 accounted for 69 % of the variation, PC-2 accounted for 8 %, and PC-3 accounted for 6 %. A grouping of mixed diet samples was observed for PC-1 when compared with PC-2 and PC-3. Diet has a significant effect on PC-1 (P< 0.001), but not for PC-2 (P= 0.371) and PC-3 (P=0.317). Regression analysis of diet, CH_4 yield and interactions with PC scores are summarised in Table 4.7. Diet, CH_4 yield and interactions were significantly different for PC-1, but not for PC-2 (Table 4.7). Figure 4.9 showed a linear regression between PC-1 score and CH_4 (g/kg of DMI) for concentrate diet.

Table 4.7 P-values of regression analysis between diet, CH_4 yield and interactions with PC scores

	PC-1	PC-2	PC-3
CH_4 (g/ kg DMI)	0.001	0.062	0.215
Diet	0.001	0.943	0.052
Interaction	0.002	0.368	0.049

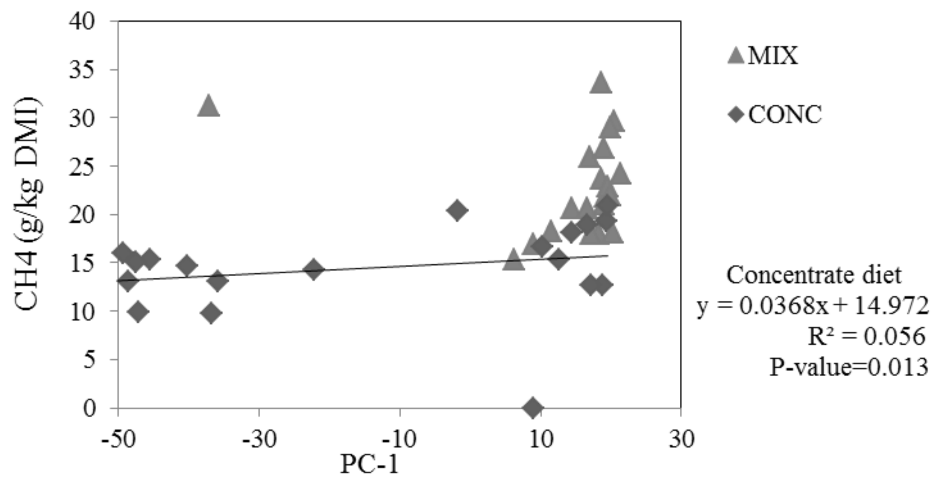


Figure 4.9 Correlation between CH_4 yield and PC-1 score for mixed and concentrate diet

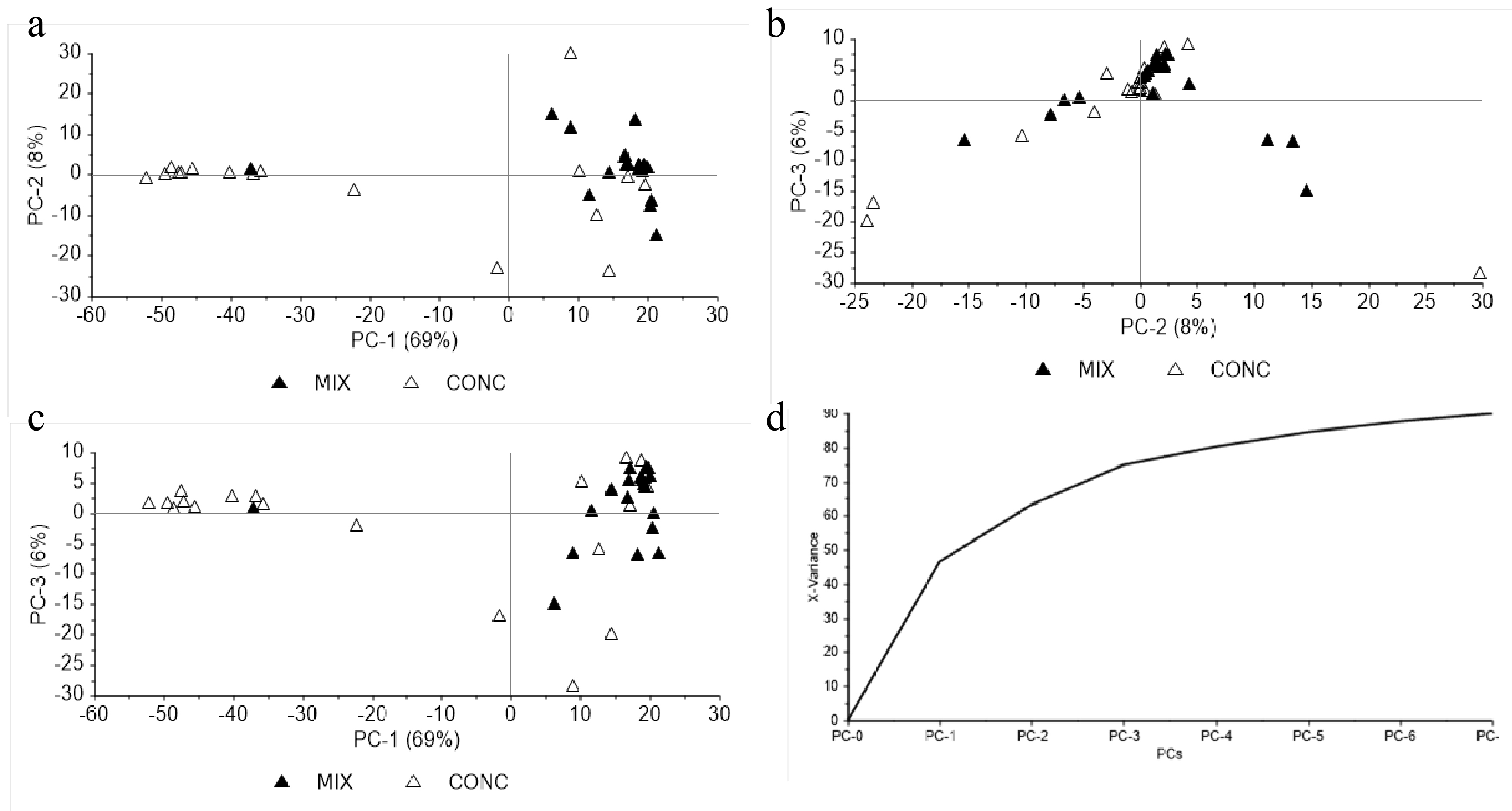


Figure 4.10 PCA overview of 16S rRNA gene analysis of concentrate and mixed diets (a) scores plot for PC-1 vs. PC-2 (b) scores plot for PC-3 vs. PC-2 (c) scores plot for PC-1 vs PC-3 (d) explained variance plot

Family: When families presented at abundance greater than 1% were selected, 16 families remained which accounted for more than 90% relative abundance of microbial populations across all samples (Table 4.8). Families *Paraprevotellaceae* and *Prevotellaceae* were present at greater abundances in mixed diets compared with concentrate diets. Similarly, some families belonging to Firmicutes (*Ruminococcaceae, Clostridiaceae,* Unknown (order Clostridiales*)* and *Mogibacteriaceae*) had greater relative abundances in mixed diets compared with control diets. More details are presented in Table 4.8.

Table 4.8 Relative abundances of families in samples from steers fed concentrate and mixed diets

Phylum	Family	Concentrate	Mixed	SED	P-Value
Bacteroidetes	*Prevotellaceae*	31.0	38.9	4.78	0.007
	Unknown (order Bacteroidales*)*	1.8	2.9	0.55	0.045
	Paraprevotellaceae	1.8	3.6	0.53	0.002
Firmicutes	*Veillonellaceae*	10.1	9.7	1.82	
	Ruminococcaceae	3.3	6.1	1.02	0.008
	Lachnospiraceae	3.6	4.4	0.78	
	Clostridiaceae	0.9	2.1	0.22	0.001
	Unknown (order Clostridiales)	1.0	3.0	0.32	0.001
	Mogibacteriaceae	0.4	0.8	0.11	0.001
	Erysipelotrichaceae	0.7	0.7	0.18	
Proteobacteria	*Succinivibrionaceae*	35.6	11.4	6.75	0.001
Fibrobacteres	*Fibrobacteraceae*	0.5	2.1	0.36	0.001
Spirochaetes	*Spirochaetaceae*	3.6	1.7	2.10	
Verrumicrobia	*RFP12*	0.2	1.1	0.18	0.001

Genus: Genera presented at abundances of greater than >1% were further studied. Relative abundance of genus *Ruminobacter*, *Ruminoccocus*, genera belonging to Clostridiales and Bacteroidales, and genus *Fibrobacter* were higher in mixed diet compared with concentrate diet (P<0.05). The relative abundances of *Prevotella, Treponema* and *Succiniclasticum* were not different between concentrate and mixed diets (P>0.05) (Table 4.9).

Table 4.9 Relative abundances of genera in samples from steers fed concentrate and mixed diets

Family	Genus	Concent	Mixed	SED	P-value
Prevotellaceae	*Prevotella*	30.7	38.0	4.83	
Succinivibrionaceae	Unknown *g*	35.9	9.4	6.76	0.001
	Ruminobacter	0.0	1.9	0.55	0.002
Veillonellaceae	*Succiniclasticum*	5.5	7.6	1.24	
Lachnospiraceae	*Butyrivibrio*	5.5	7.6	1.24	
Ruminococcaceae	*Ruminococcus*	2.5	5.0	0.91	0.010
Spirochaetes	*Treponema*	3.6	1.6	2.12	
Fibrobacteraceae	*Fibrobacter*	0.5	2.1	0.36	0.001
Unknown (Order Clostridiales)		1.0	3.0	0.32	0.001
Unknown (Order Bacteroidales)		1.8	2.9	0.55	0.045

Archaea community composition between mixed and concentrate diet

At the family and genus levels, the relative abundance of methanogenic populations and the methanogenic community structure were different between mixed and concentrate diets (Table 4.10) with greater relative abundance of *Methanobacteriacea* in mixed diet compared with concentrate diet. This result was mainly explained by the most abundant methanogen (*Methanobrevibacter*, 97.5%) that was more abundant in samples from the mixed diet compared with concentrate diet. In contrast, methylotrophic methanogenic population represented by family *Methanomassiliicoccaceae* (genus *VadinCA11*) were more abundant in samples from the concentrate diet.

OTUs: OTUs 964 (identified as genus *Vadin CA11*), and OTU 26 (identified as genus *Methanobrevibacte*r) were less abundant in samples from the concentrate diet (Figure 4.11).

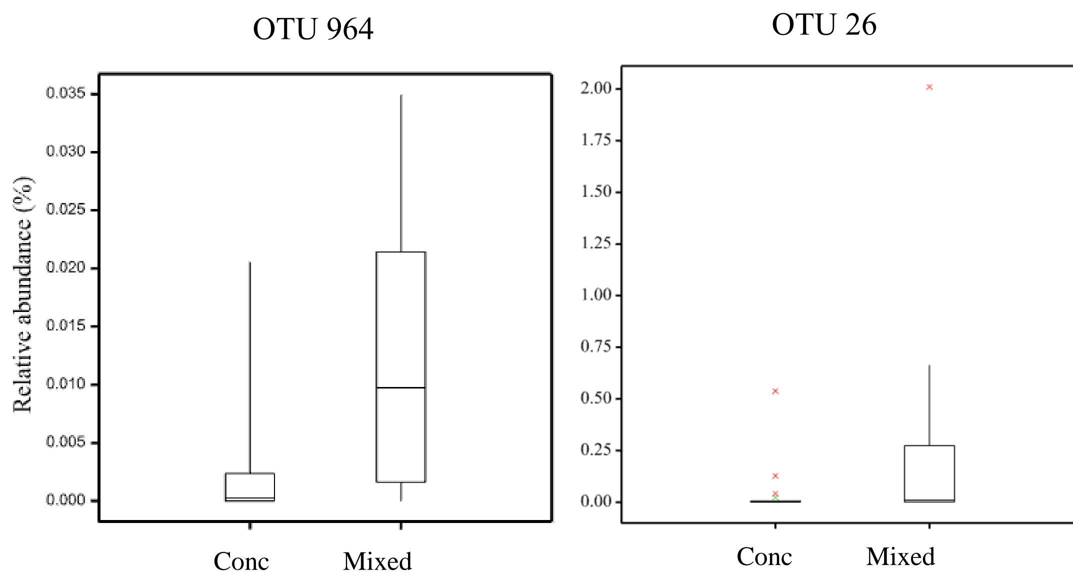


Figure 4.11 Relative abundances of OTUs 964 (genus *VadinCA11*) and OTU 26 (genus *Methanobrevibacter*) in samples from steers fed the concentrate and mixed diets

Table 4.10 Differences in relative abundances of archaeal populations between mixed and concentrate diets

Family	Genus	OTU	Concentrate	Mixed	SED	P-value
Methanobacteriaceae			0.81	2.03	0.43	0.007
	Methanobrevibacter		0.79	1.98	0.42	0.007
		OTU 26	0.04	0.22	0.111	<0.001
	Methanosphaera		0.02	0.05	0.01	0.001
Methanomassiliicoccaceae	*vadinCA11*		0.04	0.02	0.01	0.016
		OTU 964	0.002	0.013	0.0025	<0.001
Total Archaea			0.82	2.07	0.43	0.006

4.3.1.4 Effect of nitrate on microbial community

Phylum: The relative abundance of Bacteroidetes was not different between mixed diets but it was present at smaller relative abundance in nitrate concentrate diet compared with concentrate control diet (29.8 vs 43.5 %; P=0.028), showing an interaction between basal diet and nitrate. Actinobacteria relative abundance was greater with nitrate addition to mixed diet (1.9 vs 0.7 %, P=0.039). No significant changes were observed in mean relative abundance for the rest of representative phyla with the addition of nitrate to control diets (Table 4.5 above).

OTUs: 27% of all OTUs were significantly different for mixed nitrate diets compared with control diets, and 14% were significantly different for concentrate nitrate diets compared with concentrate control diet (Appendix 4.5). PC scores plots showing overall differences between treatments are presented in Figure 4.12 and 4.13. PC-1 accounted for 46 % of the variation, PC-2 accounted for 17 %, and PC-3 accounted for 12 % in mixed diets (Figure 4.12). A slight grouping of mixed nitrate samples was observed for PC-2. In concentrate diet PC-1 accounted for 69% of the variance. No grouping was observed for concentrate diet treatment groups (Figure 4.13). Nitrate addition (diet effect) to mixed and concentrate diets had no significant effect on any of the PC-scores ($P > 0.05$). The regression analysis showed that CH_4 yield were significantly different for PC-2 in mixed and concentrate diets. In addition an interaction between diet and CH_4 was observed for PC-3 within concentrate diet ($P<0.05$) (Table 4.11).

Table 4.11 The effects of diet and CH_4 production on 16S results using PC scores (P-values)

	Mixed diet			Concentrate diet		
	PC-1	PC-2	PC-3	PC-1	PC-2	PC-3
CH_4 yield	0.879	0.007	0.443	0.293	0.040	0.253
Diet (+nitrate)	0.958	0.369	0.668	0.143	0.093	0.065
Interaction	0.822	0.507	0.531	0.174	0.059	0.037

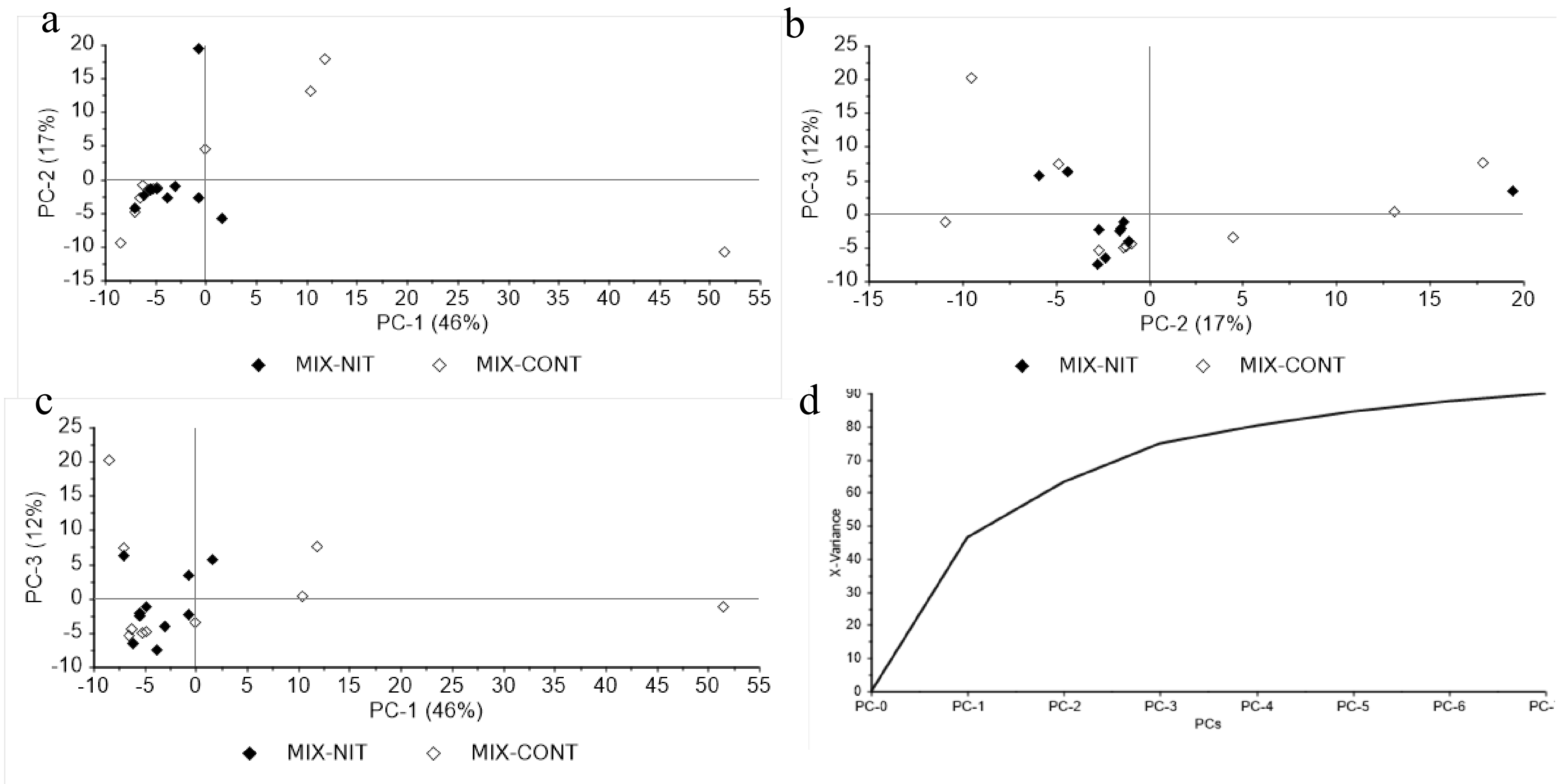


Figure 4.12 PCoA showing the relationship of samples from mixed diet based on all OTUs with or without addition of nitrate to diets (a) scores plot for PC-1 vs. PC-2 (b) scores plot for PC-3 vs. PC-2 (c) scores plot for PC-1 vs PC-3 (d) explained variance plot.

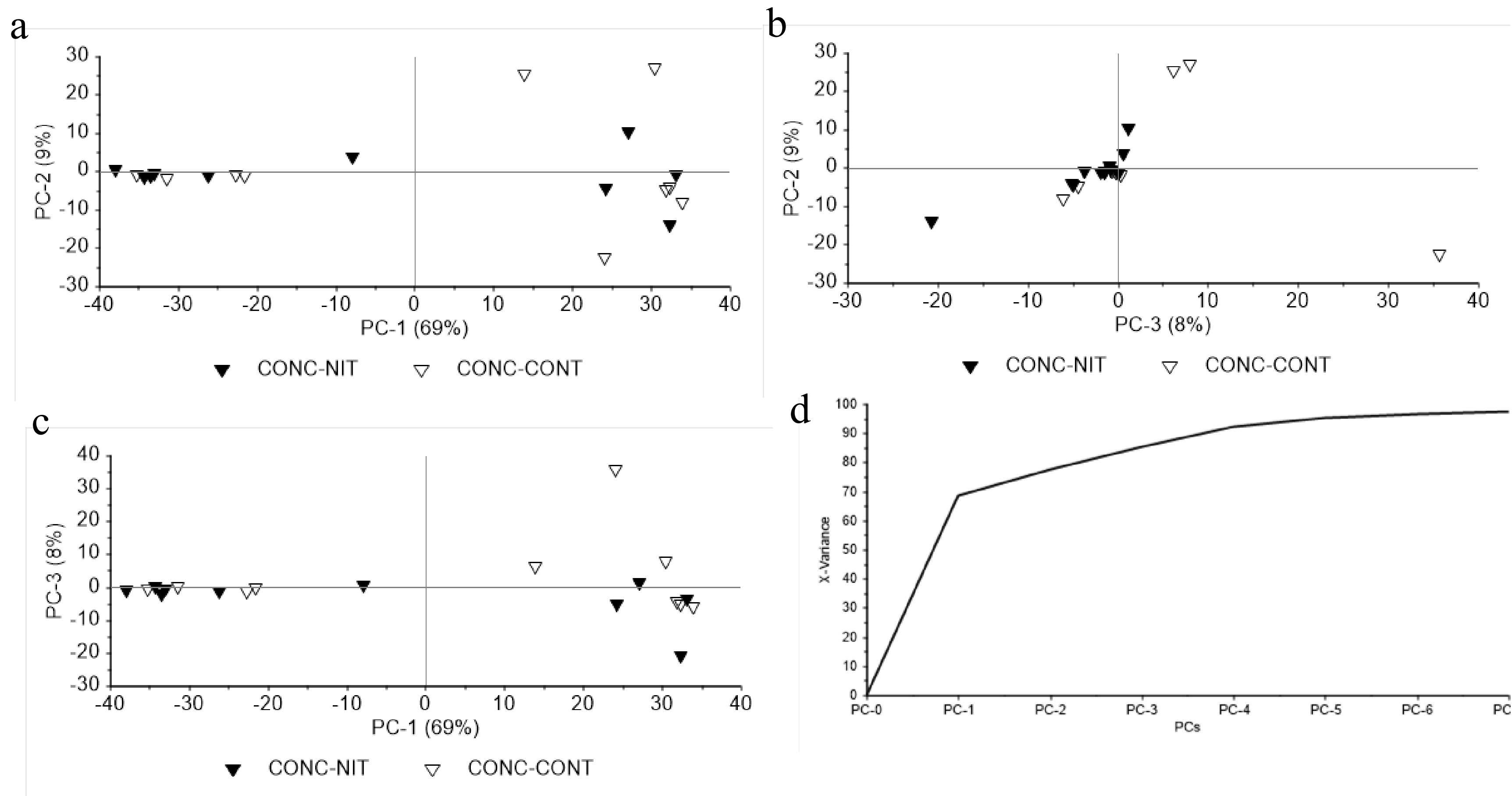


Figure 4.13 PCoA showing the relationship of samples from concentrate diets based on all OTUs with or without addition of nitrate to diets (a) scores plot for PC-1 vs. PC-2 (b) scores plot for PC-3 vs. PC-2 (c) scores plot for PC-1 vs PC-3 (d) explained variance plot.

Bacterial community composition affected by nitrate

Relative abundance of *genera* present at >0.5% were studied (Table 4.11) *Ruminoccocus* was higher (P<0.05) with diets containing nitrate compared with controls (4.8 vs 2.7 % on average).

Table 4.11 Main genus relative abundance on samples from steers fed the concentrate and mixed diets with and without nitrate addition

	Con-Cont	Con-Nit	Mix-Cont	Mix-Nit	SED	P-value
Prevotella	38.1	23.4	39.5	36.5	6.56	
Succiniclasticum	6.1	5.0	7.8	7.4	1.78	
Ruminococcus	1.4[a]	3.5[b]	3.9[b]	6.0[c]	1.23	0.008
Unknown	30.0[a]	40.5[a]	12.5[b]	6.3[b]	9.63	0.004
Butyrivibrio	2.9	1.3	1.4	1.6	0.48	
Treponema	1.4	5.8	1.6	1.7	3.00	
Fibrobacter	0.2[a]	0.7[a]	2.0[b]	2.1[b]	0.51	0.001

Archaeal community composition affected by nitrate

Nitrate did not affect the total number of archaea. Overall nitrate had little effect on the methanogenic community structure. Only a smaller abundance of *Methanosphaera* was found when nitrate was included in the concentrate diet (P=0.042; Table 4.12).

OTUs: When looking at OTUs assigned to archaea phyla, 2 OTUs were affected by nitrate addition across all diets (P<0.05); OTU 961: family *Methanobacteriaceae* and OTU 215: genus *Methanosphaera* had lower relative abundance when nitrate was added to the diets (concentrate and mixed). In addition, the relative abundance of some methylotrophics OTUs archaea were affected by nitrate depending on the basal diet; OTU 897 (genus *vadinCA11)* was presented at higher abundance with nitrate addition to mixed diets (P=0.013) and OTU 692 (genus *vadinCA11)* had lower relative abundance with nitrate addition to concentrate diets (P=0.007).

Table 4.12 Families, genus and some OTUs archaeal populations from samples from steers fed the concentrate and mixed diets with and without nitrate addition

Family	Genus	OTU	Conc Control	Conc Nitrate	SED	P-value	Mixed Control	Mixed Nitrate	SED	P-value
Methanobacteriaceae			1.0	0.6	0.32		2.3	1.8	0.80	
		OTU 961	0.002	0.000	0.0013	0.005	0.006	0.000	0.002	<0.001
	Methanobrevibacter		1.0	0.6	0.32		2.2	1.7	0.79	
	Methanosphaera		0.02	0.01	0.007	0.042	0.06	0.04	0.014	
		OTU 215	0.023	0.008	0.0067	0.003	0.058	0.036	0.0142	0.033
Methanomassiliicoccaceae	*vadinCA11*		0.02	0.01	0.010		0.03	0.04	0.012	
		OTU 897	0.000	0.002	0.0021		0.000	0.007	0.0042	0.013
		OTU 692	0.008	0.001	0.0035	0.007	0.011	0.008	0.004	
Total Archaea			1.0	0.6	0.33		2.3	1.8	0.81	

Nitrate reducers

Organisms which have been identified as nitrate reducers were investigated. *Wolinella succinogenes, Veillonella parvula* and *Mannheimia succiniciproducens* were not identified in the current study. Only one OTU was identified as *Selenomonas ruminantum* (OTU 86) which did not present different relative abundances (P>0.05) with nitrate addition to mixed and concentrate diets.

Potential bacterial denitrifiers such as *Pseudomonas aeruginosa*, *Propionibacterium* and *Nitrosomonas spps.* were not identified in this study. *Campylobacter* species have been identified as potential nitrate-reducer populations and some species as food borne pathogens and it was found that the relative abundance of *Campylobacter* was differently affected by nitrate addition to mixed diet than to concentrate diet, with a significantly greater relative abundance when nitrate was present in mixed diets (P=0.001) but with no differences in relative abundance with nitrate addition to concentrate diets (P=0.150) (Table 4.13 and Figure 4.14). OTU 686 identified as genus *Campylobacter* was present at greater abundance (P=0.001) in nitrate diet samples compared with control samples within concentrate and mixed diets.

Table 4.13 Relative abundances (%) of genus *Campylobacter* and OTU 686 identified as Campylobacter genus

Taxonomies	Conc Cont	Conc Nit	SED	P-value	Mixed Cont	Mixed Nit	SED	P-value
Campylobacter	0.005	0.045	0.242		0.004	0.030	0.004	<0.001
OTU 686	0.004	0.015	0.009	<0.001	0.004	0.029	0.009	<0.001

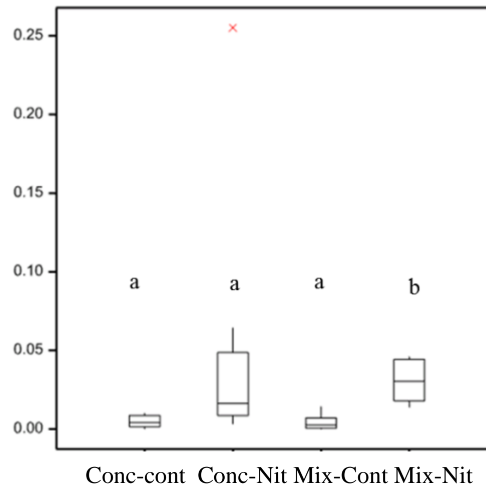


Figure 4.14 Relative abundance of genus *Campylobacter* from steers fed the concentrate and mixed diets with and without nitrate addition

4.3.1.5 Taxonomic populations correlated with methane production and nitrate metabolism

Selemonas species have been observed to play an important role in nitrate and nitrite reduction in the rumen (Asanuma et al., 2015) and *Succinivibrionaceae* has been negatively correlated with CH_4 emission (Danielsson et al., 2017; Tapio et al., 2017; Wallace et al., 2015). Accordingly, a general screening was performed to look for differences between treatments in taxonomies potentially related to CH_4 and nitrate metabolism (genus *Selenomonas*, Family *Succinivibrionaceae* and genus *Succinivibrio*). The relative abundance of the family *Succinivibrionaceae* was greater ($P<0.001$) in concentrate diet (35.6 %) compared with mixed diet (11.4 %). However, the results showed no statistically significant differences for most of them as a consequence of nitrate addition (Table 4.14). Genus *Succinivibrio* was significantly higher with nitrate addition to the mixed diet (0.07 vs 0.20±0.05, P=0.02). OTUs: OTU 3685 identified as *Succinivibrio* was significantly lower with nitrate addition to all diets whilst OTU 201, also belonging to genus *Succinivibrio*, was significantly higher with nitrate addition to concentrate diets. OTU 1 identified as family *Succinivibrionaceae* was lower with nitrate addition to mixed diets (Table 4.14).

Table 4.14 Relative abundance of taxonomic populations potentially correlated with CH_4 production from concentrate and mixed basal diets with and without addition to nitrate.

Family	Genus	OTU	Con-Cont	Con-Nit	SED	P-value	Mix-Cont	Mix-Nit	SED	P-value
	Selenomonas		0.13	0.35	0.210		0.37	0.50	0.160	
Succinivibrionaceae			30.6	44.8	12.57		14.3	8.5	5.12	
		OTU 1	28.9	39.5	13.19		8.4	2.3	5.49	0.011
	Succinivibrio		0.08	0.06	0.050		0.07	0.20	0.050	0.020
		OTU 3685	0.012	0.006	0.0107	0.001	0.002	0.000	0.0011	0.014
		OTU 201	0.000	0.029	0.0280	<0.001	0.055	0.195	0.0517	

SED: Estandard error of differences

Correlations were assessed between main families and genera within samples from mixed and concentrate fed steers with CH_4 yields (Appendix 4.6). Overall, no correlations were found between main bacterial family and genera in concentrate diets. Interestingly, *Prevotella* was positively correlated with CH_4 production within concentrate diet (0.521, 0.022) and negatively within mixed diets (-0.441, 0.001) although the relative abundances was not different between treatments.

In addition, correlations between all OTUs and CH_4 data were assessed. OTUs correlated with CH_4 and present at higher abundance (>0.1%) are presented in Appendix 4.7 .When correlations between OTUs and CH_4 were checked within mixed and concentrate diet only 5 OTUs were correlated with CH_4 production (Table 4.15).

Table 4.15 OTUs correlated with CH_4 in both mixed and concentrate diets

		Concentrate		Mixed	
OTUs	Taxonomy	R	P-value	R	P-value
OTU_574	Order Bacteroidales	0.563	0.009	0.445	0.043
OTU_449	*Clostridium*	0.444	0.048	0.512	0.020
OTU_3579	*Prevotella*	0.466	0.037	0.503	0.023
OTU_961	*Methanobacteriaceae*	0.485	0.029	0.445	0.048
OTU_708	*Ruminococcaceae*	0.454	0.043	0.519	0.018

Correlations between archaea taxonomies and CH_4 are presented in Table 4.16. Total archaea was correlated with CH_4 production in mixed and concentrate diets. In addition, genus *Methanobrevibacter* and OTU 9 belonging to that one were positively correlated with CH_4 across mixed and concentrate diets (Table 4.16). Linear regression was studied between total archaea (Figure 4.15), genus *Methanobrevibacter* and OTU 9 (Figure 4.16).

Table 4.16 Correlation between archaea population and CH_4 production within mixed and concentrate diets

Samples		OTUs	Concentrate		Mixed	
Family	Genus		Ro	P-value	Ro	P-value
Methanobacteriaceae			0.842	0.002		
	Methanobrevibacter		0.764	0.010	0.427	0.028
		OTU 9	0.796	0.006	0.537	0.015
	Methanosphaera					
Methanomassiliicoccaceae	*vadinCA11*		0.855	0.002		
Total Archea			0.774	0.009	0.415	0.025
Ratio A:B			0.773	0.009	0.415	0.025

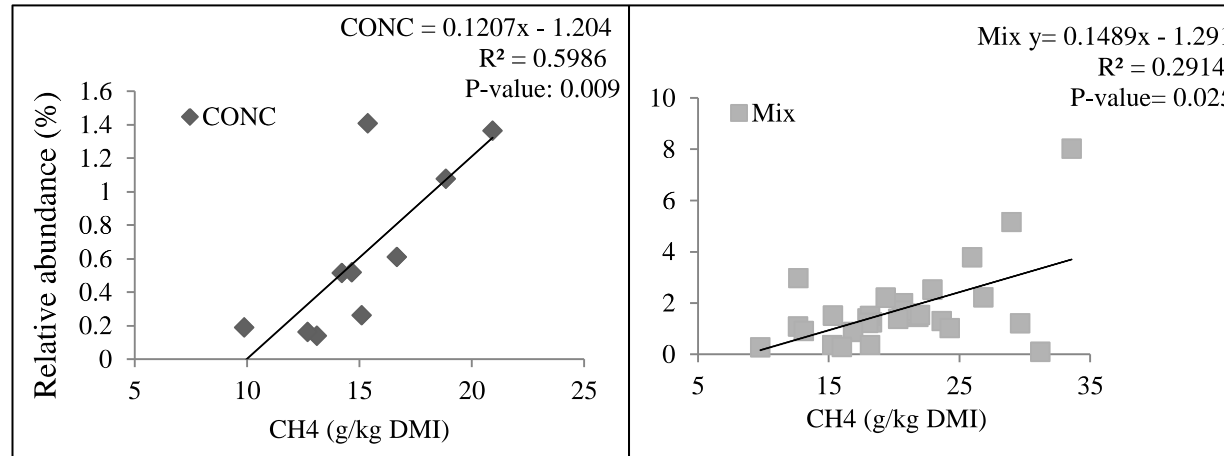
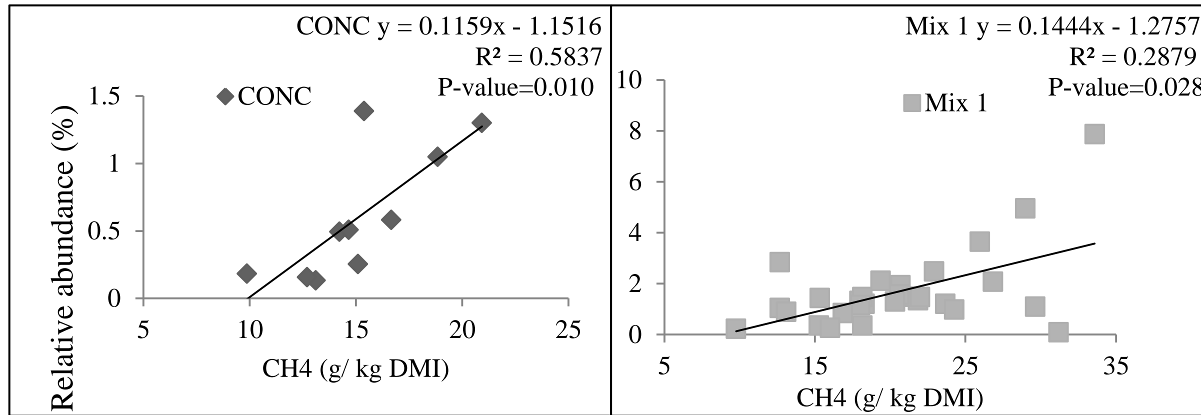


Figure 4.15 Linear regression between Total Archaea with CH_4 (g/kg of DMI) within mixed and concentrate basal diets

Methanobrevibacter



OTU 9 (*Methanobrevibacter*)

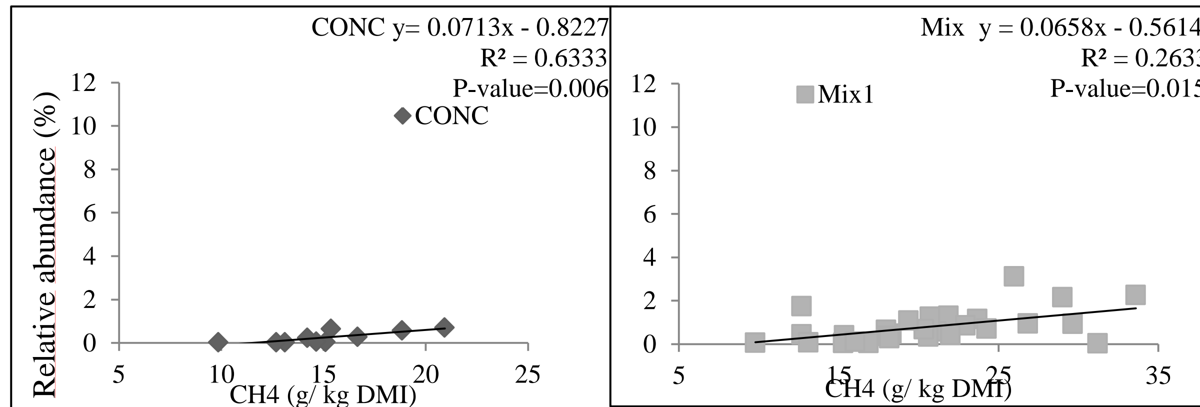


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Figure 4.16 Linear regression between *Methanobrevibacter* genus and OTU 9 with CH_4 (g/kg of DMI) within mixed and concentrate basal diets

4.3.2 Experiment 2

4.3.2.1 CH_4 yield (g/kg DMI)

Methane emission was significantly lower in nitrate mixed diets (n=17) compared with control mixed diets (n=17) (P<0.05) (Figure 4.17). Steers receiving nitrate produce more H_2 (0.09 vs 0.04 g/kg DMI) and had greater acetate to propionate ratio than animals in the control diets (Duthie et al., 2016).

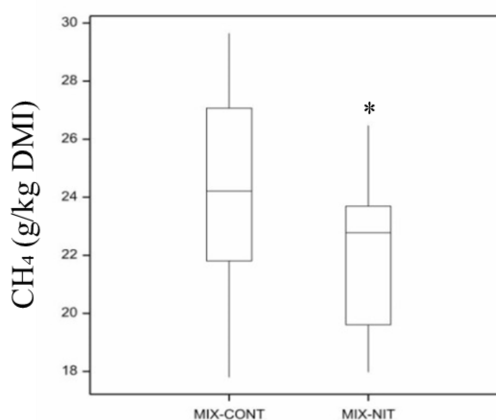

Figure 4.17 Boxplot of CH_4 produced (g/kg DMI) by steers offered mixed diets with and without nitrate addition

4.3.2.2 Analysis of Microbial composition (16S rRNA gene)

After filtering, a total of 9,470,573 reads were obtained from 34 samples, giving an average of 278,546 ±105,330 reads per sample that were assigned to an OTU. The number of archaeal sequences was 635,437 (6.7% of total OTUs) with an average of 18,689 ±10,562 sequences per sample.

4.3.2.3 Effect of nitrate on microbial community composition

Phylum: Once unclassified taxonomies were removed, 22 taxonomies were recorded at the phylum level. Spirochaetes, Firmicutes, Bacteroidetes, Proteobacteria, Euryarchaeota, Actinobacteria and Verrumicrobia were present at relative abundances above 1% across all dietary treatments. Bacteroidetes was the most abundant phylum, representing average of 42.9±9.6% (mean ± stdev) of the total community, followed by Firmicutes (37.3±7.2%), Proteobacteria (4.5±4.3%) and Spirochaetes (2.1±1.7%). A summary of the phyla with highest abundance within group can be found in Table 4.17. Overall, phylum relative abundances were not different with nitrate addition to mixed diets. Only, Spirochaetes relative abundances were significantly greater with nitrate addition to mixed diets. Figure 4.18 represents relative abundances at the phylum level for all samples from steers fed the mixed diet with and without addition of nitrate.

Diversity within group (alpha diversity) was within the expected range (1.8-2.9) for all groups. There were no differences ($P>0.05$) in Shannon diversity indexes between mixed control diet (2.8±0.12) and mixed nitrate diet (2.8±0.07) (Shannon index values calculated using log base 10).

Table 4.17 Mean relative abundance of phyla most abundant and A: B ratio on samples from steers fed mixed diets with and without addition of nitrate.

Taxonomy (Phylum)	Mixed Control	Mixed Nitrate	SED	P-value
Bacteroidetes	42.9	39.3	3.38	
Firmicutes	35.8	37.8	2.34	
Euryarchaeota	8.4	9.0	1.15	
Proteobacteria	6.5	4.8	1.24	
Verrucomicrobia	1.3	1.6	0.26	
Spirochaetes	1.3	3.1	0.73	0.018
Actinobacteria	1.1	1.7	0.28	
Tenericutes	0.7	0.9	0.13	
Ratio A:B	0.09	0.10	0.009	

OTUs: When nitrate was added to diets, 27% of all OTUs detected were significantly different in samples from the mixed nitrate diets compared with controls (Appendix 4.8). PC scores plots showing overall differences between treatments in microbial communities are presented in Figure 4.19. PC-1 accounted for 27% of the variation, PC-2 accounted for 21%, and PC-3 accounted for 10%. Nitrate addition to mixed diet had no significant effect on any of the PC-scores (P> 0.05). Correlation analysis of diet, CH_4 yield and interactions showed that CH_4 yield were significantly different for PC-2 (Table 4.18).

Table 4.18 Correlation between CH_4 yield, diet and interaction with PC scores

	PC-1	PC-2	PC-3
CH_4 yield	0.900	<0.001	0.676
Diet	0.810	0.190	0.056
Interaction	0.815	0.405	0.065

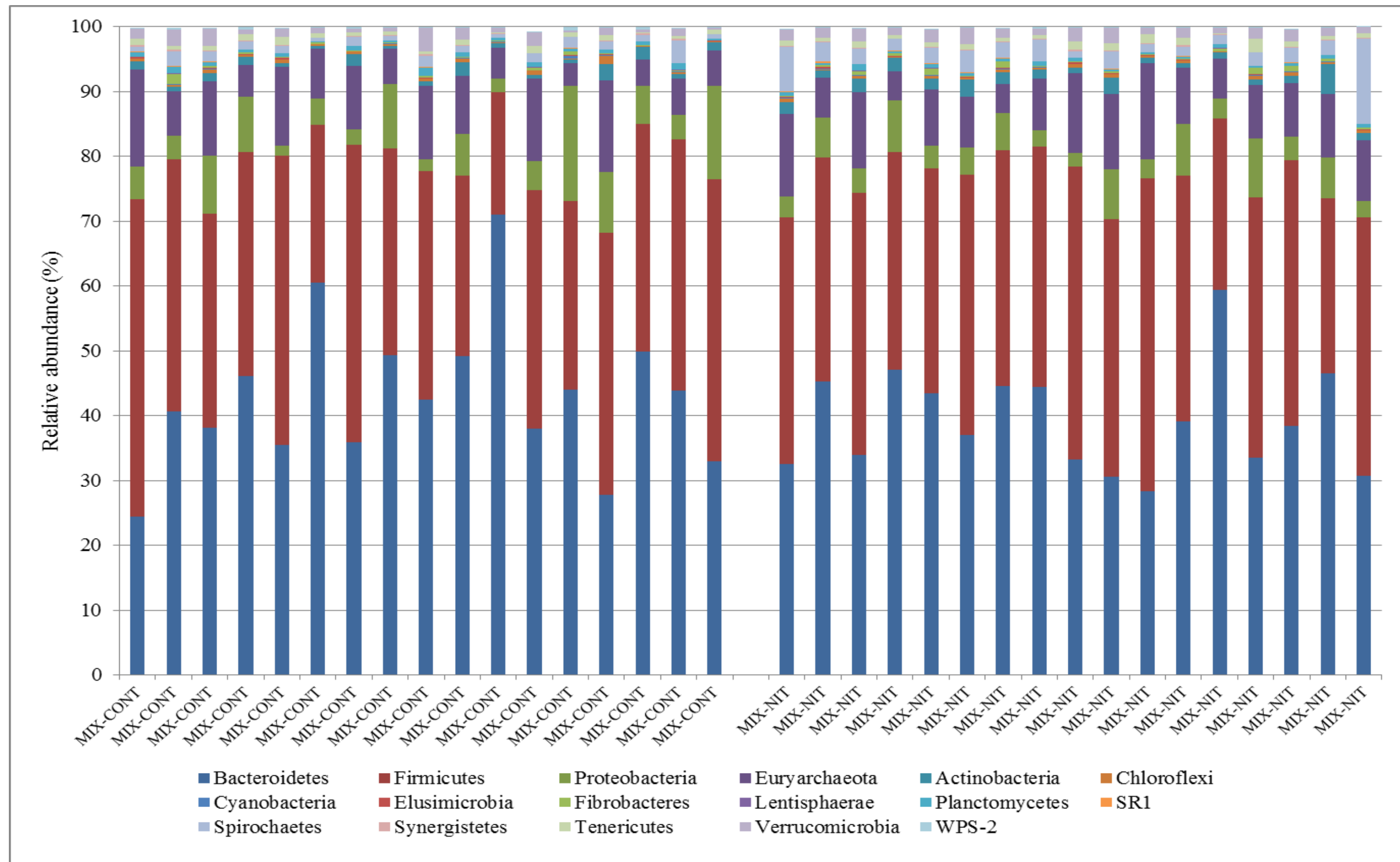

Figure 4.18 Plot of relative abundances for individual rumen samples at phylum level from steers fed mixed diets with and without addition of nitrate

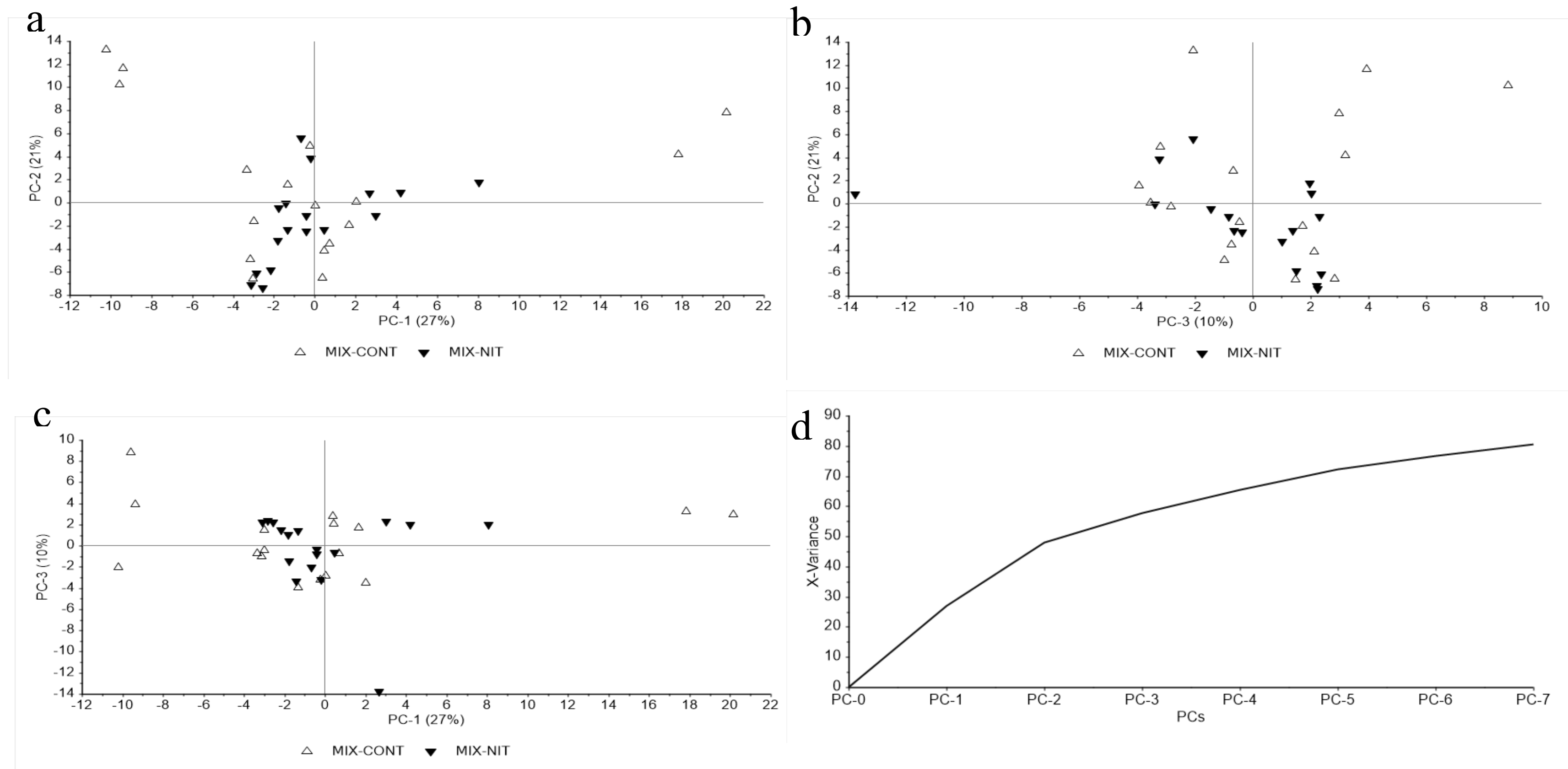

Figure 4.19 PCoA for relative abundance of all OTUs in samples from steers fed mixed diets with and without nitrate (a) scores plot for PC-1 vs. PC-2 (b) scores plot for PC-3 vs. PC-2 (c) scores plot for PC-1 vs PC-3 (d) explained variance plot

Bacterial community composition affected by nitrate

Genus: At the genus level, 113 genera were classified. *Prevotella* dominated, representing on average 33.5±2.7% of all sequences, with 257 OTUs assigned to that genus. Other abundant genera were *Succiniclasticum* (12.9±4.1%), *Ruminococcus* (6.6±2.1%), *Butyrivribio* (2.6±0.2%) and *Treponema* (2.0±0.7%). When genera present at high abundances (>1%) were studied, overall nitrate did not affect the relative abundances. Only the relative abundance of genus *Treponema* was higher (P<0.05) in diets containing nitrate compared with control (Table 4.19).

Table 4.19 Relative abundance of main genera in samples from mixed diet with and without nitrate

	Mixed-Control	Mixed-Nitrate	SED	P-Value
Prevotella	34.7	31.3	2.34	
Succiniclasticum	13.1	11.9	1.42	
Methanobrevibacter	6.2	7.7	0.93	
Ruminococcus	5.8	5.7	0.80	
Unknown (f *Succinovibroniaceae)*	3.9	3.1	0.96	
Unknown (o Clostridiales)	3.1	3.6	0.34	
Butyrivibrio	2.6	2.7	0.31	
Unknown (o Bacteroidales)	2.4	2.6	0.24	
Treponema	1.5	2.6	0.40	0.012
Fibrobacter	0.9	0.7	0.16	

Archaeal community composition affected by nitrate

The archaeal community was represented by the phylum Euryarchaeota. The relative abundance of the archaea community across all samples was 7.1±3.8% of all sequences. The class Methanobacteria was formed by the order Methanobacteriales (99.3±2.2) and the class Thermoplasmata by the order E2 (0.6±0.0 %). Methanobacteriales order included the family *Methanobacteriaceae*, which was dominated by the genus *Methanobrevibacter*, which represented 97.9±2.2% of all archaea sequences, followed by *Methanosphaera* (1.4±0.01%), and an unclassified group (0.05±0.00%). The order Thermoplasmata was represented by the family *Methanomassiliicoccaceae*, identified at the genus level as *VadinCA11*. Eleven OTUs identified at the genus level were assigned to archaea, including 5 OTUs identified as *VadinCA11*, 4 OTUs as *Methanobrevibacter* and 1 OTU as *Methanosphaera*. Overall, nitrate did not affect the archaeal community structure. At the family and genus levels, the relative abundance of methanogenic populations and the methanogenic community structure were not different between treatments (Table 4.20).

OTUs: When OTUs archaeal populations were studied, 4 OTUs were affected by nitrate and associated with a lower relative abundance compared to the control treatment; OTU 1742 and OTU 950 (genus *vadinCA11*), and OTU 961 (family *Methanobacteriaceae)* and OTU 215 (genus *Methanosphaera)* presented lower relative abundance when nitrate was added to the diets (Table 4.20).

Table 4.20 Relative abundance of archaeal populations with and without addition of nitrate

Family	Genus	OTU	Mix Cont	Mix Nit	SED	P-value
Methanobacteriaceae			8.4	9.0	1.15	
		OTU 961	0.007	0.001	0.0016	<0.001
	Methanobrevibacter		8.3	8.9	1.15	
	Methanosphaera		0.11	0.09	0.012	
		OTU 215	0.115	0.095	0.0115	0.028
Methanomassiliicoccaceae	*vadinCA11*		0.04	0.05	0.011	
		OTU 1742	0.0025	0.0006	0.00009	<0.001
		OTU 950	0.008	0.005	0.0029	0.028
Total Archaea			8.5	9.0	1.15	0.63

Nitrate reducers

Wolinella succinogenes, Veillonella parvula and *Mannheimia succiniciproducens* were not identified in the current study. The only one OTU identified as *Selenomonas. ruminantum* (OTU 86) did not presented different relative abundances with nitrate addition to mixed diets . Only one OTU was identified as genus *Pseudomonas* (OTU 567), a potential denitrifier, and was present at lower abundance with nitrate addition to diets (Table 4.21). The relative abundance of *Campylobacter* and OTU 686 identified as genus *Campylobacter* were more abundant (P<0.05) (Figure 4.20) in nitrate diet samples compared with control samples (Table 4.21).

Table 4.21 Relative abundance of the genus Campylobacter, OTU 686 and OTU 567 in the presence or absence of supplementary nitrate

Taxonomy	Mixed-Control	Mixed-Nitrate	SED	P-Value
OTU 567 *(Pseudomonas)*	0.008	0.014	0.0039	<0.001
OTU 686 (*Campylobacter*)	0.003	0.017	0.0027	<0.001
Campylobacter	0.005	0.019	0.003	<0.001

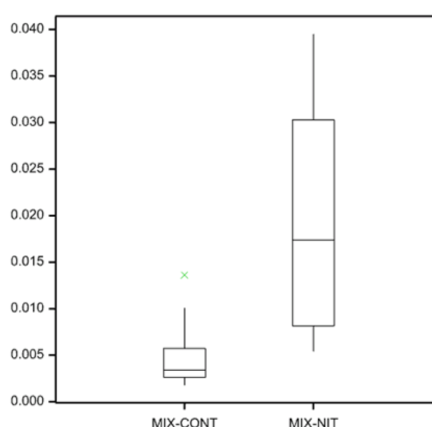

Figure 4.20 Relative abundance of the genus Campylobacter in the presence or absence of supplementary nitrate

4.3.2.4 Taxonomies correlated with methane production

Populations considered to have an influence on CH_4 production were studied (Table 4.22). At family and genus level, only *Succinivibrio* was significantly more abundant with nitrate addition.

OTUs: OTU 3685 identified as genus *Succinivibrio* and OTU 1 identified as family *Succinivibrionaceae* relative abundances were significantly smaller with nitrate addition.

Correlations were assessed between main genera across all samples. Genus that were correlated with CH_4 (g/ kg DMI) across all samples are presented in Table 4.23. Although there were no between treatment differences in relative abundances *Prevotella* and *Succiniclasticum* were negatively correlated with CH_4 production. Genus *Methanobrevibacter* and family *Prevotellaceae* were positively correlated with CH_4 production. Pearson correlation between all OTUs and CH_4 data were assessed (Appendix4.9).

Table 4.22 Relative abundance of taxonomies potentially correlated with CH_4 production from mixed diets with and without addition of nitrate

Family	Genus	OTU	Mix Cont	Mix Nit	SED	P-value
	Selenomonas		0.26	0.36	0.06	
Succinivibrionaceae			6.21	4.42	1.24	
		OTU 1	3.37	1.55	1.34	0.006
	Succinivibrio		0.06	0.16	0.05	0.040
		OTU 3685	0.00066	0.00005	0.00064	<0.001

Table 4.23 Correlation between main genera and CH_4 production across all samples

Taxonomy	R	P-value
Methanobrevibacter	0.626	0.000
Unknown (o Bacteroidales)	0.380	0.032
Prevotellaceae	0.377	0.033
Ruminococcaceae	0.307	
Prevotella	-0.370	0.037
Succiniclasticum	-0.468	0.007

Correlations between archaea taxonomies and CH_4 are presented in Table 4.24. Total archaea was correlated with CH_4 production. In addition, genus *Methanobrevibacter* and OTU 9 belonging to that one were positively correlated with CH_4 across mixed diets (Table 4.24). Linear regression were studied between total Archaea, *Methanobrevibacter* and OTU 9 (*Methanobrevibacter*) (Figure 4.21).

Table 4.24 Correlation between archaea population and CH_4 production

Taxonomy			R	P-value
Methanobacteriaceae			0.630	<0.001
	Methanobrevibacter		0.630	<0.001
		OTU 9	0.515	0.002
	Methanosphaera		0.350	0.046
Methanomassiliicoccaceae	*vadinCA11*		0.256	0.151
Total Archea			0.640	<0.001
Ratio A:B			0.640	<0.001

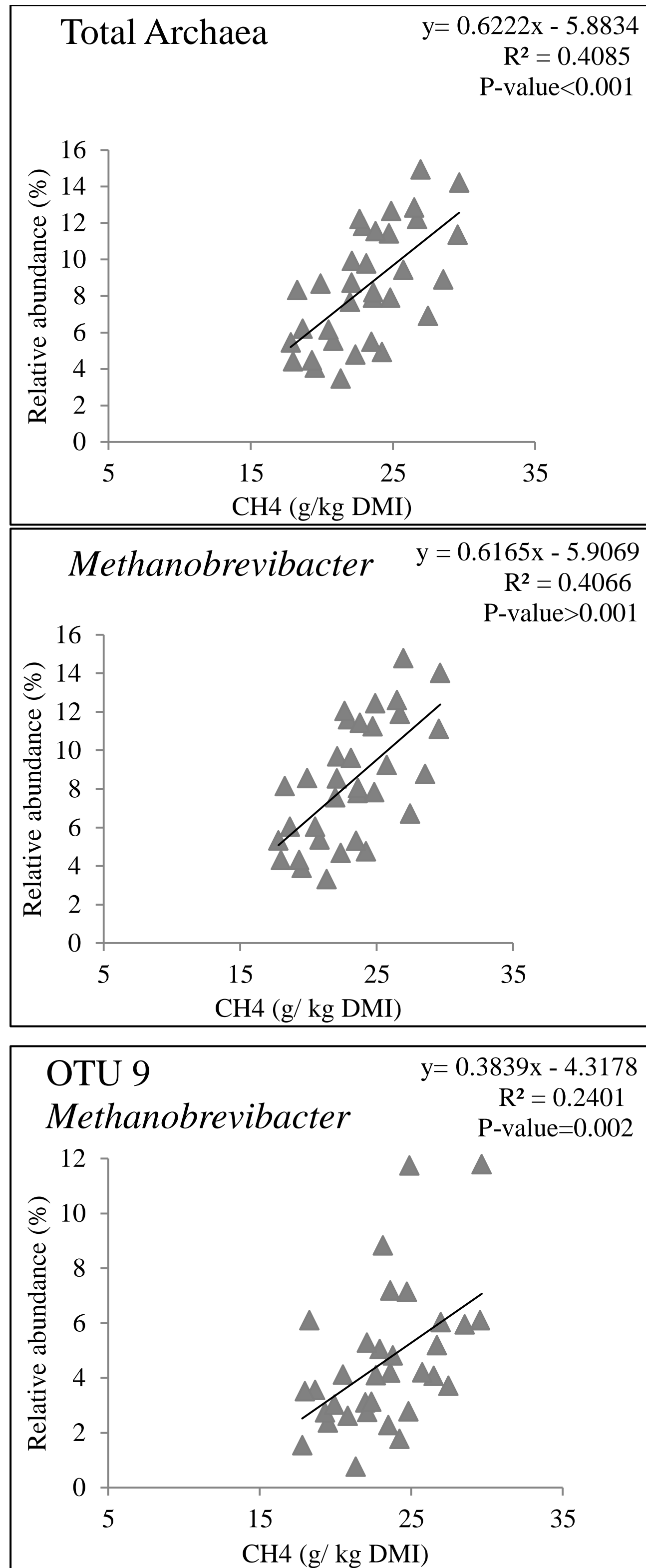

Figure 4.21 Linear regression between Total Archaea, *Methanobrevibacter* genus and OTU 9 (*Methanobrevibacter*) with CH_4 (g/kg of DMI) in mixed diets

4.3.3 Effects of nitrate: Results Summary

4.3.3.1 CH_4 and H_2 production (g/kg DMI)

Nitrate addition tended to reduce CH_4 production when added to mixed diets (16%), with no effect observed when it was added to the concentrate diet in Experiment 1 (Exp 1), and reduced CH_4 production when added to mixed diet in Experiment 2 (Exp 2). Nitrate addition to mixed diet increased H_2 production in both experiments, where addition of nitrate to the concentrate diet did not change H_2 production. The relative abundance of bacterial taxonomies that were affected with nitrate addition to mixed diets in any of the experiments and main archaeal taxonomies in control mixed diets from both experiments are presented in Table 4.25.

Table 4.25 Relative abundances of taxonomies in control mixed diets with significant different abundances with nitrate addition from Exp 1 and Exp 2

Family	Genus	Exp1	Exp 2
	Succiniclasticum	7.8	13.1
	Ruminococcus	3.9	5.8
	Unknown	12.5	3.9
	Butyrivibrio	1.4	2.6
	Treponema	1.6	1.5
	Fibrobacter	2.0	0.9
Methanobacteriaceae		2.3	8.4
	Methanobrevibacter	2.2	8.3
	Methanosphaera	0.06	0.11
Methanomassiliicoccaceae	*vadinCA11*	0.03	0.04
Total Archaea		2.3	8.5

4.3.3.2 Bacterial population affected by nitrate

Overall, bacterial taxonomies were not significantly affected by nitrate addition with few exceptions. The phylum Actinobacteria increased with nitrate in the mixed diets in Exp 1 (P=0.039). *Ruminoccocus* was higher with nitrate addition in mixed and concentrate diets in Exp 1 (P=0.008), but did not have different relative abundance with nitrate addition in Exp 2 (P>0.05). In Exp 2, the relative abundance of genus *Treponema* was higher with nitrate addition (P=0.012).

Selected bacterial taxa were studied in detail because of their potential relationship with CH_4 production and/or nitrate reduction. *Selenomonas* and OTUs assigned to this genus and family *Succinovibrionaceae* relative abundances did not change with addition of nitrate to diets. While there were few changes in taxonomies relative abundance with nitrate addition, OTU 1 (family *Succinovibrionaceae*) was lower with nitrate addition in mixed diets in both experiments (P=0.011 and P=0.006) and *Succinivibrio* was more abundant in the presence of nitrate in mixed diet in both experiments but with no change in concentrate diet (P=0.020 and P=0.060). OTU 3685 was significantly lower with nitrate addition across all diets while (P<0.001) OTU 201 was significantly higher with nitrate addition within concentrate diets (<0.001). Overall, the relative abundance of the genus *Campylobacter* was higher (P<0.001) with nitrate in mixed diets and the *Campylobacter* OTU 686 was higher with nitrate addition across all diets (P<0.001) (Figure 4.22).

4.3.3.3 Archaeal population affected by nitrate

The total number of archaea did not change with nitrate addition to diets (P>0.05). Interestingly, OTU 215 (genus *Methanosphaera*) and OTU 961 (family *Methanobacteriaceae* (unidentified at genus level) relative abundance were lower with nitrate across all experimental diets and years (P<0.001 for OTU 961, and P=0.033 and 0.028 for OTU 215) (Figure 4.23).

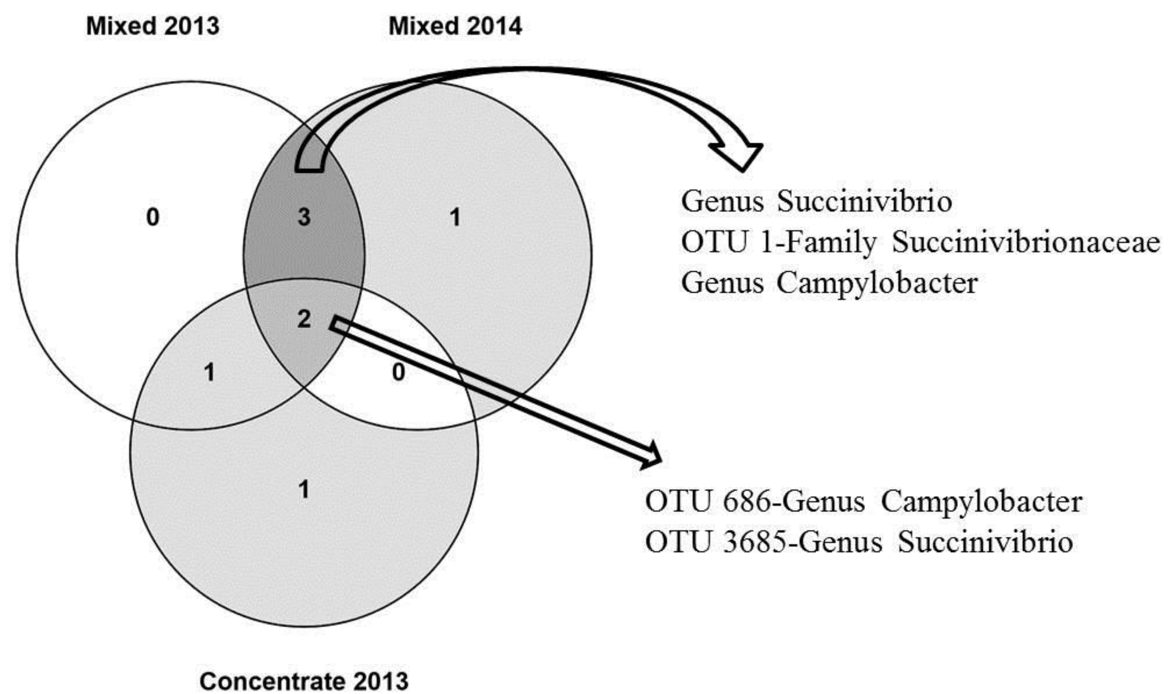

Figure 4.22 Venn diagram showing bacterial taxonomies of interest affected by nitrate across different diets and experiments.

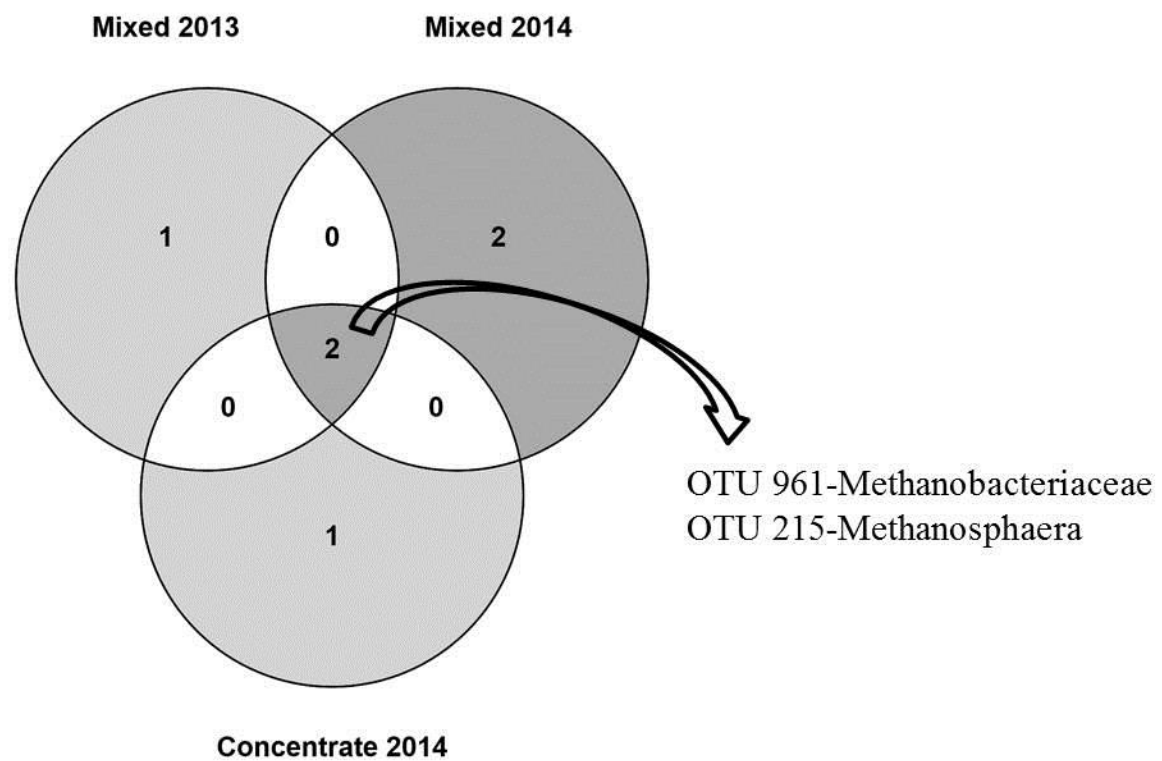

Figure 4.23 Venn diagram showing archaeal OTUs affected by nitrate across diets and experiments

4.3.3.4 Correlations between bacterial and archaeal populations and CH_4 across different basal diets

Interestingly, *Prevotella* was positively correlated with CH_4 production within concentrate diet (0.521, 0.022) and negatively within mixed diets in Exp 1 (-0.441, 0.001) and Exp 2 (-0.370, 0.037) but did not present different relative abundances with nitrate addition to diets. In addition in Exp 2, *Prevotellaceae* was positively correlated with CH_4 production (0.377, 0.033).

Remarkably, the total population of archaea (Figure 4.24) and the ratio A: B were positively correlated with CH_4 emission across all basal diets (Table 4.26). Genus *Methanobrevibacter* and OTU 9 belonging to it presented a linear positive correlation with CH_4 yield when studied within each diet and experiment (concentrate Exp 1, mixed Exp 1 and mixed Exp 2). The correlation was stronger when mixed diets groups from both experiments were combined. OTU 26 (genus *Methanobrevibacter*) was correlated with CH_4 production within samples from the mixed diets but not in samples from the concentrate diet. Genus *Vadin CA11* was positively correlated with CH_4 production within mixed diet but not in concentrate diet. OTU 964 (genus *Vadin CA11*) was positively correlated with CH_4 emissions for the concentrate diet, but not for the mixed diet.

Genus *Methanobrevibacter* relative abundance presented a strong and positive correlation with genus *Ruminoccocus* within all dietary treatments (ro>0.6; P-value<0.001).

4.3.3.5 Correlation between H_2 released from the rumen, CH_4 production, main bacterial genus and archaeal populations

Spearmen correlation between H_2 released from the rumen and archaeal taxonomies was assessed within basal diets and across experiments. No correlations were found between main archaeal taxonomies and H_2 (P>0.05). Hydrogen was not correlated with CH_4 production and neither with main bacterial communities in mixed diets (P>0.05). Opposing, H_2 was positively correlated with CH_4 production in concentrate diets (0.746, <0.000).

Table 4.26 Spearman correlation between archaeal populations and CH_4 production across all samples and within concentrate and mixed (combining data from the two experiments) basal diets.

Samples		All samples		Concentrate		Mixed	
Family	Genus	Ro	P-value	Ro	P-value	Ro	P-value
Methanobacteriaceae		0.594	0.000			0.377	0.005
	Methanobrevibacter	0.594	0.000	0.461	0.047	0.387	0.004
	Methanosphaera	0.516	0.000			0.339	0.013
Methanomassiliicoccaceae	*vadinCA11*	0.485	0.000			0.305	0.026
Total Archaea		0.596	0.000	0.774	0.009	0.393	0.004
Ratio A:B		0.476	0.000	0.773	0.009	0.393	0.004

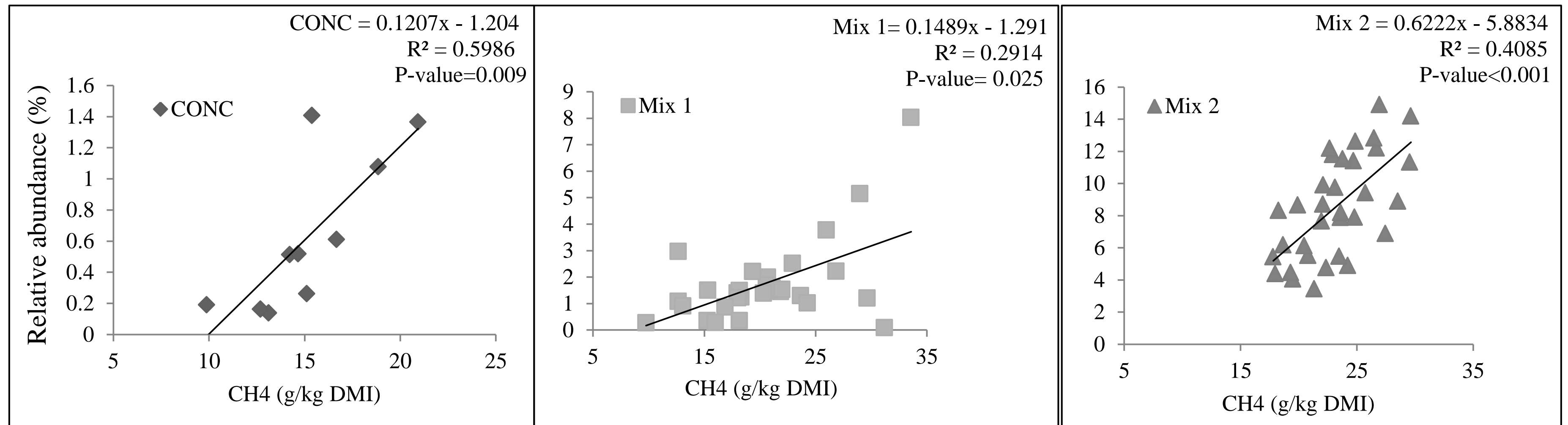

Figure 4.24 Plot of relative abundance of total archaea, against CH_4 yield within mixed diet and concentrate diet in Exp 1 (conc, mix 1) and within mixed diet in Exp 2 (mix 2). Regression equations and P-values for each group diet are presented

4.4 DISCUSSION

The objective of this study was to determine the differences in the rumen populations associated with nitrate addition to diets with different concentrate to forage ratio and to explore any potential links with CH_4 emission. For this study, the V4 region of the gene 16S rRNA was selected to analyse the microbial community within the rumen of steers. Others rumen microbial community studies have utilized 16S variable regions of this gene (Myer et al., 2015; Shabat et al., 2016; Veneman et al., 2015). Differences in hypervariable regions used can influence the relative abundances of some taxonomies. To this extent, the comparison of data regarding specific microbial community can be influenced by the variable region selected (Baker et al., 2003; Myer et al., 2016). The selection of the V4 region in this study seems to be adequate to the study purposes and directly comparable with other most 16S rRNA studies (Danielsson et al., 2017; Popova et al., 2017). The primers selected for this study (Caporaso et al., 2012) were the same for archaea and bacteria communities although it has been argued that these primers are more specific for bacteria communities and different ones could be used for archaea (Popova et al., 2017). It has been reported that primers selection for methanogen community amplification could exhibit biased amplification of certain taxa (Malmuthuge, 2017). For example, this study reported that *Methanobacteriales* represented on average 98 % of all archaea, whether in the study of Popova et al. (2017) *Methanobacteriales* represented 70% of the archaeal sequences. Nevertheless, it is not possible to elucidate if dissimilarities between studies are real and caused by genotypes or/and diets differences, or because primers and sequencing bias.

Different sequencing techniques and pyrosequencing platforms employed between studies could also affect differences in results. In this study OTUs have been used as the units to assess the variations in the rumen microbial composition. But it is still not clear whether these identified OTUs are biologically relevant or whether they are the results of artificial bioinformatics analyses (Malmuthuge, 2017).

4.4.1 Effect of basal diet on CH_4 production and microbial community structure

As previously reported (Sauvant and Giger-Reverdin, 2007, Mitsumori and Sun, 2008, Cottle et al., 2011; Hristov et al., 2013;Rooke et al., 2014; Troy et al., 2015), animals fed high concentrate diet (900 g/kg DM) produced less CH_4 than animals fed diets with lower content of concentrate (500g/ kg DM) diet. A reduction in H_2 production and increase in propionate molar proportions with concentrate diets compared with mixed diets was observed on samples from this study (Troy et al., 2015) and others (Rooke et al., 2014). As expected, the bacterial and archaeal composition between mixed and concentrate diet were different. Relative abundance of genus *Ruminobacter*, *Ruminoccocus*, genera belonging to Clostridiales and Bacteroidales, and genus *Fibrobacter* well known as H_2 producer were higher in mixed diet compared with concentrate diet. *Prevotella* relative abundance was not different between diets. Similar results have been previously reported with quantitative PCR community analysis from steers fed similar diets (Rooke et al., 2014) . This is in contrast to the results presented by others where no differences were observed in *Ruminoccocus sps* between high concentrate diets (700 g/kg of DM) and forage diets (0 g/kg of concentrate) and increase in *Prevotella* with higher amounts of concentrate was reported (Carberry et al., 2014). Probably differences are caused by different diets between studies. The increase in propionate molar proportion in the diets could be associated with a decrease in pH which may reduce the activity of rumen methanogens (Hegarty, 1999; Van Kessel and Russell, 1996). Nevertheless, the response to increase in concentrate for CH_4 production is not linear (Martin et al., 2010b; Troy et al., 2015). The level of concentrate in the diets should be considered regarding the effects in VFA, pH, microbial populations and therefore CH_4 (reviewed in chapter 1). For example, level of concentrate up to 500 g/ kg of DM may favour protozoa populations that can protect somehow methanogens (Danielsson et al., 2012). However when the proportion of concentrate increased from 500 g/kg of DM to 900 g/ kg of DM, protozoa population were inhibited (Rooke et al., 2014, Duthie et al., 2017) and CH_4 production was lower.

Concentrate diets that lower ruminal pH may provide a practical means of decreasing ruminal CH_4 production. However, it has been argued that pH is not strongly correlated to CH_4 emissions from beef cattle and other factors such passage rate and feeding pattern contribute to CH_4 production (Hunerberg et al., 2015). Also the type of forage fed affects the proportion of propionate formed and therefore the amount of H_2 available for CH_4 production. Importantly, the H_2 concentration is higher under conditions that also result in an increased passage rate, decreased CH_4 formation, and an increased importance of propionate formation (Janssen, 2010). When concentrate diets are fed the passage rate is higher than with mixed diets and, due to the higher ruminal turnover rate, the extent of ruminal degradation also decreases. At high passage rates, resident microbes require a higher growth rate to maintain themselves in the rumen. This effect influences the maintenance of protozoal, bacterial and archaeal population (Franzolin and Dehority, 1996) and protozoa are postulated to be washed out of the rumen when passage rates are high.

An alternative, but not exclusive explanation, for the differences in CH_4 production between diets is the lowest microbial diversity (Shannon diversity index) in rumen samples from steers receiving concentrate diet compared to mixed diet observed in this study. McCann et al. (2014) stated similar observations and showed that a high level of forage in diet tends to increase both microbial richness and alpha diversity. In agreement with the results of the present study, higher microbial diversity has been observed in animals thought to produce more CH_4 than those with lower CH_4 productions (Shabat et al., 2016; Zhou and Hernandez-Sanabria, 2009). Opposing, Myer et al.(2015) reported no differences in Shannon diversity between efficient and inefficient animals. From ecological perspective, low abundance taxonomic groups as methanogenic archaea are more sensitive to changes in diversity and richness (Shabat et al, 2016). This result was contrasting with Wallace et al. (2015) showing no significant change in microbial genus richness between low and high CH_4 emitters. In addition to the diversity analyses, the PCA examining the phylogenetic divergence between the OTUs separated the groups supporting differences between the microbial communities within each group.

The phylum Proteobacteria was significantly more abundant in samples from the concentrate diet. Proteobacteria was mainly represented by family *Succinivibrionaceae,* which has been associated with low CH_4 emissions (Pope et al., 2011). As expected, the relative abundance of this family was higher in concentrate diet samples which produced lower CH_4 compared with mixed diet. *Succinivibrionaceae* produces succinate as their principal fermentation end product which captures H_2 decreasing the amount of H_2 available for methanogenesis (Wallace et al., 2015). Genera *Ruminobacter, Ruminoccocus* and *Fibrobacter* were present at higher relative abundance in mixed diets than concentrate diets, which was to be expected as these genera are well known cellulolytic bacteria and acetate producers (Hegarty and Gerdes, 1999).

Archaeal community structure and total abundance were different between mixed and concentrate diets in the present study. Total numbers of archaea were higher for diets with 500 g of concentrate /kg of DM compared with diets with 900 g of concentrate/kg of DM. This result is in agreement with previous studies with similar ratios concentrate forage in the mixed diets (50%) (Duthie et al., 2017; Lillis et al., 2011; Rooke et al., 2014). Hook et al. (2011) reported no effect on the total numbers of methanogens with the level of concentrate in diets when comparing forage diets (100%) with diets with 650 g of concentrate/kg of DM. The lack of effect in the total number of methanogens in that study could be explained because the decrease in pH observed when concentrate was included in the diets was not severe enough to affect total methanogens population. Alternative, an increase in protozoa population could have protected methanogens from a decrease in pH. Danielsson et al. (2012) found higher total archaea in cows fed higher amounts of concentrate compared with lower amount of concentrate (500 vs 100 g/kg DM) diet, but with no concomitant increase in CH_4 production. The results from that study suggested that the changes in particular species of methanogens with changes in diets have more impact in CH_4 production than the total density of methanogens. Danielsson et al. (2012) argued that a specific *Methanobrevibacter* group was responsible for most of the CH_4 produced. *Methanobrevibacter* genus was the dominant methanogen in the rumen fluid across all dietary treatments as previously confirmed in several studies (Carberry et al., 2014; Danielsson et al., 2012; Wallace

et al., 2015; Zhou and Hernandez-Sanabria, 2009). *Methanobrevibacter* is known as the main hydrogenotrophic methanogen in the rumen and this genus was higher in mixed diet compared with concentrate supporting the idea that hydrogenotrophic methanogens are more active, accounting for most of the proportion of CH_4 formed in the rumen (Hook et al., 2010). In contrast, genus *Vadin CA11* belonging to the methylotrophic methanogens was detected at higher abundance in concentrate diet compared to mixed diet. This group may be stimulated by methylated substrates such as glycine betain (present in beet pulp), methanol, trimethylamine, dimethyl sulphide, dimethylsulfoniopropionate suggesting that methylotrophs will be more abundant and potentially more active in specific experimental conditions and diets (Poulsen et al., 2013). The apparent increase in the relative abundance of methylotrophic group with concentrate diet in this study may be explained by a higher concentration of substrate for methylotrophic populations, a higher salt concentration (Zhuang et al., 2016) or acidic tolerance of this group. It might be that rapeseed meal, which was present at higher concentration in concentrate diets and contains precursors for trimethylamine, could have promoted methylotrophic populations. Also, thinking about ecological niche and co-ocurrence of microbial populations, the lower number of hydrogenotrophic methanogens in concentrate diets may favour the presence of methylotrophics. In this study, the relative abundance of methylotrophs (*Vadin CA11*) was below 1% accounting likely for little amount of CH_4 formed. Interestingly, the lower relative abundance of *Vadin CA11* in mixed diet compared with concentrate diet was not generalized across all OTUs belonging to the specific archaea genera, and particular OTUs such as OTUs 964 (genus *VadinCA11*), were less abundant in samples from the concentrate diet.

Tapio et al. (2017) identified two clades within *Methanobrevibacter* with different affinities for H_2 and therefore different CH_4 emissions. Similarly, Danielsson et al. (2012) identified from T-RFP analysis two main groups of *Methanobrevibacter* based in the phylogenetic distribution elaborated by King et al. (2011) and found higher amount of *Methanobrevibacter* SGMT group in animals with high CH_4 emissions. These results support the idea that the study of individual OTUs within main genera and families could give better explanation for differences in CH_4 than relative abundances of genus or families. The different CH_4 emissions

may be partly due to varying relative abundances within specific minor community of methanogenic archaea (Tapio et al., 2017).

4.4.2 Effect of nitrate on CH_4 production and microbial community structure

Microbial mechanisms of CH_4 reduction when nitrate is fed have been scarcely studied (Yang et al., 2016). Some studies have identified microbial population affected by nitrate *in vitro* (Lin et al., 2013; Yoshii et al., 2003; Zhou et al., 2012) and *in vivo* (Asanuma et al., 2015; Lin et al., 2013) but there are only four published studies employing 16S rRNA amplicon sequencing for rumen microbial populations with animals fed nitrate (Asanuma et al., 2015; Popova et al., 2017; Veneman et al., 2015; Zhao et al., 2015). Overall, the effects of nitrate on microbiome in this study were minor in agreement with previous research (Popova et al., 2017; Veneman et al., 2015). However, in the study of Popova et al, (2017) nitrate did not affect CH_4 production and consequently the microbial population was not significantly affected, likely because the low level of nitrate used in that study (10 g/kg DM) and Zhao et al. (2015) did not study CH_4 production with nitrate addition.

Concentrate versus mixed basal diets in Experiment 1

In presence of nitrate, a decrease in CH_4 production from mixed diet fed steers was confirmed, but no effect was observed for the steers fed the concentrate diet. As stated before, different composition of microbial community were expose to nitrate depending on the diet. Iwamoto et al. (2001) demonstrated that nitrate- and nitrite-reducing activity was faster at neutral pH than at pH 6.0 or lower. The inhibitory activity of the lower pH on nitrate- and nitrite-reducing activity within mixed rumen populations was attributed to lower availability of electron-donating substrates such as H_2, formate, or lactate resulting from an inhibition of fermentation caused by the low pH. Therefore, nitrate added to concentrate diet is less effective in decreasing ruminal CH_4 emissions than when added to mixed diets in beef cattle (Latham et al., 2016). An increase in H_2 release with nitrate addition in mixed diets

was observed with no change of H_2 release with nitrate in concentrate diet. Increased H_2 release with the addition of nitrate to the diet has been also recorded in previous studies when mixed diets were fed (van Zijderveld et al., 2011; Veneman et al., 2015). In agreement with different effects of nitrate in CH_4 production within basal diets, the effects of nitrate addition on microbial populations were different depending on the basal diet. Microbial population were somehow more altered by nitrate in mixed diets with 27% of OTUs affected by nitrate in mixed diets compared with 14% of OTUs in concentrate diet. Microbial diversity (Shannon diversity index) was not affected by nitrate addition in this study, in agreement with Veneman et al. (2015). In addition, no obvious clustering was observed between group diets with addition of nitrate when looking at PCA. The relative abundance of the genus *Ruminococcus* was higher with nitrate addition in mixed and concentrate diets in Exp 1. Similar results have been reported elsewhere (Zhao et al., 2015) with an increase in relative abundance of some *Ruminoccocus* spp. *Ruminococus* is known as the main H_2 producers and has been reported to be inhibited by nitrate *in vitro* (Marais et al., 1988). Others studies reported a decrease in some representative members of celullolytic bacteria including *Ruminoccocus* (Asanuma et al., 2002; Asanuma et al., 2015; Iwamoto et al., 2002).. It has to be considered that the studies of Asanuma et al. (2002), Marais et al. (1988) and Iwamoto et al. (2002) were based in cultured techniques and therefore not directly comparable with the results reported in this study (16S rRNA gene). They reported absolute counts, whilst in this study the changes in relative abundances of a specific community may be due to a change in the community itself or an increase or decrease in other communities. Others H_2 producing bacteria not identified may have been affected as well. *Ruminoccocus* genus was positively correlated with *Methanobrevibacter* genus in this study, in agreement with Kittelmann et al. (2013). This may be explained by a case of H_2 interspecies, with H_2 produced by *Ruminoccocus* used by *Methanobrevibacter* to produce CH_4 or by sharing similar ecological niches that are favoured by specific rumen conditions and/or diets. The identification of specific *Ruminococcus* populations in presence of nitrate will need to be investigated.

Succinivibrio is known to be associated with low CH_4 emissions (Pope et al., 2011) and is a fibre-digesting bacteria which produce large amounts of succinate,

which is finally converted to acetate capturing H_2. In this study, genus *Succinivibrio* had a higher relative abundance in presence of nitrate in mixed diets in both experiments, but this genus was not affected by nitrate addition to concentrate diet, which was in agreement with the results obtained for CH_4 emissions. However, *Succinivibrio* relative abundance was not correlated with CH_4 emissions. To the best of my knowledge, the effect of nitrate enhancing the relative abundance of the genus *Succinivibrio* in mixed diet has never been reported. The study of known populations related to CH_4 showed no significant differences for most of them as a consequence of nitrate addition (Pope et al., 2011).

Some known nitrate reducers, as *Wolinella succinogenes, Veillonella parvula* and *Mannheimia succiniciproducens* (Iwamoto et al., 2002) were not identified in the current study and the only OTU identified as *Selenomonas ruminantum* (OTU 86) was not different between dietary treatments. Latham et al. (2016) reported that potential bacterial denitrifiers such as *Pseudomonas aeruginosa* and certain species of *Propionibacterium* and *Nitrosomonas* may increase in the rumen with the addition of nitrate. In the current study, these bacterial taxa were not identified. Latham et al. (2016) also suggested that nitrate addition to the diet may increase the relative abundance of Campylobacter microbes. In the present study, relative abundances of *Campylobacter*, increased with nitrate in the mixed diet and OTU 686, identified as the main OTU within Campylobacter genus, increased with nitrate in all diets. Many *Campylobacter* species contain the nitrate reductase gene and the increase in this genus with nitrate addition was observed in others studies (Lin et al., 2013; Zhao et al., 2015). The importance of *Campylobacter* species as food pathogen (Silva et al., 2011) should be considered when feeding nitrate due to the possible risk of enhancing *Campylobacter* pathogens strains abundance.

An increase in H_2 release with nitrate addition to mixed diets could be attributed to the fact that nitrite is highly toxic for rumen methanogens (Iwamoto et al., 2002). However, the total abundance of archaea did not change with nitrate addition, suggesting that nitrite did not negatively affect methanogens population. A possible explanation is that the sudden increase in H_2 production after feeding mixed diets (Rooke et al., 2014), accompanied by an increase of H_2 producers as

Ruminoccocus sps. induced by nitrate, exceeded the capacity of methanogens to utilise the H_2 produced. Therefore, higher values of H_2 released may imply that less H_2 was captured by methanogens. This idea was supported by a numerical increase in methylotrophic methanogens with nitrate addition to mixed diets and a decrease in specific hydrogenotrophic methanogen OTUs. In agreement, no change in the total abundance of archaea with nitrate addition has been observed in previous studies (Popova et al., 2017). Contrarily, a reduction in methanogen growth *in vitro* with nitrate addition was observed (Iwamoto et al., 2002) and their numbers were reduced *in vivo* (Asanuma et al., 2015; van Zijderveld et al., 2010; Veneman et al., 2015).

In this study, nitrate appears to have an inhibitory and selective effect in some hydrogenothrophic archaea, as archaea specific OTUs were affected by nitrate across all diets. For example, OTU 961 (*Methanobacteriaceae*) and OTU 215 *(Methanosphaera)* decreased with nitrate in all diets, which was consistent with a decrease in CH_4 production in mixed diets, but was not explained in the concentrate diet. The genus *Methanosphaera* was lower with nitrate in the concentrate diet, and tended to be lower in the mixed diet with nitrate addition. Family *Methanobacteriaceae* is hydrogenotrophic and seems to account for most of the reduction in CH_4 observed with nitrate addition to mixed diets.

Some OTUs belonging to *vadinCA11* genera were affected by nitrate but differently across diet and experiments. Therefore, it seems to be clear the importance to combine results obtained at both the genus and OTU levels to get a clear idea of the important populations affected by the different treatments.

Mixed diet in Exp 1 versus mixed diet in Exp 2

CH_4 production was reduced in mixed with nitrate addition diet by a 71% and 37%, in Exp 1 and Exp 2 respectively, of the theoretical CH_4 reduction potential of the added nitrate. Consistently, the increase in acetate to propionate ratio and H_2 release observed may suggest that the excess of H_2 that was not capture for methanogenesis was not redirect to propionic metabolic pathways (McAllister and Newbold, 2008). As previously explained in chapter 3, a possible explanation for the lower efficiency of dietary nitrate to reduce CH_4 than theoretically expected is that

nitrate stimulates H_2 producers with more H_2 available for CH_4 production (Leng, 2014). An alternative explanation is that nitrate is excreted rather than reduced to NH_3-N if the rate of nitrate ingestion overcomes the capacity for reduction. Depending on the diet, there is a balance between the amount of nitrate required by the ruminal microbiota and the potential of CH_4 reduction that can be achieved. Thirdly, nitrate stimulates formate production by methanogens, which is converted to H_2 that is release (Leng, 2014; van Zijderveld et al., 2011; Veneman et al., 2015). The reason for the difference in CH_4 reduction efficiency in presence of nitrate in mixed diets between different experiments implied that nitrate was more effective in reducing CH_4 in specific experimental conditions than in others. One important factor that could greatly contribute to variations in the reduction potential of nitrate in the animals is the specific individual rumen microbiome that could be affected by phenotype and/or diet. Metabolomics analysis has revealed large effect of roughage types on rumen microbial metabolic profile in dairy cows. It could be that differences in silage composition between experiments affect somehow microbial composition and the sensitive to nitrate addition (Zhao et al., 2014). In experiment 2 the amount of grass silage was slightly higher and could have accounted for part of the higher population of methanogens (8% vs 2%) from animals in Exp 2 compared with Exp 1. Other possible explanation is that basal diet in Exp 2 had more nitrate content than in Exp 1, and therefore, bacteria nitrate reducers in animals from Exp 2 were not able to reduce the exceed nitrate added into the diets, and more nitrite was excreted in the urine, without contributing to decrease CH_4 production.

An exploratory screening was done to look for differences in microbial communities in steers fed the mixed control diet between experiments. *Butyribivrio*, *Methanobrevibacter* and *Ruminococcus* were present at higher abundance in steers in Exp 2 were nitrate was less effective in reducing CH_4. *Fibrobacteres* relative abundance was higher in steers from Exp 1. In addition, the relative abundance of archaea population was significantly higher in Exp 2 compared with Exp 1. The more abundant population of archaea in Exp 2 could have been less vulnerable to addition of nitrate. However, this screening is questionable as animals were not their own control. A different case would have been if the experiment design would have been a Latin square, were the animals act as their control.Nevertheless, differences in

basal diets between experiments could have influenced the microbial populations exposed to nitrate.

At microbial taxonomies level, little differences were observed in population affected by nitrate between different experiments within mixed diets. The relative abundance of the genus *Ruminococcus* was higher with nitrate addition in mixed diets in Exp 1 but was not affected by nitrate in Exp 2. *Ruminococcus* spp are H_2 producers and therefore could have account for the lower potential of nitrate to reduce CH_4 production than theoretically expected in Exp 1.

4.4.3 Correlation between CH_4 production, H_2 production and microbial community structure

Interestingly, *Prevotella* was positively correlated with CH_4 production in concentrate diets, and negatively within mixed diets in both experiments. *Prevotella* relative abundance was not affected by nitrate addition. In support, a metagenomics study showed that *Prevotella* could increase in parallel to more propionate production in presence of compounds inhibiting CH_4 (Denman et al., 2015).

No more consistent bacterial taxonomy was related to CH_4 production across the different diets and experiments. This may be due to the ability of diet to modify microbial populations which may have neglect any variation in taxonomies caused by differences in CH_4 production (Carberry et al., 2012). No bacterial or archaeal community were able to explain a significant amount of variability in H_2 production in agreement with previous study (Rooke et al., 2014). Surprisingly, but in agreement with previous study (Rooke et al., 2014) no correlation was observed between *Ruminococcus* (well-known H_2 producers) and H_2 production, and neither with CH_4 poduction.

The total number of archaea and A:B ratio were correlated with CH_4 emissions across all diets and experiments without been affected by nitrate. Overall abundance of total archaea has been correlated with the production of CH_4 in some studies (Wallace et al., 2014; Wallace et al., 2015) but not in others (Danielsson et al., 2017; Kittelmann et al., 2014; Shi et al., 2014; Zhou et al., 2011). It may be that

the composition of archaeal community with different methanogenic pathways are more related to CH_4 production than total abundance (Tapio et al., 2017). *Methanobrevibacter* and OTU 9 (identified as *Methanobrevibacter)*, was positively correlated with CH_4 in both experiments and diets. In agreement, *Methanobrevibacter* has been found to be positively correlated with CH_4 emissions in some studies (Danielsson et al., 2012; Shi et al., 2014; Zhou et al., 2011). Interestingly OTU 9 and OTU 26, identified as *Methanobrevibacter*, were positively correlated with CH_4 and highly abundant in samples from the mixed diets. OTU 9 (*Methanobrevibacter*) had a strong positive correlation with CH_4 for the concentrate diet also, where it was present at low abundance, suggesting that this population is important to explain CH_4 emissions. The stronger correlation between *Methanobrevibacter* and CH_4 emissions in concentrate diet than in mixed diet may be explained by greater peaks of H_2 production after feed consumption when feeding the mixed diet (Rooke et al., 2014). When estimates of total H_2 produced in mixed and concentrate diets have been calculated not significant differences were observed between mixed and concentrate diets (Rooke et al., 2014), although H_2 released was higher with mixed dies compared with concentrate diets (Troy et al., 2015). The increased production of H_2 after consumption may exceeds the capacity of *Methanobrevibacter* to capture the H_2 produced. In addition, no correlation between H_2 released and archaea relative abundance was observed in this study. In support of this idea, the H_2 released was correlated with CH_4 produced in concentrate diet but not in mixed diets.

The relative abundance of OTU 964 identified as methylotrophic belonging to the genus *Vadin CA11*, was positively correlated with CH_4 emissions from the concentrate diet. However, this population was present at lower abundance in concentrate diet compared with mixed diet. The correlation with CH_4 emission in the concentrate diet may suggest that this population were more active in the concentrate diet. The contribution of methylotrophic archaea to methanogenesis will depend to some extent of the concentration of methylamine in the diet (Borrel et al., 2013; Poulsen et al., 2013; Tapio et al., 2017). However, the content of methylamine or other compounds that may be presented in the concentrate diet and promote methylotrophic archaea were not known in this study.

The different microbial communities interact and it will be interested to address the co-ocurrence of microbial patterns. For example, a negative correlation between different clades of *Methanobrevibacter* has been observed in previous studies. (Kittelmann et al., 2013) and presumably both H_2 utilizing groups compete in the rumen. One of this clade was found to be correlated with *Fibrobacteres* while the other was correlated with *Ruminoccocu*s. This co-variation suggests that methanogens may adapt to different H_2 concentrations or respond to differences in precursors for CH_4. For example, one of the *Methanobrevibacter* clade seems to be specialized in low H_2 concentrations. Also, high specific interactions could exist between certain methanogens and bacteria. In this study, genus *Methanobrevibacter* presented a strong and positive correlation with genus *Ruminoccocus* within all dietary treatments suggesting a cooperation between them or that they shared the same niche that is favoured under specific conditions.

Moreover, previous microarrays (mcrA) studies reported that although *Methanobrevibacter* was the dominant methanogen population in the rumen, they only contributed to a third of the RNA-derived mcrA sequences, while other minority methanogen group (*M. luminensis* specie) mcrA sequences represented the majority and may contribute highly to CH_4 formation. Therefore, less abundant but highly active methanogens may contribute greatly to CH_4 formation than it is thought when assessed with quantification of genomic DNA (Kang et al, 2013).

4.5 CONCLUSIONS AND IMPLICATIONS

To the best of my knowledge, no study has addressed the changes in microbial community with nitrate addition to different basal diets. This was one of the first studies to directly examine the effect of nitrate in rumen microbial communities correlated with CH_4 production of steers fed different basal diets at the 16S rRNA gene level utilizing NGS technologies. The main conclusions of this study were:

- The alteration in the composition of the rumen microbiome by nitrate was different depending on the basal diet. Microbial population were more altered by nitrate addition in mixed diets compared with concentrate diets.
- Consistent changes were not noted in microbial taxonomies at genus or family level with nitrate addition to diets but some consistent changes were observed in some OTUs with nitrate addition.
- *Methanobrevibacter* was the dominant methanogen genus in the rumen.
- Total archaea, *Mehanobrevibacter* and OTU 9 (*Mehanobrevibacter*) were positively correlated with CH_4 production.
- *Prevotella* was positively correlated with CH_4 production in concentrate diets and negatively in mixed diets.
- The necessity to combine the results obtained at the genus level and OTUs was confirmed by the opposite results obtained for some important microbial genera and specific OTUs and impacting on CH_4 production.

4.5.1 Implications of the study

The reason for the difference in CH_4 reduction efficiency of nitrate depending on basal diet was not clarified. Nitrate and nitrite reducing activity is widespread across different bacterial populations and phyla and therefore it is hard to target all this populations with this sequencing technique. It is also likely, that all ruminal nitrate reducers have not yet been identified (Popova et al., 2017) and the study of all OTUs affected by nitrate could recognize new members involve in nitrate metabolism. Also, some microbial populations may have affected the efficiency of nitrate in reducing CH_4 as *Succinivibrio*. It seems that *Succinivibrio* OTUs have great variability being affected differently by nitrate depending on basal diet and related somehow to CH_4 production. The results may imply that different methanogens have different sensitivity to nitrate as a result of genes differently expressed that make some species more tolerant to changes in the rumen induced by nitrate.

In this study, some methodological issues could likely impact the quality of the results. First of all, 16S rRNA Gene Clone Libraries technique has some limitations: it does not provide information about microbial activity and only provide a structural analysis of the ecosystem and rumen microbiome may have similar composition but differ in metabolic activity (McCann et al., 2014). The gene 16S rRNA is present across all prokaryotic taxonomic. The major disadvantages of this method are that bacteria with more 16S rRNA genes will be over-represented. In addition, the sequencing depth of this study may not have been sufficient to identify minor populations such as important nitrate reducers; *Wolinella succinogenes, Veillonella parvula and Mannheimia succiniciproducens.* Also, the database used is important for a better identification of microbial populations. Currently available taxonomic frameworks such as Greengenes used in this study offer limited resolution beyond the genus level for taxonomic assignments (Seedorf et al., 2014). The statistic method chosen may not have been the most adequate and alternative statistical methods such as Tukey's HSD could be more adequate. The study had sufficient power to identify some changes likely induced by nitrate at OTU level. The lack of differences observed between dietary groups at the level of diversity analyses may simply indicate that the important variation in microbial communities

lie at a finer resolution. For example, variation among or within specific taxa and OTUs provided by partial 16S rRNA gene sequencing may be more informative rather than the number and diversity of all taxa and OTUs. Although, great differences in microbiome because nitrate addition were not detected at Phylum or genus level, some correlations between CH_4 and OTUs were identified.

Other genomic analysis may better determine nitrate effect on microbial population that are correlated with changes in CH_4 production. Different suggestions will be addressed in Future work section.

4.6 FUTURE WORK.

Due to the role of protozoa in methanogenesis and nitrate metabolism, the study of protozoa population should be addressed in conjunction with bacteria and archaea members involve in nitrate metabolism and contributing to CH_4 mitigation effect.

The study of basal microbiome composition before addition of nitrate and after long term nitrate feeding will give a better understanding of changes in microbial community induced by nitrate and not related to animal variation, feed pattern, or breed. More information could be gathered with different time points sampling, during and after adaptation to nitrate. Ruminal microorganisms do not exist in isolation and network analyses of taxa interactions across complex and diverse communities may help to ascertain the functional roles of uncultured microorganisms. There can be substantial animal to animal variation in the rumen microbial community, so a greater number of animals may be needed to observe an association between the rumen microbiome and CH_4 production. In order to obtain samples from a large data set, the use of different samples as an alternative to rumen content would be desirable. Recent studies have demonstrated that the study of microbiota in buccal swabs and bolus are comparable to microbial population identified in the rumen content (Kittelmann et al., 2015; Tapio et al., 2016). Therefore, buccal swabs samples could be an alternative to rumen content samples for future studies, allowing a non invasive and less costly sampling for analysis of rumen microbial communities in large numbers of animals. The study of rumen content samples at slaughter could be studied, as microbial population from slaughter samples have been highly correlated with the microbial population *in vivo*. For the experiment 2 of this study, rumen samples from slaughter were sequenced and it would be of interest to look for correlations between microbial populations *post-mortem* with samples from animals before slaughtering.

Massively parallel sequencing (MPS) can be applied to have a better understanding of microbial populations affected by nitrate and observe the functional changes directly (Ross et al., 2013). MPS may allow for identification of biomarkers

significantly affected by nitrate that lead to lower CH_4 productions. A more complete database would have the advantage of using a greater proportion of the sequence for the analysis that is based on alignments to the rumen database. After, a comprehensive characterization of microbial populations and genes would bring together more information about nitrate metabolism.

Furthermore, metagenomic analysis predicts metabolic function better than only a taxonomic description, because different taxa share genes with the same function (Roberts and Ingham, 2008) and metatranscriptomics may give more information about the dynamic of microbial activity (Wallace et al., 2017). Therefore, the use of Omics techniques and its combinations are likely to improve the understanding of functions and interactions between rumen microbial populations and provide insight into the functional pathways involve in nitrate metabolism and nitrate mode of action in CH4 reduction.

CHAPTER 5

General discussion

Chapter 5. General discussion

Ruminants account for the largest part of global livestock emissions and a substantial share of global anthropogenic GHG. When considering total supply chain emissions, cattle production generates 4.6 Gt and 3.3 Gt when only the direct CH_4 and N_2O emissions from enteric fermentation and manure are considered (FAO, 2013b).

Despite the extensive research on feed additives to decrease CH_4 emissions from ruminants, there are factors that are not yet well understood and there is a need for further research in this area to actually develop feasible sources of additives to adopt at a farm level or refine existing technologies to increase their applicability. The main objective of this thesis was to assess effective additives to reduce rumen CH_4 production.

5.1 SUMMARY OF MAIN FINDINGS

In *Chapter 2* biochar was tested as a novel antimethanogenic compound *in vitro* using buffered rumen fluid from beef cattle. The main results were:

- Biochar at 10 g/kg of substrate reduces CH_4 by 5% and total gas by 3% compared with control.

- Biochar reduces NH_3-N concentration in rumen fluid *in vitro.*

- When biochar produced from miscanthus straw at the lower pyrolysis temperature (550° C) was incubated *in vitro*, the lowest amount of CH_4 and NH_3-N was recorded.

Overall, adding biochar with a range of compositions to *in vitro* assays produced small reductions in CH_4 production which in isolation are unlikely to be useful in practice. Accordingly biochar was not pursued for further studies in this thesis.

In *Chapter 3* EN plus elemental S were tested *in vivo* as antimethanogenic compounds in beef cattle fed forage diets. The main results were:

- CH_4 production was numerically lower with addition of EN (20 g/kg of DM, equivalent to 14.3 g nitrate /kg DM) to forage diets from steers compared with diets with urea or true protein.

- Encapsulated nitrate fed *ad libitum* and with a time of adaptation of 14 days did not produce evident signs of toxicity and MtHeb values were below the threshold for toxicity.

- As hypothesised, EN did not show any effects on N metabolism and MPS compared with true protein or urea.

- The addition of elemental S to NPN sources did not affect fermentation parameters or gas production.

The current study demonstrated for the first time that nitrate can replace part of protein of the ration without adverse effects on N metabolism and rumen fermentation and reducing CH_4 production. Furthermore, encapsulated nitrate added with stepwise adaptation did not produce any adverse effects in animal health.

For *Chapter 4* the effects of nitrate addition to beef cattle diets on rumen microbial population and correlations with CH_4 production were studied. The main results were:

- Bacterial and archaeal composition between concentrate and mixed diets were different.
- Nitrate did not produce significant changes in microbiota composition but was confirmed to affect specific archaeal and bacterial OTUs consistently between studies.
- Microbial composition was more affected with nitrate addition to forage diets than to concentrate diets.
- Some methanogen OTUs were affected with nitrate addition across all diets. Specifically, OTU 215 and OTU 961 belonging to *Methanobrevibacter* were present at lower relative abundance with nitrate addition.
- A direct and strong correlation between total archaea, *Methanobrevibacter* archaea genus and OTU 9 (*Methanobrevibacter*) with CH_4 production was observed.

Nitrate added to beef diets produce minor changes in microbial composition. The results suggest that more information could be highlighted with the study of microbial function.

5.2 PRACTICAL CONSIDERATIONS OF METHODOLOGIES

5.2.1 Sampling of rumen fluid

There are different options for rumen sampling. The adequacy of the method would be determined by the analysis purpose, numbers of animal, ethics and resources available. Sampling of rumen fluid *in vivo* can be done through cannula in cannulated animals or using less invasive technique such as oesophagus rumen tube in intact animals. Both sampling methods have received considerable attention. For Experiment 2, steers were rumen cannulated and for experiment 1 and 3 rumen samples were taken with oesophagus rumen tube from intact steers. In the second experiment, the use of cannulated steers allowed to take rumen content from different sites of the rumen, maybe obtaining a more representative sample. Nevertheless, for the experiment 2, microbial populations were not directly studied and rumen fluid samples were taken to estimate rumen fermentation parameters. Due to the increase of animal welfare considerations, the availability of cannulated animals in UK is limited and not broadly accepted. Therefore, for the last experiment, rumen fluid from steers was taken with oesophagus rumen tube. When rumen fluid is taken from intact cows, the greatest challenge is to achieve a representative sample in terms of microbial population and concentration. Oesophagus rumen tubing may underestimate some members of fibrolytic communities in the rumen, which are attached to solid particles as less solid content is obtained with this method (Henderson et al., 2013). Another negative aspect of this technique is the possible contamination of samples with saliva. On the contrary, some studies reported stomach tubing as a feasible alternative to rumen cannulation for rumen microbiome studies, and observed the same taxa affected by dietary treatment across both types of samples (Ramos-Morales et al., 2014; Terre et al., 2013). In addition, it is often possible to sample a greater number of intact, compared to cannulated animals.

Another alternative for rumen sampling is obtaining *post-mortem* digesta content. For the last experiment of this study, rumen samples from a group of animals (samples from mixed diets from 2014) were collected at the slaughter house and available. DNA extraction, amplicon libraries preparation and sequencing were

carried out with the same methods and simultaneously with samples from *in vivo*. Due to time limitation, a complete study of the sequencing analysis from *post-mortem* samples was not performed and not presented in the results section. Nevertheless, some preliminary analyses are presented here. When samples from mixed control group and mixed nitrate group were compared, 20 and 25% of all OTUs were significantly different in *in vivo* and *post-mortem* samples, respectively. Approximately one third (33%) of OTUs presented at different relative abundance between treatments were common across both samples types (*in vivo* and *post-mortem*) (Figure 5.1).

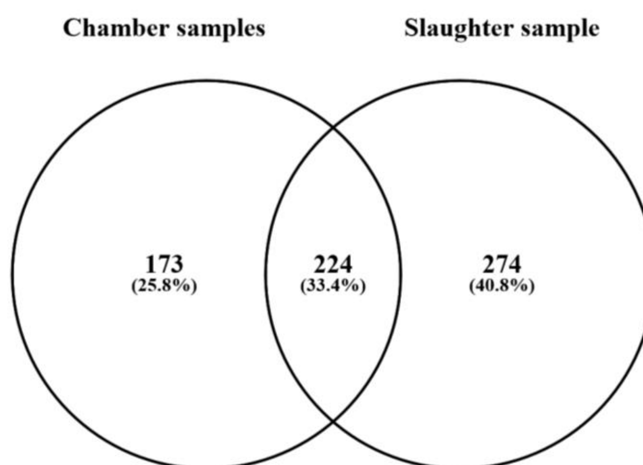

Figure 5.1 Number of OTUs with different relative abundance between mixed control and mixed nitrate diets in chamber (*in vivo*) and slaughter (*post mortem*) samples

When archaea specific OTUs were investigated, two OTUs with relative abundances significantly different between treatment groups in *post mortem* samples were common in rumen fluid *in vivo* samples (OTU 961, *Methanobacteriaceae* and OTU 950, *vadin CA11*). Relative abundance of total archaea was higher in *in vivo* samples than *post-mortem*, but similar percentage was shared between the different archaea taxonomies in *in vivo* and *post-mortem* samples, with *Methanobrevibacter* as the dominant genera. An overall correlation was found between CH_4 emissions per DMI from individual animals and total archaea relative abundance with the correlation being similar in samples from live animals (R^2 = 0.39) and *post mortem*

(R^2 = 0.46) (Table 5.1). Splitting according to sample type (*post-mortem* or *in vivo*) resulted in similar regressions with both types of sample (Figure 5.2) with a significant regression coefficient for both types of samples ($P < 0.05$). The results seem to imply that *in vivo* samples will differ from the *post-mortem* ones, but the shifts due to dietary treatment are similar and can be detected in both sample types.

Table 5.1 Mean relative abundance of archaea taxonomies and correlation with CH_4 (g/kg DMI) in *in vivo* and *post-mortem* samples

	Mixed (*In vivo*)			Mixed (*Post-mortem*)		
Taxonomy	Ro	P-value	Mean	Ro	P-value	Mean
Methanobacteriaceae	0.377	0.005	8.69	0.458	0.008	5.41
G *Methanobrevibacter*	0.387	0.004	8.58	0.454	0.009	5.32
G *Methanosphaera*	0.339	0.013	0.10			0.08
Methanomassiliicoccaceae Genus *vadinCA11*	0.305	0.026	0.05	0.449	0.010	0.04
Total Archaea	0.393	0.004	8.74	0.461	0.008	5.45
Ratio A:B	0.393	0.004	0.10	0.461	0.008	0.06

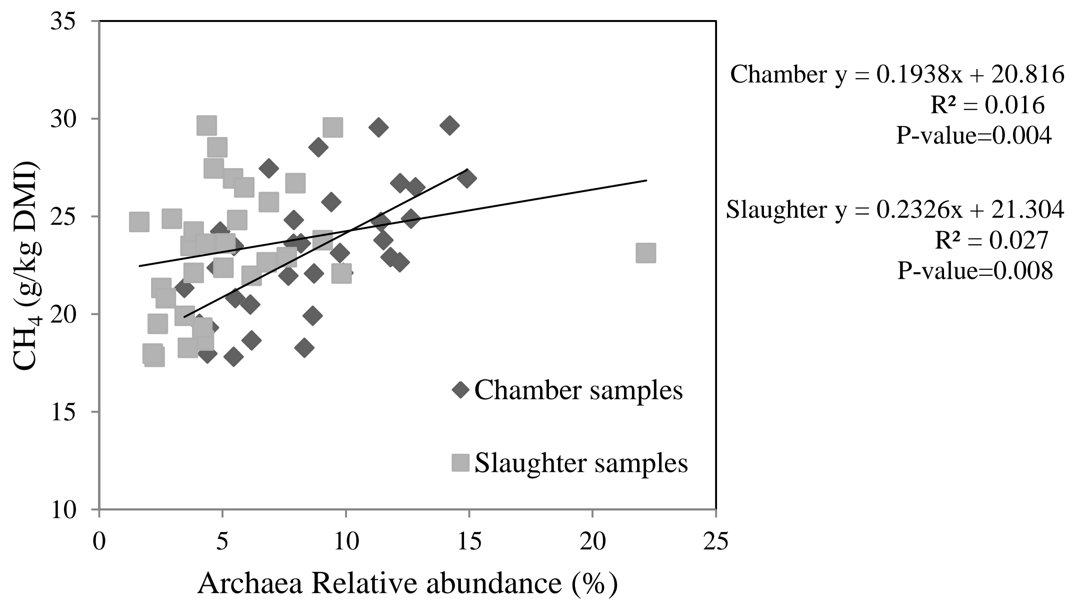

Figure 5.2 Correlation between CH_4 emissions of steers in respiration chambers and archaea relative abundance in ruminal digesta in live animals and at slaughter

Similar correlations between number of archaea and CH_4 emission from *in vivo* samples and *post-mortem* have been reported in a previous study (Wallace et al., 2014). Protozoal numbers were reported to be lower in *post-mortem* samples than *in vivo*. The authors hypothesized that this difference in protozoa number could be due to starvation of animals before slaughter (Wallace et al., 2014).

From the analysis reported here and relevant literature review the rumen samples taken with oesophagus tube for the last experiment of this thesis were considered adequate for the study purpose. *Post-mortem* samples seem promising as an alternative to *in vivo* samples but further analysis would be needed.

5.3 GENERAL IMPLICATIONS

5.3.1 Strategies to reduce methane emission from ruminants

The major focus of this research was to assess effective strategies to reduce enteric CH_4 emission from beef cattle but the results reported here could be likely applied to other domestic ruminants. Globally, optimising productivity is by far the most important strategy for lowering enteric CH_4 production from ruminants. Currently, there are a great number of ruminant production systems below their productivity potential due to a lack of resources (Steinfeld et al., 2006). In developed countries, the reality is different as ruminant production systems have been improved. In this context, the possibility for more refined approaches to reduce CH_4 production from individual animals is feasible and of paramount importance. From all the methods to decrease CH_4 emission from ruminants studied so far, there is no consensus in the literature on which method has more advantages over the others, given that different factors such as species, production system, geographic location and economic level interfere with the aplicability of the method. Dietary additives to mitigate enteric CH_4 have different mechanisms of action and the effectiveness of these compounds might be strongly linked to ruminal fermentation conditions being affected by substrate (Castro-Montoya et al., 2012). Accordingly, this research assess antimethanogenic additives within specific production system and/or diets.

In addition, it should be taken into account that additives used to decrease CH_4 production could have an influence on the form and amount of N excreted in urine and faeces. An increase in N portioning towards faecal excreta is desirable as N in manure is more stable and less prone to denitrification and nitrification than NH_3-N from urinary urea. Although not a direct GHG, NH_3-N affects the earth's radiation balance through aerosol formation and cloud forming processes and if NH_3-N is deposited may suffer from denitrification releasing N_2O to the atmosphere (Denmead et al., 2008; Loh et al., 2008). However, the interactions involving N_2O and NH_3-N need to be studied in detail in specific conditions because the conditions that support nitrification and denitrification processes are highly variable (Gerber et al., 2013).

5.3.2 Practical implications of nitrate and biochar addition to beef cattle diets

Nitrate addition: Based on the literature and the results from the current study nitrate added to high forage diets (>50%) is an efficient feeding strategy to reduce long term CH_4 emission from ruminants. For practical implementation, the inclusion of nitrate in the animal diet would need to be studied on whole farm basis, with measures of N_2O and H_2 release by the animal, the excretion of N, and the costs of feeding accounted. Nitrate should be included in high forage diets (>50%) because nitrate has been ineffective when included in intensive finishing beef diets (Troy et al., 2015). However, it is not feasible to administer nitrate to grazing animals since access should be continued and the intake of nitrates must be controlled to avoid adverse effects. Nitrate must be thoroughly mixed and diluted with other feed constituents. This is probably best achieved by the use of total mixed rations or by inclusion of nitrate in pelleted compound feeds. Nitrate should replace protein sources in the diet to avoid excess excretion of N in manure. The amount of nitrate naturally present and from fertilisers applied in the forages would need to be calculated prior to include this additive in the diets.

Encapsulated Nitrate fed in Experiment 2 decreased the excretion of N in urine compared with soybean meal as the main protein source. van Zijderveld et al. (2011) reported that feeding nitrate as a substitute for urea did not change the amount of N excreted in urine but may alter the N composition of urine (*e.g.*, greater nitrate but less urea). If urea excreted in urine is decreased NH_3-N emissions from excreta would be lower as urinary urea is the major source of NH_3-N volatilized from manure (Lee et al., 2012). On the other hand, the possible release of N_2O when nitrate is fed should be studied. N_2O could be released directly from the animal or after denitrification of nitrate excreted in faeces and urine in soils.

As a practical implication, most of the major types of beef production system in UK use a high proportion of grass compared with other regions of the world (Wilkinson, 2011). Therefore, the addition of nitrate to farms that fed conserved grass could be effective in decreasing CH_4 yield from cattle. Nevertheless, because

of the possible animal health implications nitrate is likely to be classified as a feed additive under EU Regulations and cannot be legally included in animal feeds at present.

In the current study energy balance was not studied but DMI and body weight gain were not affected by treatments. Previous studies have observed mild negative effects in animal efficiency with nitrate addition (21 g/kg of DM), such as a decrease in FCR (Duttie et al., 2017), ADG (Hegarty et al., 2016) and decrease in DMI (Zijderveld et al., 2011, Newbold et al., 2014, Guyader et al., 2015, Hegarty et al., 2016, Klop et al, 2016). Hegarty et al. (2016) reported reduced DMI, ADG and FCR when nitrate at high dose (45 g/kg DM) replaced urea in feedlot using high grain diets (700 g / kg DM). Guyader et al. (2016) reported reduced fat and protein corrected milk yield when both nitrate (18 g/kg of DM) and extruded linseed were added to the diet. Feeding nitrate at 21 g/kg DM resulted in a small, but significant reduction in milk protein content and yield (Klop et al., 2016). In this thesis, I argue that it can not be assumed that the increase in metabolizable energy from lowering CH_4 emissions will be retained and use by the animal. An important factor when including nitrate into rations is the level of inclusion. If the dose exce ssed the microbial growth requirement it is likely that nitrate will reduce DMI and more N will be excreted and N_2O released from manure storage.

Decreasing the rapidity of nitrate reduction in the rumen by feeding nitrate with a slow-release coating is a strategy that is now under investigation (de Raphaelis-Soissan et al., 2017; Lee et al., 2015). Encapsulated nitrate used in this study is a product in development which aims to provide a slower release source of nitrate in comparison with nitrate salts with expected lower risk of toxicity of nitrate salts. In support, a recent study carried out by Raphaelis-Soissan et al. (2017) showed that nitrate coated with paraffin improves the safety of nitrate supplementation, as shown by the lower blood MetHb concentrations but without apparent reductions in CH_4 mitigation.

In addition, the persistency of CH_4 lowering effect with nitrate was not well investigated with encapsulated nitrate. Otherwise, Troy et al. (2015) observed a reduction in CH_4 with calcium nitrate after a period of 24 weeks implying a long

term reduction of CH_4 production. Others have also reported long term reduction in CH_4 production with nitrate addition (Guyader et al., 2016; van Zijderveld et al., 2011). The additional benefit of nitrate as a NPN source should be considered. Since protein continues to be a costly component of ruminant rations, decreasing quantities of protein required has become a strategy to reduce production costs (Kalscheur et al., 1999). In addition, protein sources currently used in livestock feeding may be used directly as vegetable protein sources for human feed consumption. Therefore, formulation of ruminant diets with lower true protein concentrations may have an economical, social and environmental impact. However, considering the risk of toxicity of nitrate and levels needed to achieve substantial effects in CH_4 production, promote the idea of combining nitrate with another antimethanogenic additive with different mechanism of action in CH_4 reduction. Reducing the dose of nitrate required would increase the safety of feeding nitrate but may reduce its protein nutritional value.

Biochar addition: Biochar as antimethanogenic compound could be applied to animals fed under different production systems as the interaction with diets and feeding method has not yet been explored. The absence of any negative effects of biochar on rumen fermentation coupled with the observed reduction in NH_3-N concentrations makes it possible that feeding biochar to livestock could be a low cost means of applying biochar to pasture and soil, and a way to reduce NH_3-N excretion from ruminants. The rumen CH_4 reduction potential is still questionable.

Economical implications: The cost of production, environmental and purchase of biochar and nitrate should also be considered. Nitrate is not yet used as a feed additive, but is sold at present as fertiliser, with highly variable price. The cost of feeding nitrate at the level required to reduce CH_4 production would need to be studied. In the hypothesis that nitrate were regulated as feed additive for animals, an increase in cost will affect the use of nitrate as a CH_4 mitigating strategy (Callaghan et al., 2014), especially if no additional gains are obtained feeding nitrate to finishing cattle. Therefore, up to date nitrate could only be considered and recommended following some change to income streams that would provide farmers with an incentive to mitigate CH_4 (Doreau et al., 2014).

CO_2 emission due to the industrial process of nitrate production is similar to that of urea production (Doreau et al., 2014) and therefore not significant when the environmental costs of production are considered.

The production of biochar reduces the wastes on agricultural land, with less CO_2 from natural decomposition or open combustion and N_2O from inappropriate use of wastes on agricultural land. Moreover, it helps to sequester carbon in a stable form in biochar compound. From the previous, it could be concluded that the use of biochar as ruminant additive could bring environmental benefits but probably not as an enteric CH_4 mitigation strategy per se.

Combination of additives: An interesting option would be the study of the addition of biochar and nitrate in combination into beef cattle diets. If biochar is effective in reducing NH_3-N concentration in the rumen, less urea will be excreted in urine, and is likely that less N_2O would be release from slurry. As an example, supplementing the diet with tannins or feeding tanniferous forages can also decrease N release rate from manure, and thus affect manure-N availability for plant growth (Hristov et al., 2013). In support, Grainger et al. (2009) showed that feeding CT to dairy cows reduced urinary N excretion by 50% with 20% more N excreted in the faeces. However, this option would need further study before practical recommendations because supplementation with tannins may impair protein utilization, reducing animal performance and not all sources of tannin have shown to decrease CH_4 production. Others promising options that have been under study are the combination of nitrate with sulphate sources or lipids (Troy et al, 2015, Guyader et al, 2015, van Zijderveld et al, 2010). Other combinations of additives should be further assessed.

5.3.3 Microbial community analysis

Understanding the role of methanogens and bacteria in ruminal CH_4 production and their interaction with diets will be important for development and implementation of ruminant CH_4 abatement strategies. There is currently a broad range of sequencing technologies studying the rumen microbiome by characterising the rumen microbial community structure and linking this with the functions of the rumen microbiota (Morgavi et al., 2013). Most molecular techniques for identification and classification of bacteria and archaea have been based on the nucleotide sequences of the 16S ribosomal RNA gene (Ozutsumi et al., 2005).

It has been suggested that the rumen microbiome and rumen function might be disturbed if CH_4 production in the rumen is decreased (Veneman et al., 2015). However, in this study rumen fermentation was not significantly affected by nitrate, although some minor changes were observed in microbial populations. The sequencing of 16S rRNA gene showed that the effects of nitrate addition on microbial populations were different depending of the basal diet. In addition individual differences appear to influence the effectiveness of nitrate to reduce CH_4. Understanding individual responses and interaction with rumen microbiome is essential to increase the safety of feeding nitrate (Yang et al., 2016).

Future studies combining diets, breeds, and sires and results from deep sequencing techniques may help to get a better understanding of the factors that modify the microbial composition, host-microbiome interactions and effectiveness of antimethanogenic compounds. In addition, genes expression involve in methanogenesis pathways would need to be studied in conjunction with microbial composition.

5.4 FUTURE WORK

5.4.1 Biochar

Firstly, the effects of biochar in rumen fermentation and gas production need to be validated with more *in vitro* work. Only after accurate *in vitro* studies have been carried out the prospect to develop *in vivo* studies could be determined. The *in vivo* experiments would be able to certify the antimethanogenic effects of biochar in ruminants. If *in vivo* work is design, the partitioning of N excreta with biochar inclusion in ruminants diets would need to be studied.

- To confirm the greatest CH_4 reduction with biochar produced from miscanthus straw and at lower pyrolysis temperature (550° C), a preliminary study with MSC550 included should be carried out.

- Biochar compounds available, prioritising the ones from lower pyrolysis temperature and from miscanthus straw, at 10g/kg DM substrate level of addition will be incubated with different substrates (high and low forages diets).

- If biochar compounds are able to reduce CH_4 production by more than 5% further *in vitro* test would be of interest. For following trials the compounds that reduce CH_4 by more than 5% will be the ones to be pursue.

- To answer if biochar is contributing to fermentation, a combination of biochar compounds (from different feedstuffs) and different substrates (high and low forages diets) with the study of substrate degradability will bring together more information about the mode of action and the effects on rumen fermentation.

5.4.2 Nitrate

A recent *in vivo* study (de Raphaelis-Soissan et al., 2017) has shown that peaks of nitrate and nitrite in rumen and plasma occur 30 mins after feeding and that nitrite returned to pre experimental levels within 3 h and nitrate after 6 h of administration. In agreement, Veneman et al. (2015) detected both peak of rumen fluid nitrate and nitrite concentrations approximately 30 min after the sheep were dosed with nitrate. Plasma MetHb showed a peak 60 mins after feeding, suggesting a quick response in the conversion of Hb to MetHb due to plasma nitrite.

- To add information about the absorption and metabolism of nitrate compounds, an experiment will be design with inclusion of different forms of nitrate (slow release forms vs nitrate salts). The concentration of nitrate, nitrite, and NH_3-N in the rumen and plasma, blood MetHb concentration during the day after feeding both additives, and the daily pattern of CH_4 production will be measured. A start point before 30 mins will be set as nitrite half life in blood is only 11 mins, and prior conversion to NH_3-N may happen. No study has recorded the levels of the different forms of N before 30 mins after feeding. Due to practical implications of blood sampling, catheter will be used to allow for multiple sampling in a day. Cannulated animals would be desirable if the purpose is to measure rumen N contents at different times over the day. The different forms of nitrate will be dosed intra ruminally in order to provide identical nitrate supply without any effects of individual feeding pattern.

- An alternative to the use of cannulated animals would be to take rumen contents at different times from different animals (one measure per animal) but a large number of animals would be needed to account for differences in nitrate reduction between animals.

- To corroborate the no effect of nitrate in CH_4 production in concentrate diets a confirmatory experiment with nitrate included to diets with different levels of concentrate would be appropriate. For CH_4 measurement the Cal method would be the desired method.

- To explore the effects of nitrate on feeding behaviour, a different study with nitrate included in the ration will be develop with *ad libitum* feeding. The study of the daily feed intake pattern with automatic feeders during adaptation to nitrate will be studied.

- For future application, the emission of N_2O would need to be accounted, to address whether a decrease in CH_4 production is not counteracted by a significant increase in N_2O emissions.

- To validate the use of nitrate as a source of NPN, control diet should be designed to be below the minimum levels of protein required by the animal and urea could be used as a NPN control source. For beef cattle diets CP should be below 13% of DM and nitrate should not be included at levels greater than 25g/kg of DM and urea will be included at level of 10 g/kg of DM to have a positive control. If an improvement of MPS is observed with nitrate inclusion compared with protein deficient diets, the use of nitrate as a source of NPN would be validated.

- To calculate the metabolizable energy, the energy excreted with faeces and urine, as well as energy losses in the form of CH_4 and H_2 will be measured and subtract them from the gross energy ingested (GEI).

- To accurately determine the effects of nitrate in animal performance, DMI, ADG and RFI will be measure. The protocol of adaptation to nitrate could be similar as the one used in the current study (with 25% increase of the final dose of nitrate every 4 days) as it was successful to avoid nitrate toxicity and effects in DMI.

- To increase both nitrate and nitrite reductions, the inclusion of sulphur compounds will be studied. From results of the current study, it seems likely that organic sulphur compounds, or sulphate would be the sulphur compounds selected.

5.4.3 Microbial community analysis.

The inclusion of microbial analysis would bring together information about the effects of nitrate in rumen fermentation, microbial composition and CH_4 production.

a) Further analysis on existing data:

- To bring more information about unknown nitrate reducers or population directly correlated with CH_4 production, the study of all OTUs identified affected by nitrate will be of interest.

- To validate the use of slaughter rumen content samples, the data available from microbial composition of slaughter samples can be further studied and check for correlations with samples *in vivo* and if treatment effects on *post mortem* microbial communities were similar to those observed *in vivo*.

a) Further analysis on existing samples:

- To obtain a better resolution when assigning taxonomies, the study of multiple hypervariable regions of 16S rRNA gene will be of interest.

- To identify protozoa populations and their potential role in nitrate metabolism and CH_4 production, samples will be used for WGS to identify 18S rRNA gene.

- To have better information about functionality, metagenomic approach would be better to highlight the genes involve in nitrate metabolism and CH_4 production.

c) New experiments

- To explore the changes that are directly induced by nitrate inclusion and not host specific, the microbial composition before addition of nitrate to animal diets should be studied. Then, the study of microbial community at different time points during adaptation could highlight the population likely affected by nitrate.

- Buccal swabs samples could be an alternative to rumen content samples for future studies. To confirm whether microbial community present in buccal samples represent the rumen content, a study should be designed taking rumen content and saliva samples from the same animals at the same time point and compared the sequences obtained.

- For rumen microbial community analysis, the study of rumen content in the slaughter seems a good alternative to sampling *in vivo* when animals are already adapted to nitrate.

- For future work with rumen cannulated animals or with samples taken at the slaughter, the fractions of rumen content could be separated and look for differences in rumen liquid and solid fractions.

5.5 CONCLUDING REMARKS

To summarise, many dietary strategies to reduce CH_4 emissions are under study, with differences in effectiveness and applicability. From the present study, biochar was not proved to be a good candidate for lowering CH_4 production from ruminants for extensive deliver on farms, but further research would be needed. Nitrate demonstrated to reduce CH_4 emission in beef cattle fed forage based diets and did not produce any toxicity effect with stepwise adaptation. Despite CH_4 reductions, no improvements in protein synthesis or energetic gains were observed with nitrate addition. Some minor but consistent changes were observed at OTU level with nitrate addition to steers diets. In addition, archaea specific population were correlated with CH_4 production across all samples.

Reference List

Abecia, L., Toral, P.G., Martin-Garcia, A.I., Martinez, G., Tomkins, N.W., Molina-Alcaide, E., Newbold, C.J., Yanez-Ruiz, D.R., 2012. Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. Journal of Dairy Science 95, 2027-2036.

Alaboudi, A.R., Jones, G.A., 1985. Effect of acclimation to high nitrate intakes on some rumen fermentation parameters in sheep. Canadian Journal of Animal Science 65, 841-849.

Allison, M.J., 1969. Biosynthesis of amino acids by ruminal microorganisms. Journal of animal science 29, 797-807.

Allison, M.J., Reddy, C.A., 1984. Adaptations of gastrointestinal bacteria in response to changes in dietary oxalate and nitrate.

Allison, M.J., Reddy, C.A., Cook, H.M., 1981. The effects of nitrate and nitrite on VFA and CH_4 production by ruminal microbes. J. Anim. Sci 53, 391.

Aluwong, T., Wuyep, P.A., Allam, L., 2011. Livestock-environment interactions: Methane emissions from ruminants. African Journal of Biotechnology 10, 1265-1269.

Alves, E.M., Magalhúes, D.R., Freitas, M.A., Santos, E.d.J.d., Pereira, M.L.c.A., Pedreira, M.d.S., 2014. Nitrogen metabolism and microbial synthesis in sheep fed diets containing slow release urea to replace the conventional urea. Acta Scientiarum. Animal Sciences 36, 55-62.

Ammerman, C.B., Goodrich, R.D., 1983. Advances in mineral nutrition in ruminants. Journal of animal science 57, 519-533.

Anderson, R.C., Krueger, N.A., Stanton, T.B., Callaway, T.R., Edrington, T.S., Harvey, R.B., Jung, Y.S., Nisbet, D.J., 2008. Effects of select nitrocompounds on *in vitro* ruminal fermentation during conditions of limiting or excess added reductant. Bioresource Technology 99, 8655-8661.

Asanuma, N., Iwamoto, M., Kawato, M., Hino, T., 2002. Numbers of nitrate–reducing bacteria in the rumen as estimated by competitive polymerase chain reaction. Animal Science Journal 73, 199-205.

Asanuma, N., Yokoyama, S., Hino, T., 2015. Effects of nitrate addition to a diet on fermentation and microbial populations in the rumen of goats, with special reference to *Selenomonas ruminantium* having the ability to reduce nitrate and nitrite. Animal Science Journal 86, 378-384.

Ascensao, A.M.D., 2010. Effects of nitrate and additional effect of probiotic on methane emissions and dry matter intake in Nellore bulls.

Attwood, G.T., Altermann, E., Kelly, W.J., Leahy, S.C., Zhang, L., Morrison, M., 2011. Exploring rumen methanogen genomes to identify targets for methane mitigation strategies. Animal Feed Science and Technology 166, 65-75.

Attwood, G., McSweeney, C., 2008a. Methanogen genomics to discover targets for methane mitigation technologies and options for alternative H_2 utilisation in the rumen. Anim. Prod. Sci. 48, 28-37.

Attwood, G., McSweeney, C., 2008b. Methanogen genomics to discover targets for methane mitigation technologies and options for alternative H_2 utilisation in the rumen. Aust. J. Exp. Agric. 48, 28-37.

Baker, G.C., Smith, J.J., Cowan, D.A., 2003. Review and re-analysis of domain-specific 16S primers. Journal of microbiological methods 55, 541-555.

Baldwin, R.L., Allison, M.J., 1983. Rumen metabolism. Journal of animal science 57, 461-477.

Ball, P.N., MacKenzie, M.D., DeLuca, T.H., Montana, W.E.H., 2010. Wildfire and Charcoal Enhance Nitrification and Ammonium-Oxidizing Bacterial Abundance in Dry Montane Forest Soils All rights reserved. 39, 1243-1253.

Barton, H.A., Taylor, N.M., Lubbers, B.R., Pemberton, A.C., 2006. DNA extraction from low-biomass carbonate rock: an improved method with reduced contamination and the low-biomass contaminant database. J Microbiol Methods 66.

Beauchemin, K.A., Kreuzer, M., O Mara, F., McAllister, T.A., 2008. Nutritional management for enteric methane abatement: a review. Aust. J. Exp. Agric. 48, 21-27.

Beauchemin, K.A., McGinn, S.M., 2005. Methane emissions from feedlot cattle fed barley or corn diets. Journal of animal science 83, 653-661.

Beauchemin, K.A., McGinn, S.M., 2006a. Methane emissions from beef cattle: effects of fumaric acid, essential oil, and canola oil. Journal of animal science 84, 1489-1496.

Beauchemin, K.A., McGinn, S.M., 2006b. Effects of various feed additives on the methane emissions from beef cattle. International Congress Series 1293, 152-155.

Belanche, A., de la Fuente, G., Newbold, C.J., 2014. Study of methanogen communities associated with different rumen protozoal populations. FEMS Microbiology Ecology 90, 663-677.

Benchaar, C., Greathead, H., 2011. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. Animal Feed Science and Technology 166–167, 338-355.

Berndt, A., Boland, T.M., Deighton, M.H., Gere, J.I., Grainger, C., Hegarty, R.S., Iwaasa, A.D., Koolaard, J.P., Lassey, K.R., Luo, D., 2014. Guidelines for use of sulphur hexafluoride (SF 6) tracer technique to measure enteric methane emissions from ruminants. Ministry of Primary Industries: Wellington, New Zealand.

Bird, P.R., 1972. Sulphur Metabolism and Excretion Studies in Ruminants IX. Sulphur, Nitrogen, and Energy Utilization by Sheep Fed a Sulphur-Deficient and a Sulphate-Supplemented, Roughage-Based Diet. Aust. Jnl. Of Bio. Sci. 25, 1073-1086.

Boadi, D.A., Wittenberg, K.M., Kennedy, A.D., 2002. Validation of the sulphur hexafluoride (SF6) tracer gas technique for measurement of methane and carbon dioxide production by cattle. Canadian Journal of Animal Science 82, 125-131.

Boadi, D.A., Wittenberg, K.M., Scott, S.L., Burton, D., Buckley, K., Small, J.A., Ominski, K.H., 2004. Effect of low and high forage diet on enteric and manure pack greenhouse gas emissions from a feedlot. Canadian Journal of Animal Science 84, 445-453.

Bodas, R., Lopez, S., Fernandez, M., Garcia-Gonzalez, R., Rodriguez, A.B., Wallace, R.J., Gonzalez, J.S., 2008. *In vitro* screening of the potential of numerous plant species as antimethanogenic feed additives for ruminants. Animal Feed Science and Technology 145, 245-258.

Borrel, G., PW. O'Toole, P.W., Harris, H.M., Peyret, P., Brugere, J.F., Gribaldo, S., 2013. Phylogenomic data support a seventh order of methylotrophic methanogens and provide insights into the evolution of methanogenesis. Genome biology and evolution 5, 1769-1780.

Bradford, G.E., 1999. Contributions of animal agriculture to meeting global human food demand. Livestock Production Science 59, 95-112.

Broderick, G.A., Reynal, S.M., 2009. Effect of source of rumen-degraded protein on production and ruminal metabolism in lactating dairy cows1. Journal of Dairy Science 92, 2822-2834.

Brown, E.G., Anderson, R.C., Carstens, G.E., Gutierrez-Bañuelos, H., McReynolds, J.L., Slay, L.J., Callaway, T.R., Nisbet, D.J., 2011. Effects of oral nitroethane administration on enteric methane emissions and ruminal fermentation in cattle. Animal Feed Science and Technology 166, 275-281.

Brown, P.; Broomfield, M.; Buys, G.; Cardenas, L.; Kilroy, E.; MacCarthy, J.; Murrells, T.; Pang, Y.; Passant, N.; Ramirez Garcia, J.; Thistlethwaite, G.; Webb, N.. 2016 UK greenhouse gas inventory, 1990 to 2014: annual report for submission under the Framework Convention on Climate Change. Energy & Environment, 568pp.

Bruning-Fann, C.S., Kaneene, J.B., 1993. The effects of nitrate, nitrite, and N-nitroso compounds on animal health. Veterinary and Human Toxicology 35, 237-253.

Callaghan, M.J., Tomkins, N.W., Benu, I., Parker, A.J., 2014. How feasible is it to replace urea with nitrates to mitigate greenhouse gas emissions from extensively managed beef cattle? Anim. Prod. Sci. 54, 1300-1304.

Calsamiglia, S., Busquet, M., Cardozo, P.W., Castillejos, L., Ferret, A., 2007. Invited review: essential oils as modifiers of rumen microbial fermentation. Journal of Dairy Science 90, 2580-2595.

Calvelo Pereira, R., Muetzel, S., Camps Arbestain, M., Bishop, P., Hina, K., Hedley, M., 2014. Assessment of the influence of biochar on rumen and silage fermentation: A laboratory-scale experiment. Animal Feed Science and Technology 196, 22-31.

Caporaso, J.G., Lauber, C.L., Walters, W.A., Berg-Lyons, D., Huntley, J., Fierer, N., Owens, S.M., Betley, J., Fraser, L., Bauer, M., 2012. Ultra-high-throughput microbial community analysis on the Illumina HiSeq and MiSeq platforms. The ISME journal 6, 1621-1624.

Carberry, C.A., Kenny, D.A., Han, S., McCabe, M.S., Waters, S.M., 2012. Effect of phenotypic residual feed intake and dietary forage content on the rumen microbial community of beef cattle. Applied and Environmental Microbiology 78, 4949-4958.

Carberry, C.A., Waters, S.M., Kenny, D.A., Creevey, C.J., 2014. Rumen Methanogenic Genotypes Differ in Abundance According to Host Residual Feed Intake Phenotype and Diet Type. Applied and Environmental Microbiology 80, 586-594.

Cardozo, P.W., Calsamiglia, S., Ferret, A., Kamel, C., 2005. Screening for the effects of natural plant extracts at different pH on *in vitro* rumen microbial fermentation of a high-concentrate diet for beef cattle. Journal of animal science 83, 2572-2579.

Carro, M.D., Ranilla, M.J., 2003. Effect of the addition of malate on *in vitro* rumen fermentation of cereal grains. British journal of nutrition 89, 181-188.

Casey, J.W., Holden, N.M., 2006. Quantification of GHG emissions from sucker-beef production in Ireland. Agricultural Systems 90, 79-98.

Castro-Montoya, J., De Campeneere, S., Van Ranst, G., Fievez, V., 2012. Interactions between methane mitigation additives and basal substrates on in *vitro* methane and VFA production. Animal Feed Science and Technology 176,47-60.

Change, I.P.O.C., 2007. Climate change 2007: the physical science basis. Agenda 6.

Change, I.I.P.O.C., 2006. Guidelines for national greenhouse gas inventories. CD ou no site: www. ipcc. ch. Guia de Boas Praticas.

Chen, X.B., Orskov, E.R., Hovell, F.D., 1990. Excretion of purine derivatives by ruminants: endogenous excretion, differences between cattle and sheep. British journal of nutrition 63, 121-129.

Chung, Y.H., Walker, N.D., McGinn, S.M., Beauchemin, K.A., 2011. Differing effects of 2 active dried yeast (*Saccharomyces cerevisiae*) strains on ruminal acidosis and methane production in nonlactating dairy cows. Journal of Dairy Science 94, 2431-2439.

Chuntrakort, P., Otsuka, M., Hayashi, K., Takenaka, A., Udchachon, S., Sommart, K., 2014. The effect of dietary coconut kernels, whole cottonseeds and sunflower seeds on the intake, digestibility and enteric methane emissions of Zebu beef cattle fed rice straw based diets. Livestock Science 161, 80-89.

Clark, H., 2013. Nutritional and host effects on methanogenesis in the grazing ruminant. animal 7, 41-48.

Cockburn, A., Brambilla, G., Fernandez, M.L., Arcella, D., Bordajandi, L.R., Cottrill, B., van Peteghem, C., Dorne, J.L., 2013. Nitrite in feed: from animal health to human health. Toxicology and applied pharmacology 270, 209-217.

Cockrum, R., Austin, K., Ludden, P., Cammack, K., 2008. Effects of elevated dietary nitrate on production and reproduction parameters in Suffolk ewes. In: pp. 332-335.

Conrad, R., Wetter, B., 1990. Influence of temperature on energetics of hydrogen metabolism in homoacetogenic, methanogenic, and other anaerobic bacteria. Archives of Microbiology 155, 94-98.

Cottle, D.J., Nolan, J.V., Wiedemann, S.G., 2011. Ruminant enteric methane mitigation: a review. Anim. Prod. Sci. 51, 491-514.

Crawford, R.F., Kennedy, W.K., Davison, K.L., 1966. Factors influencing the toxicity of forages that contain nitrate when fed to cattle. Cornell Veterinarian 56, 3-17.

Czerkawski, J.W., Harfoot, C.G., Breckenridge, G., 1972. The relationship between methane production and concentrations of hydrogen in the aqueous and gaseous phases during rumen fermentation *in vitro*. Journal of Applied Bacteriology 35, 537-551.

Danielsson, R., Schnurer, A., Arthurson, V., Bertilsson, J., 2012. Methanogenic population and CH4 production in Swedish dairy cows fed different levels of forage. Applied and Environmental Microbiology 78, 6172-6179.

Danielsson, R., Dicksved, J., Sun, L., Gonda, H., Muller, B., Schnirer, A., Bertilsson, J., 2017. Methane production in dairy cows correlates with rumen methanogenic and bacterial community structure. Frontiers in microbiology 8.

de Almeida Rufino, L.M., Detmann, E., Gomes, D., dos Reis, W.L.S., Batista, E.D., de Campos Valadares Filho, S., Paulino, M.F., 2016. Intake, digestibility and nitrogen utilization in cattle fed tropical forage and supplemented with protein in the rumen, abomasum, or both. Journal of animal science and biotechnology 7, 1.

de Raphaelis-Soissan, V., Nolan, J.V., Godwin, I.R., Newbold, J.R., Perdok, H.B., Hegarty, R.S., 2017. Paraffin-wax-coated nitrate salt inhibits short-term methane production in sheep and reduces the risk of nitrite toxicity. Animal Feed Science and Technology 229, 57-64.

Demeyer, D.I., Fiedler, D., De Graeve, K.G., 1996. Attempted induction of reductive acetogenesis into the rumen fermentation *in vitro*. Reproduction Nutrition Development 36, 233-240.

Denman, S.E., Martinez Fernandez, G., Shinkai, T., Mitsumori, M., McSweeney, C.S., 2015. Metagenomic analysis of the rumen microbial community following inhibition of methane formation by a halogenated methane analog. Frontiers in microbiology 6, 1087.

Denman, S.E., Tomkins, N.W., McSweeney, C.S., 2007. Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. FEMS Microbiology Ecology 62, 313-322.

Denmead, O.T., Chen, D., Griffith, D.W.T., Loh, Z.M., Bai, M., Naylor, T., 2008. Emissions of the indirect greenhouse gases NH3 and NOx from Australian beef cattle feedlots. Aust. J. Exp. Agric. 48, 213-218.

Dewhurst, R.J., Kim, E.J., Evans, R.T., Cabrita, A.R.J., Fonseca, A.J.M., 2007. Effects of dietary sulphur sources on concentrations of hydrogen sulphide in the rumen head-space gas of dairy cows.

Dewhurst, R., 2013. Milk production from silage: comparison of grass, legume and maize silages and their mixtures. Agricultural and food science 22, 57-69.

Di Bella, J.M., Bao, Y., Gloor, G.B., Burton, J.P., Reid, G., 2013. High throughput sequencing methods and analysis for microbiome research. Journal of microbiological methods 95, 401-414.

Diaz, N.N., Krause, L., Goesmann, A., Niehaus, K., Nattkemper, T.W., 2009. TACOA: taxonomic classification of environmental genomic fragments using a kernelized nearest neighbor approach. BMC Bioinformatics 10.

Dijkstra, J., Oenema, O., Bannink, A., 2011. Dietary strategies to reducing N excretion from cattle: implications for methane emissions. Current Opinion in Environmental Sustainability 3, 414-422.

Doreau, M., Bamire, L., Pellerin, S., Lherm, M., Benoit, M., 2014. Mitigation of enteric methane for French cattle: potential extent and cost of selected actions. Anim. Prod. Sci. 54, 1417-1422.

Drewnoski, M.E., Pogge, D.J., Hansen, S.L., 2014. High-sulfur in beef cattle diets: a review. Journal of animal science 92, 3763-3780.

Drewnoski, M.E., Richter, E.L., Hansen, S.L., 2012. Dietary sulfur concentration affects rumen hydrogen sulfide concentrations in feedlot steers during transition and finishing. Journal of animal science 90, 4478-4486.

Duin, E.C., Wagner, T., Shima, S., Prakash, D., Cronin, B., Yanez-Ruiz, D.R., Duval, S., Rambeli, R., Stemmler, R.T., Thauer, R.K., 2016. Mode of action uncovered for the specific reduction of methane emissions from ruminants by the small molecule 3-nitrooxypropanol. Proceedings of the National Academy of Sciences 113, 6172-6177.

Duthie, C.A., Rooke, J.A., Troy, S., Hyslop, J.J., Ross, D.W., Waterhouse, A., Roehe, R., 2016. Impact of adding nitrate or increasing the lipid content of two contrasting diets on blood methaemoglobin and performance of two breeds of finishing beef steers. animal 10, 786-795.

Duthie, C.A., Haskell, M., Hyslop, J.J., Waterhouse, A., Wallace, R.J., Roehe, R., Rooke, J.A., 2017. The impact of divergent breed types and diets on methane emissions, rumen characteristics and performance of finishing beef cattle. animal, 1-10.

Eckard, R.J., Grainger, C., De Klein, C.A.M., 2010. Options for the abatement of methane and nitrous oxide from ruminant production: A review. Livestock Science 130, 47-56.

El-Zaiat, H.M., Araujo, R.C., Soltan, Y.A., Morsy, A.S., Louvandini, H., Pires, A.V., Patino, H.O., Correa, P.S., Abdalla, A.L., 2014. Encapsulated nitrate and cashew nut shell liquid on blood and rumen constituents, methane emission, and growth performance of lambs. Journal of animal science 92, 2214-2224.

Eryavuz, A., Dundar, Y., Ozdemir, M., Aslan, R., Tekerli, M., 2003. Effects of urea and sulfur on performance of faunate and defaunate Ramlic lambs, and some rumen and blood parameters. Animal Feed Science and Technology 109, 35-46.

Eugene, M., Archimede, H., Sauvant, D., 2004. Quantitative meta-analysis on the effects of defaunation of the rumen on growth, intake and digestion in ruminants. Livestock Production Science 85, 81-97.

FAOSTAT (FAO, accessed March 2016) http://www.fao.org/faostat/en/#data.

Farra, P.A., Satter, L.D., 1971. Manipulation of the ruminal fermentation. III. Effect of nitrate on ruminal volatile fatty acid production and milk composition. Journal of Dairy Science 54, 1018-1024.

Farrell, M., Kuhn, T.K., Macdonald, L.M., Maddern, T.M., Murphy, D.V., Hall, P.A., Singh, B.P., Baumann, K., Krull, E.S., Baldock, J.A., 2013. Microbial utilisation of biochar-derived carbon. Science of The Total Environment 465, 288-297.

Fenner, H., 1965. Method for determining total volatile bases in rumen fluid by steam distillation. Journal of Dairy Science 48, 249-251.

Feng, Y.X., 2012. Y Yu Y, Xie Z and Lin Xi 2012 Mechanisms of biochar decreasing methane emission from Chinese paddy soils. Journal of Soils Biology and Biochemistry 46, 80-88.

Fiorentini, G., Carvalho, I.P.C., Messana, J.D., Castagnino, P.S., Berndt, A., Canesin, R.C., Frighetto, R.T.S., Berchielli, T.T., 2014. Effect of lipid sources with different fatty acid profiles on the intake, performance, and methane emissions of feedlot Nellore steers. Journal of animal science 92, 1613-1620.

Firkins, J.L., Yu, Z., 2015. Ruminant Nutrition Symposium: How to use data on the rumen microbiome to improve our understanding of ruminant nutrition. Journal of animal science 93, 1450-1470.

Foley, P.A., Kenny, D.A., Callan, J.J., Boland, T.M., Omara, F.P., 2009a. Effect of- malic acid supplementation on feed intake, methane emission, and rumen fermentation in beef cattle. Journal of animal science 87, 1048-1057.

Foley, P.A., Kenny, D.A., Lovett, D.K., Callan, J.J., Boland, T.M., Ömara, F.P., 2009b. Effect of dl-malic acid supplementation on feed intake, methane emissions, and performance of lactating dairy cows at pasture. Journal of Dairy Science 92, 3258-3264.

Fonty, G., Joblin, K., Chavarot, M., Roux, R., Naylor, G., Michallon, F., 2007. Establishment and development of ruminal hydrogenotrophs in methanogen-free lambs. Applied and Environmental Microbiology 73, 6391-6403.

Franzolin, R., Dehority, B.A., 1996. Effect of prolonged high-concentrate feeding on ruminal protozoa concentrations. Journal of animal science 74, 2803-2809.

Fron, M.J., Boling, J.A., Bush, L.P., Dawson, K.A., 1990. Sulfur and nitrogen metabolism in the bovine fed different forms of supplemental sulfur. Journal of animal science 68, 543-552.

Fujihara, T., Orskov, E.R., Reeds, P.J., Kyle, D.J., 1987. The effect of protein infusion on urinary excretion of purine derivatives in ruminants nourished by intragastric nutrition. The Journal of Agricultural Science 109, 7-12.

Gai, X., Wang, H., Liu, J., Zhai, L., Liu, S., Ren, T., Liu, H., 2014. Effects of feedstock and pyrolysis temperature on biochar adsorption of ammonium and nitrate. PloS one 9, e113888.

Gerber, P.J., Steinfeld, H., Henderson, B., Mottet, A., Opio, C., Dijkman, J., Falcucci, A., Tempio, G., 2013. Tackling climate change through livestock: a global assessment of emissions and mitigation opportunities. Food and Agriculture Organization of the United Nations (FAO).

Getachew, G., Blummel, M., Makkar, H.P.S., Becker, K., 1998. *In vitro* gas measuring techniques for assessment of nutritional quality of feeds: a review. Animal Feed Science and Technology 72, 261-281.

Gilbert, J.A., Field, D., Swift, P., Thomas, S., Cummings, D., Temperton, B., Weynberg, K., Huse, S., Hughes, M., Joint, I., Somerfield, P.J., Muhling, M., 2010. The taxonomic and functional diversity of microbes at a temperate coastal site: a 'multi-omic' study of seasonal and diel temporal variation. PloS one 5.

Gill, M., Smith, P., Wilkinson, J.M., 2010. Mitigating climate change: the role of domestic livestock. animal 4, 323-333.

Global Livestock Environmental Assessment Model (GLEAM). (accesed February 2017); http://www.fao.org/gleam/results/en/.

Goel, G., Makkar, H.P.S., Becker, K., 2008. Changes in microbial community structure, methanogenesis and rumen fermentation in response to saponin–rich fractions from different plant materials. Journal of applied microbiology 105, 770-777.

Goel, G., Makkar, H.P., 2012. Methane mitigation from ruminants using tannins and saponins. Tropical animal health and production 44, 729-739.

Goel, G., Makkar, H.P., Becker, K., 2009. Inhibition of methanogens by bromochloromethane: effects on microbial communities and rumen fermentation using batch and continuous fermentations. British journal of nutrition 101, 1484-1492.

Gould, D.H., 1998. Polioencephalomalacia. Journal of animal science 76, 309-314.

Godwin, I., Li, L., Luijben, K., Oelbrandt, N., Velazco, J., Miller, J., Hegarty, R., 2014. The effects of chronic nitrate supplementation on erythrocytic methaemoglobin reduction in cattle. Anim. Prod. Sci.

Grainger, C., Beauchemin, K.A., 2011. Can enteric methane emissions from ruminants be lowered without lowering their production? Animal Feed Science and Technology 166–167, 308-320.

Grainger, C., Clarke, T., Auldist, M.J., Beauchemin, K.A., McGinn, S.M., Waghorn, G.C., Eckard, R.J., 2009. Mitigation of greenhouse gas emissions from dairy cows fed pasture and grain through supplementation with Acacia mearnsii tannins. Can. J. Anim. Sci 89, 241-251.

Grainger, C., Williams, R., Eckard, R.J., Hannah, M.C., 2010. A high dose of monensin does not reduce methane emissions of dairy cows offered pasture supplemented with grain. Journal of Dairy Science 93, 5300-5308.

Guan, H., Wittenberg, K.M., Ominski, K.H., Krause, D.O., 2006. Efficacy of ionophores in cattle diets for mitigation of enteric methane. Journal of animal science 84, 1896-1906.

Guo, W.S., Schaefer, D.M., Guo, X.X., Ren, L.P., Meng, Q.X., 2009. Use of nitrate-nitrogen as a sole dietary nitrogen source to inhibit ruminal methanogenesis and to improve microbial nitrogen synthesis *in vitro*. Asian Aust. J. Anim. Sci 22, 542-549.

Gurwick, N.P., Moore, L.A., Kelly, C., Elias, P., 2013. A systematic review of biochar research, with a focus on its stability in situ and its promise as a climate mitigation strategy. PloS one 8, e75932.

Guyader, J., Doreau, M., Morgavi, D.P., Gerard, C., Loncke, C., Martin, C., 2016. Long-term effect of linseed plus nitrate fed to dairy cows on enteric methane emission and nitrate and nitrite residuals in milk. animal 10, 1173-1181.

Guyader, J., Eugene, M., Noziere, P., Morgavi, D.P., Doreau, M., Martin, C., 2014. Influence of rumen protozoa on methane emission in ruminants: a meta-analysis approach. animal 8, 1816-1825.

Haisan, J., Sun, Y., Beauchemin, K., Guan, L., Duval, S., Barreda, D.R., Oba, M., 2013. Effects of feeding 3-nitrooxypropanol, at varying levels, on methane emissions and rumen fermentation in lactating dairy cows. Adv. Anim. Biosci 4, 326.

Hammond, K.J., Burke, J.L., Koolaard, J.P., Muetzel, S., Pinares-Patino, C.S., Waghorn, G.C., 2013. Effects of feed intake on enteric methane emissions from sheep fed fresh white clover (Trifolium repens) and perennial ryegrass (Lolium perenne) forages. Animal Feed Science and Technology 179, 121-132.

Hammond, K.J., Pacheco, D., Burke, J.L., Koolaard, J.P., Muetzel, S., Waghorn, G.C., 2014. The effects of fresh forages and feed intake level on digesta kinetics and enteric methane emissions from sheep. Animal Feed Science and Technology 193, 32-43.

Hansen, H.H., Storm, I.M.L.D., Sell, A.M., 2012. Effect of biochar on *in vitro* rumen methane production. Acta Agriculturae Scandinavica, Section Animal Science 62, 305-309.

Hedderich, R., Klimmek, O., Kruger, A., Dirmeier, R., Keller, M., Stetter, K.O., 1998. Anaerobic respiration with elemental sulfur and with disulfides. FEMS microbiology reviews 22, 353-381.

Hegarty, R.S., 1999. Mechanisms for competitively reducing ruminal methanogenesis. Aust. J. Agric. Res. 50, 1299-1306.

Hegarty, R.S., Gerdes, R., 1999. Hydrogen production and transfer in the rumen. Recent Advances in Animal Nutrition in Australia 12, 37-44.

Hegarty, R.S., Goopy, J.P., Herd, R.M., McCorkell, B., 2007. Cattle selected for lower residual feed intake have reduced daily methane production. Journal of animal science 85, 1479-1486.

Hegarty, R.S., Miller, J., Oelbrandt, N., Li, L., Luijben, J.P.M., Robinson, D.L., Nolan, J.V., Perdok, H.B., 2016. Feed intake, growth, and body and carcass attributes of feedlot steers supplemented with two levels of calcium nitrate or urea. Journal of animal science 94, 5372-5381.

Hegarty, R.S., Miller, J., Robinson, D.L., Li, L., Oelbrandt, N., Luijben, K., McGrath, J., Bremner, G., Perdok, H.B., 2013. Growth, efficiency and carcass attributes of feedlot cattle supplemented with calcium nitrate or urea. Greenhouse Gas and Agriculture Animals (GGAA), Dublin, Ireland.

Helmer, L.G., Bartley, E.E., 1971. Progress in the utilization of urea as a protein replacer for ruminants. A review. Journal of Dairy Science 54, 25-51.

Henderson, G., Cox, F., Kittelmann, S., Miri, V.H., Zethof, M., Noel, S.J., Waghorn, G.C., Janssen, P.H., 2013. Effect of DNA extraction methods and sampling techniques on the apparent structure of cow and sheep rumen microbial communities. PloS one 8, e74787.

Henning, P.H., Horn, C.H., Steyn, D.G., Meissner, H.H., Hagg, F.M., 2010. The potential of Megasphaera elsdenii isolates to control ruminal acidosis. Animal Feed Science and Technology 157, 13-19.

Herd, R. M., et al. "Genetic variation for methane traits in beef cattle." Proceedings of the 10th world congress of genetics applied to livestock production'. Paper. Vol. 38. 2014.

Holder, V.B., Tricarico, J.M., Kim, D.H., Kristensen, N.B., Harmon, D.L., 2015. The effects of degradable nitrogen level and slow release urea on nitrogen balance and urea kinetics in Holstein steers. Animal Feed Science and Technology 200, 57-65.

Hook, S.E., Steele, M.A., Northwood, K.S., Wright, A.D., McBride, B.W., 2011. Impact of high-concentrate feeding and low ruminal pH on methanogens and protozoa in the rumen of dairy cows. Microbial ecology 62, 94-105.

Hook, S.E., Wright, A.D., McBride, B.W., 2010. Methanogens: methane producers of the rumen and mitigation strategies. Archaea 2010.

Hristov, A.N., Lee, C., Hristova, R., Huhtanen, P., Firkins, J.L., 2012. A meta-analysis of variability in continuous-culture ruminal fermentation and digestibility data. Journal of Dairy Science 95, 5299-5307.

Hristov, A.N., Oh, J., Firkins, J.L., Dijkstra, J., Kebreab, E., Waghorn, G., Makkar, H.P.S., Adesogan, A.T., Yang, W., Lee, C., 2013. SPECIAL TOPICS:Mitigation of methane and nitrous oxide emissions from animal operations: I. A review of enteric methane mitigation options. Journal of animal science 91, 5045-5069.

Hulshof, R.B.A., Berndt, A., Gerrits, W.J.J., Dijkstra, J., van Zijderveld, S.M., Newbold, J.R., Perdok, H.B., 2012. Dietary nitrate supplementation reduces methane emission in beef cattle fed sugarcane-based diets. Journal of animal science 90, 2317-2323.

Hunerberg, M., McGinn, S.M., Beauchemin, K.A., Entz, T., Okine, E.K., Harstad, O.M., McAllister, T.A., 2015. Impact of ruminal pH on enteric methane emissions. Journal of animal science 93, 1760-1766.

Hungate, R.E., Smith, W., Bauchop, T., Yu, I., Rabinowitz, J.C., 1970. Formate as an intermediate in the bovine rumen fermentation. Journal of Bacteriology 102, 389-397.

Iwamoto, M., Asanuma, N., 1999. Effects of nitrate combined with fumarate on methanogenesis, fermentation, and cellulose digestion by mixed ruminal microbes *in vitro*. Nihon Chikusan Gakkaiho 70, 471-478.

Iwamoto, M., Asanuma, N., Hino, T., 2001. Effects of pH and electron donors on nitrate and nitrite reduction in ruminal microbiota. The Japanese journal of zootechnical science 72, 117-125.

Iwamoto, M., Asanuma, N., Hino, T., 2002. Ability of *Selenomonas ruminantium*, *Veillonella parvula*, and *Wolinella succinogene*s to Reduce Nitrate and Nitrite with Special Reference to the Suppression of Ruminal Methanogenesis. Anaerobe 8, 209-215.

Janssen, P.H., 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Animal Feed Science and Technology 160, 1-22.

Janssen, P.H., Kirs, M., 2008. Structure of the archaeal community of the rumen. Applied and Environmental Microbiology 74, 3619-3625.

Jayanegara, A., Goel, G., Makkar, H.P., Becker, K., 2015. Divergence between purified hydrolysable and condensed tannin effects on methane emission, rumen fermentation and microbial population *in vitro*. Animal Feed Science and Technology 209, 60-68.

Jeyanathan, J., Martin, C., Morgavi, D.P., 2013. The use of direct-fed microbials for mitigation of ruminant methane emissions: a review. animal, 1-12.

Johnson, K.A., Westberg, H.H., Michal, J.J., Cossalman, M.W., 2007. The SF6 tracer technique: Methane measurement from ruminants. In: Measuring methane production from ruminants, Springer, pp. 33-67.

Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. Journal of animal science 73, 2483-2492.

Johnson, W.H., Goodrich, R.D., Meiske, J.C., 1971. Metabolism of radioactive sulfur from elemental sulfur, sodium sulfate and methionine by lambs. Journal of animal science 32, 778-783.

Jones, G.A., 1972. Dissimilatory metabolism of nitrate by the rumen microbiota. Canadian journal of microbiology 18, 1783-1787.

Joseph, S.D., Downie, A., Munroe, P., Crosky, A., Lehmann, J., 2007. Biochar for carbon sequestration, reduction of greenhouse gas emissions and enhancement of soil fertility; A review of the materials science. In.

Kahlon, T.S., Meiske, J.C., Goodrich, R.D., 1975. Sulfur metabolism in ruminants. Journal of animal science 41, 1154-1160.

Kajikawa, H., Valdes, C., Hillman, K., Wallace, R.J., Newbold, J., 2003. Methane oxidation and its coupled electron-sink reactions in ruminal fluid. Letters in Applied Microbiology 36, 354-357.

Kalscheur, K.F., Vandersall, J.H., Erdman, R.A., Kohn, R.A., Russek-Cohen, E., 1999. Effects of Dietary Crude Protein Concentration and Degradability on Milk Production Responses of Early, Mid, and Late Lactation Dairy Cows. Journal of Dairy Science 82, 545-554.

Kang, S.H., Evans, P., Morrison, M., McSweeney, C., 2013. Identification of metabolically active proteobacterial and archaeal communities in the rumen by DNA and RNA derived 16S rRNA gene. Journal of applied microbiology 115, 644-653.

Kaspar, H.F., Tiedje, J.M., 1981. Dissimilatory reduction of nitrate and nitrite in the bovine rumen: nitrous oxide production and effect of acetylene. Applied and Environmental Microbiology 41, 705-709.

Kertz, A.F., 2010. Review: urea feeding to dairy cattle: a historical perspective and review. The Professional Animal Scientist 26, 257-272.

Khiaosa-ard, R., Zebeli, Q., 2013. Meta-analysis of the effects of essential oils and their bioactive compounds on rumen fermentation characteristics and feed efficiency in ruminants. Journal of animal science 91, 1819-1830.

Khiaosa-ard, R., Zebeli, Q., 2014. Cattle' s variation in rumen ecology and metabolism and its contributions to feed efficiency. Livestock Science.

Kim, E.T., Moon, Y.H., Min, K.S., Kim, C.H., Kim, S.C., Ahn, S.K., Lee, S.S., 2013. Changes in microbial diversity, methanogenesis and fermentation characteristics in the rumen in response to medicinal plant extracts. Asian-Australasian Journal of Animal Sciences (AJAS) 26, 1289-1294.

King, E.E., Smith, R.P., St-Pierre, B., Wright, A.D., 2011. Differences in the rumen methanogen populations of lactating Jersey and Holstein dairy cows under the same diet regimen. Applied and Environmental Microbiology 77, 5682-5687.

Kipling, R.P., Bannink, A., Bellocchi, G., Dalgaard, T., Fox, N.J., Hutchings, N.J., Kjeldsen, C., Lacetera, N., Sinabell, F., Topp, C.F., 2016. Modeling European ruminant production systems: Facing the challenges of climate change. Agricultural Systems 147, 24-37.

Kittelmann, S., Kirk, M.R., Jonker, A., McCulloch, A., Janssen, P.H., 2015. Buccal swabbing as a noninvasive method to determine bacterial, archaeal, and eukaryotic microbial community structures in the rumen. Applied and Environmental Microbiology 81, 7470-7483.

Kittelmann, S., Pinares-Patino, C.S., Seedorf, H., Kirk, M.R., Ganesh, S., McEwan, J.C., Janssen, P.H., 2014. Two Different Bacterial Community Types Are Linked with the Low-Methane Emission Trait in Sheep. PloS one 9, e103171.

Kittelmann, S., Seedorf, H., Walters, W.A., Clemente, J.C., Knight, R., Gordon, J.I., Janssen, P.H., 2013. Simultaneous Amplicon Sequencing to Explore Co-Occurrence Patterns of Bacterial, Archaeal and Eukaryotic Microorganisms in Rumen Microbial Communities. PloS one 8, e47879.

Klevenhusen, F., Muro-Reyes, A., Khiaosa-ard, R., Metzler-Zebeli, B.U., Zebeli, Q., 2012. A meta-analysis of effects of chemical composition of incubated diet and bioactive compounds on *in vitro* ruminal fermentation. Animal Feed Science and Technology 176, 61-69.

Klop, G., Hatew, B., Bannink, A., Dijkstra, J., 2016. Feeding nitrate and docosahexaenoic acid affects enteric methane production and milk fatty acid composition in lactating dairy cows. Journal of Dairy Science 99, 1161-1172.

Klug, M.J., Reddy, C.A., 1984. Current perspectives in microbial ecology : proceedings of the third International Symposium on Microbial Ecology, Michigan State University, 7-12 August 1983.

Krumholz, L.R., Forsberg, C.W., Veira, D.M., 1983. Association of methanogenic bacteria with rumen protozoa. Canadian journal of microbiology 29, 676-680.

Kumar, S., Choudhury, P.K., Carro, M.D., Griffith, G.W., Dagar, S.S., Puniya, M., Calabro, S., Ravella, S.R., Dhewa, T., Upadhyay, R.C., 2014. New aspects and strategies for methane mitigation from ruminants. Applied microbiology and biotechnology 98, 31-44.

Latham, E.A., Anderson, R.C., Pinchak, W.E., Nisbet, D.J., 2016. Insights on alterations to the rumen ecosystem by nitrate and nitrocompounds. Frontiers in microbiology 7.

Leahy, S.C., Kelly, W.J., Ronimus, R.S., Wedlock, N., Altermann, E., Attwood, G.T., 2013. Genome sequencing of rumen bacteria and archaea and its application to methane mitigation strategies. animal 7, 235-243.

Lee, C., Araujo, R.C., Koenig, K.M., Beauchemin, K.A., 2015a. Effects of encapsulated nitrate on eating behavior, rumen fermentation, and blood profile of beef heifers fed restrictively or ad libitum12. 93, 2405-2418.

Lee, C., Araujo, R.C., Koenig, K.M., Beauchemin, K.A., 2015. Effects of encapsulated nitrate on enteric methane production and nitrogen and energy utilization in beef heifers. Journal of animal science 93, 2391-2404.

Lee, C., Hristov, A.N., Dell, C.J., Feyereisen, G.W., Kaye, J., Beegle, D., 2012. Effect of dietary protein concentration on ammonia and greenhouse gas emitting potential of dairy manure. Journal of Dairy Science 95, 1930-1941.

Lee, C., Beauchemin, K.A., 2014. A review of feeding supplementary nitrate to ruminant animals: nitrate toxicity, methane emissions, and production performance. Canadian Journal of Animal Science 94, 557-570.

Lehmann, J., Joseph, S., 2009. Biochar for environmental management: science and technology. Earthscan.

Lehmann, J., Rillig, M.C., Thies, J., Masiello, C.A., Hockaday, W.C., Crowley, D., 2011. Biochar effects on soil biota :
A review. Soil Biology and Biochemistry 43, 1812-1836.

Leng, R.A., 2008. The potential of feeding nitrate to reduce enteric methane production in ruminants. A Report to the Departmernt of Climate Change Commonwealth Government of Australia. ACT Canberra Australia For paper and PPT presentation see http://www. penambulbooks. com.

Leng, R.A., 2014. Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation. Anim. Prod. Sci. 54, 519-543.

Leng, R.A., Inthapanya, S., Preston, T.R., 2012a. Biochar lowers net methane production from rumen fluid *in vitro*. Livestock Research for Rural Development 24.

Leng, R.A., Preston, T.R., 2010. Further considerations of the potential of nitrate as a high affinity electron acceptor to lower enteric methane production in ruminants. Livestock Research for Rural Development 22.

Leng, R.A., Preston, T.R., Inthapanya, S., 2012b. Biochar reduces enteric methane and improves growth and feed conversion in local Yellow cattle fed cassava root chips and fresh cassava foliage. Livestock Research for Rural Development 24.

Lewis, D., 1951. The metabolism of nitrate and nitrite in the sheep. 2. Hydrogen donators in nitrate reduction by rumen micro-organisms *in vitro*. Biochemical Journal 49, 149.

Li, L., Davis, J., Nolan, J., Hegarty, R., 2012. An initial investigation on rumen fermentation pattern and methane emission of sheep offered diets containing urea or nitrate as the nitrogen source. Anim. Prod. Sci. 52, 653-658.

Li, L., Silveira, C.I., Nolan, J.V., Godwin, I.R., Leng, R.A., Hegarty, R.S., 2013. Effect of added dietary nitrate and elemental sulfur on wool growth and methane emission of Merino lambs. Anim. Prod. Sci. 53, 1195-1201.

Lila, Z.A., Mohammed, N., Yasui, T., Kurokawa, Y., Kanda, S., Itabashi, H., 2004. Effects of a twin strain of live cells on mixed ruminal microorganism fermentation *in vitro*. Journal of animal science 82, 1847-1854.

Lillis, L., Boots, B., Kenny, D.A., Petrie, K., Boland, T.M., Clipson, N., Doyle, E.M., 2011. The effect of dietary concentrate and soya oil inclusion on microbial diversity in the rumen of cattle. Journal of applied microbiology 111, 1426-1435.

Lin, M., Schaefer, D.M., Guo, W.S., Ren, L.P., Meng, Q.X., 2011. Comparisons of *in vitro* nitrate reduction, methanogenesis, and fermentation acid profile among rumen bacterial, protozoal and fungal fractions. Asian-Australas J Anim Sci 24, 471-478.

Lin, M., Guo, W., Meng, Q., Stevenson, D.M., Weimer, P.J., Schaefer, D.M., 2013. Changes in rumen bacterial community composition in steers in response to dietary nitrate. Applied microbiology and biotechnology 97, 8719-8727.

Liu, L., Xu, X., Cao, Y., Cai, C., Cui, H., Yao, J., 2017. Nitrate decreases methane production also by increasing methane oxidation through stimulating NC10 population in ruminal culture. AMB Express 7, 76.

Liu, Y., Whitman, W.B., 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Annals of the New York Academy of Sciences 1125, 171-189.

Liu, Y., Yang, M., Wu, Y., Wang, H., Chen, Y., Wu, W., 2011. Reducing CH4 and CO2 emissions from waterlogged paddy soil with biochar. Journal of Soils and Sediments 11, 930-939.

Loh, Z., Chen, D., Bai, M., Naylor, T., Griffith, D., Hill, J., Denmead, T., McGinn, S., Edis, R., 2008. Measurement of greenhouse gas emissions from Australian feedlot beef production using open-path spectroscopy and atmospheric dispersion modelling. Aust. J. Exp. Agric. 48, 244-247.

Lopez, S., McIntosh, F.M., Wallace, R.J., Newbold, C.J., 1999. Effect of adding acetogenic bacteria on methane production by mixed rumen microorganisms. Animal Feed Science and Technology 78, 1-9.

Lund, P., Dahl, R., Yang, H.J., Hellwing, A.L.F., Cao, B.B., Weisbjerg, M.R., 2014. The acute effect of addition of nitrate on *in vitro* and *in vivo* methane emission in dairy cows. Anim. Prod. Sci. 54, 1432-1435.

Machmuller, A., 2006. Medium-chain fatty acids and their potential to reduce methanogenesis in domestic ruminants. Agriculture, ecosystems & environment 112, 107-114.

Makkar, H.P., Sen, S., Blummel, M., Becker, K., 1998. Effects of fractions containing saponins from Yucca schidigera, Quillaja saponaria, and Acacia auriculoformis on rumen fermentation. Journal of Agricultural and Food Chemistry 46, 4324-4328.

Malik, P.K., Bhatta, R., Gagen, E.J., Sejian, V., Soren, N.M., Prasad, C.S., 2015. Alternate H2 Sinks for Reducing Rumen Methanogenesis. In: Climate Change Impact on Livestock: Adaptation and Mitigation, Springer, pp. 303-320.

Malmuthuge, N., 2017. Understanding host-microbial interactions in rumen: searching the best opportunity for microbiota manipulation. Journal of animal science and biotechnology 8, 8.

Marais, J.P., Therion, J.J., Mackie, R.I., Kistner, A., Dennison, C., 1988. Effect of nitrate and its reduction products on the growth and activity of the rumen microbial population. British journal of nutrition 59, 301-313.

Martin, C, Morgavi, D.P., Doreau, M., 2010a. Methane mitigation in ruminants: from microbe to the farm scale. animal 4, 351-365.

Martinez-Fernandez, G., Abecia, L., Arco, A., Cantalapiedra-Hijar, G., Martin-Garcia, A.I., Molina-Alcaide, E., Kindermann, M., Duval, S., Yanez-Ruiz, D.R., 2014. Effects of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol on ruminal fermentation, microbial abundance, and methane emissions in sheep. Journal of Dairy Science 97, 3790-3799.

Martinez-Fernandez, G., Abecia, L., Martin-Garcia, A.I., Ramos-Morales, E., Hervas, G., Molina-Alcaide, E., Yanez-Ruiz, D.R., 2013a. *In vitro–in vivo* study on the effects of plant compounds on rumen fermentation, microbial abundances and methane emissions in goats. animal 7, 1925-1934.

Martinez-Fernandez, G., Abecia, L., Martin-Garcia, A.I., Ramos-Morales, E., Denman, S.E., Newbold, C.J., Molina-Alcaide, E., Yanez-Ruiz, D.R., 2015. Response of the rumen archaeal and bacterial populations to anti-methanogenic organosulphur compounds in continuous-culture fermenters. FEMS Microbiology Ecology 91, fiv079.

Martinez-Fernandez, G., Arco, A., Abecia, L., Cantalapiedra-Hijar, G., Molina-Alcaide, E., Martin-Garcia, A.I., Kindermann, M., Duval, S., Yanez-Ruiz, D.R., 2013b. The addition of ethyl-3-nitrooxy propionate and 3-nitrooxypropanol in

the diet of sheep sustainably reduces methane emissions and the effect persists over a month. Adv Anim Biosci 4, 368.

McAllister, T.A., Newbold, C.J., 2008. Redirecting rumen fermentation to reduce methanogenesis. Aust. J. Exp. Agric. 48, 7-13.

McAllister, T.A., Beauchemin, K.A., McGinn, S.M., Hao, X., Robinson, P.H., 2011. Greenhouse gases in animal agriculture–Finding a balance between food production and emissions. Animal Feed Science and Technology 166–167, 1-6.

McCabe, M. S., Cormican, P., Keogh, K., O'Connor, A., O'Hara, E., Palladino, R. A., Kenny, D. A., Waters, S. M. 2015. Illumina MiSeq phylogenetic amplicon sequencing shows a large reduction of an uncharacterised *Succinivibrionaceae* and an increase of the *Methanobrevibacter gottschalkii* clade in feed restricted cattle. PLOS One. DOI:10.1371/journal.pone.0133234

McCann, J.C., Wiley, L.M., Forbes, T.D., Rouquette Jr, F.M., Tedeschi, L.O., 2014. Relationship between the rumen microbiome and residual feed intake-efficiency of Brahman bulls stocked on bermudagrass pastures. PloS one 9, e91864.

McGinn, S.M., Beauchemin, K.A., Iwaasa, A.D., McAllister, T.A., 2006. Assessment of the Sulfur Hexafluoride (SF) Tracer Technique for Measuring Enteric Methane Emissions from Cattle. Journal of environmental quality 35, 1686-1691.

McSweeney, C.S., Denman, S.E., Conlan, L.L., Prasad, C.S., Anandan, S., Chandrasekharaiah, M., Sampath, K.T., 2009. The stimulatory effect of the organic sulfur supplement, mercaptopropane sulfonic acid on cellulolytic rumen microorganisms and microbial protein synthesis in cattle fed low sulfur roughages. animal 3, 802-809.

McSweeney, C.S., Denman, S.E., 2007. Effect of sulfur supplements on cellulolytic rumen micro-organisms and microbial protein synthesis in cattle fed a high fibre diet. Journal of applied microbiology 103, 1757-1765.

Mehmet, O., Abdullah, E., Gülcan A., Yavuz Osman B., İsmail K.., 2014. Effects of supplementation of inorganic sulfur on some biochemical parameters in Angora goat fed diet containing high nitrate levels. Turkish Journal of Veterinary and Animal Sciences 38, 526-533.

Menke, K.H., Raab, L., Salewski, A., Steingass, H., Fritz, D., Schneider, W., 1979. The estimation of the digestibility and metabolizable energy content of ruminant feedingstuffs from the gas production when they are incubated with rumen liquor *in vitro*. The Journal of Agricultural Science 93, 217-222.

Menke, K.H., Steingass, H., 1988. Estimation of the energetic feed value obtained from chemical analysis and *in vitro* gas production using rumen fluid. Anim. Res. Dev 28, 7-55.

Mertens, D.R., 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds with refluxing in beakers or crucibles: collaborative study. Journal of AOAC international 85, 1217-1240.

Milne, A.E., Glendining, M.J., Bellamy, P., Misselbrook, T., Gilhespy, S., Casado, M.R., Hulin, A., Van Oijen, M., Whitmore, A.P., 2014. Analysis of uncertainties in the estimates of nitrous oxide and methane emissions in the UK's greenhouse gas inventory for agriculture. Atmospheric Environment 82, 94-105.

Mitsumori, M., Ajisaka, N., Tajima, K., Kajikawa, H., Kurihara, M., 2002. Detection of Proteobacteria from the rumen by PCR using methanotroph-specific primers. Letters in Applied Microbiology 35, 251-255.

Mitsumori, M., Sun, W., 2008. Control of rumen microbial fermentation for mitigating methane emissions from the rumen. ASIAN AUSTRALASIAN JOURNAL OF ANIMAL SCIENCES 21, 144.

Morgavi, D.P., Forano, E., Martin, C., Newbold, C.J., 2010a. Microbial ecosystem and methanogenesis in ruminants. animal 4, 1024-1036.

Morgavi, D.P., Forano, E., Martin, C., Newbold, C.J., 2010b. Microbial ecosystem and methanogenesis in ruminants. animal 4, 1024-1036.

Moss, A.R., Jouany, J.P., Newbold, J., 2000. Methane production by ruminants: its contribution to global warming. In: Paris: Institut national de la recherche agronomique, 1960-2000., pp. 231-254.

Mota, D.A., Messana, J.D., Canesin, R.C., Fiorentini, G., Pires, A.V., Berchielli, T.T., 2015. Different true-protein sources do not modify the metabolism of crossbred Bos taurus Bos indicus growing heifers. Revista Brasileira de Zootecnia 44, 52-59.

Mwenya, B., Santoso, B., Sar, C., Gamo, Y., Kobayashi, T., Arai, I., Takahashi, J., 2004. Effects of including 4 galacto-oligosaccharides, lactic acid bacteria or yeast culture on methanogenesis as well as energy and nitrogen metabolism in sheep. Animal Feed Science and Technology 115, 313-326.

Myer, P.R., Kim, M., Freetly, H.C., Smith, T.P.L., 2016. Evaluation of 16S rRNA amplicon sequencing using two next-generation sequencing technologies for phylogenetic analysis of the rumen bacterial community in steers. Journal of microbiological methods 127, 132-140.

Myer, P.R., Smith, T.P., Wells, J.E., Kuehn, L.A., Freetly, H.C., 2015. Rumen microbiome from steers differing in feed efficiency. PloS one 10, e0129174.

Nagadi, S., Herrero, M., Jessop, N.S., 2000. The influence of diet of the donor animal on the initial bacterial concentration of ruminal fluid and *in vitro* gas production degradability parameters. Animal Feed Science and Technology 87, 231-239.

Napasirth, P., Wachirapakorn, C., Saenjan, P., Yuangklang, C., 2013. Effect of Sulfate-Containing Compounds on Methane Production by Using an *In vitro* Gas Production Technique. Pakistan Journal of Nutrition 12, 723.

National, R.C., 1996. Nutrient requirements of beef cattle. In: National Academy Press Washington, DC.

Newbold, C.J., Lopez, S., Nelson, N., Ouda, J.O., Wallace, R.J., Moss, A.R., 2005. Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation *in vitro*. 94, 27-35.

Newbold, C.J., Rode, L.M., 2006. Dietary additives to control methanogenesis in the rumen. In: Elsevier, pp. 138-147.

Newbold, C.J., de la Fuente, G., Belanche, A., Ramos-Morales, E., McEwan, N.R., 2015. The role of ciliate protozoa in the rumen. Frontiers in microbiology 6, 1313.

Newbold, J.R., van Zijderveld, S.M., Hulshof, R.B.A., Fokkink, W.B., Leng, R.A., Terencio, P., Powers, W.J., van Adrichem, P.S.J., Paton, N.D., Perdok, H.B., 2014. The effect of incremental levels of dietary nitrate on methane emissions in Holstein steers and performance in Nelore bulls. Journal of animal science 92, 5032-5040.

Nguyen, S., Barnett, M., Hegarty, R., 2015. Use of dietary nitrate to increase productivity and reduce methane production of defaunated and faunated lambs consuming protein deficient chaff. Anim. Prod. Sci.

Nolan, J.V., Hegarty, R.S., Hegarty, J., Godwin, I.R., Woodgate, R., 2010. Effects of dietary nitrate on fermentation, methane production and digesta kinetics in sheep. Anim. Prod. Sci. 50, 801-806.

Oae, S., Okuyama, T., 1992. Organic sulfur chemistry: biochemical aspects. CRC Press.

Odongo, N.E., Bagg, R., Vessie, G., Dick, P., Or-Rashid, M.M., Hook, S.E., Gray, J.T., Kebreab, E., France, J., McBride, B.W., 2007. Long-term effects of feeding monensin on methane production in lactating dairy cows. Journal of Dairy Science 90, 1781-1788.

Oren, A., 2012. There must be an acetogen somewhere. Frontiers in microbiology 3, 22.

Ozutsumi, Y., Tajima, K., Takenaka, A., Itabashi, H., 2005. The effect of protozoa on the composition of rumen bacteria in cattle using 16S rRNA gene clone libraries. Bioscience, biotechnology, and biochemistry 69, 499-506.

Patra, A.K., Kamra, D.N., Agarwal, N., 2006. Effect of spices on rumen fermentation, methanogenesis and protozoa counts in *in vitro* gas production test. In: Elsevier, pp. 176-179.

Patra, A.K., 2016. Recent advances in measurement and dietary mitigation of enteric methane emissions in ruminants. Frontiers in veterinary science 3.

Patra, A.K., Saxena, J., 2010. A new perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochemistry 71, 1198-1222.

Patra, A.K., Kamra, D.N., Agarwal, N., 2010. Effects of extracts of spices on rumen methanogenesis, enzyme activities and fermentation of feeds *in vitro*. J. Sci. Food Agric. 90, 511-520.

Patra, A.K., Yu, Z., 2013. Effective reduction of enteric methane production by a combination of nitrate and saponin without adverse effect on feed degradability, fermentation, or bacterial and archaeal communities of the rumen. Bioresource Technology 148, 352-360.

Patra, A.K., Yu, Z., 2014. Combinations of nitrate, saponin, and sulfate additively reduce methane production by rumen cultures *in vitro* while not adversely affecting feed digestion, fermentation or microbial communities. Bioresource Technology 155, 129-135.

Patra, A., 2012. Enteric methane mitigation technologies for ruminant livestock: a synthesis of current research and future directions. Environ Monit Assess 184, 1929-1952.

Petersen, S.r.O., Hellwing, A.L.F., Brask, M., Hojberg, O., Poulsen, M., Zhu, Z., Baral, K.R., Lund, P., 2015. Dietary nitrate for methane mitigation leads to nitrous oxide emissions from dairy cows. Journal of environmental quality 44, 1063-1070.

Phetteplace, H.W., Johnson, D.E., Seidl, A.F., 2001. Greenhouse gas emissions from simulated beef and dairy livestock systems in the United States. Nutrient cycling in agroecosystems 60, 99-102.

Phuc, H.T., Do Ho Quang, P.T., Leng, R.A., 2009. Nitrate as a fermentable nitrogen supplement for goats fed forage based diets low in true protein. Livestock Research in Rural Development 21, 81.

Pickering, N.K., Chagunda, M.G.G., Banos, G., Mrode, R., McEwan, J.C., Wall, E., 2015. Genetic parameters for predicted methane production and laser methane detector measurements. Journal of animal science 93, 11-20.

Pinares-Patiño, C.S., Clark, H., 2008. Reliability of the sulfur hexafluoride tracer technique for methane emission measurement from individual animals: an overview. Anim. Prod. Sci. 48, 223-229.

Pinares-Patiño, C.S., Ulyatt, M.J., Lassey, K.R., Barry, T.N., Holmes, C.W., 2003. Persistence of differences between sheep in methane emission under generous grazing conditions. The Journal of Agricultural Science 140, 227-233.

Pope, P.B., Smith, W., Denman, S.E., Tringe, S.G., Barry, K., Hugenholtz, P., McSweeney, C.S., McHardy, A.C., Morrison, M., 2011. Isolation of Succinivibrionaceae implicated in low methane emissions from Tammar wallabies. Science 333, 646-648.

Popova, M., McGovern, E., McCabe, M.S., Martin, C., Doreau, M., Arbre, M., Meale, S.J., Morgavi, D.P., Waters, S.a.M., 2017. The Structural and Functional Capacity of Ruminal and Cecal Microbiota in Growing Cattle Was Unaffected by Dietary Supplementation of Linseed Oil and Nitrate. Frontiers in microbiology 8, 937.

Poulsen, M., Schwab, C., Jensen, B.B., Engberg, R.M., Spang, A., Canibe, N., Hojberg, O., Milinovich, G., Fragner, L., Schleper, C., 2013. Methylotrophic methanogenic Thermoplasmata implicated in reduced methane emissions from bovine rumen. Nature Communications 4, 1428.

Qingxiang, M., Liping, R., Zhenming, Z., Miao, L., Caixia, S., 2011. Nitrate Reduction to Inhibit Ruminal Methanogenesis and to Improve Microbial Nitrogen Synthesis.

Ramin, M., Huhtanen, P., 2013a. Development of equations for predicting methane emissions from ruminants. Journal of Dairy Science 96, 2476-2493.

Ramin, M., Huhtanen, P., 2013b. Development of equations for predicting methane emissions from ruminants. Journal of Dairy Science 96, 2476-2493.

Ramos-Morales, E., Arco-Perez, A., Martin-Garcia, A.I., Yanez-Ruiz, D.R., Frutos, P., Hervas, G., 2014. Use of stomach tubing as an alternative to rumen cannulation to study ruminal fermentation and microbiota in sheep and goats. Animal Feed Science and Technology 198, 57-66.

Ramos-Morales, E., de la Fuente, G., Duval, S., Wehrli, C., Bouillon, M., Lahmann, M., Preskett, D., Braganca, R., Newbold, C.J., 2017. Antiprotozoal effect of saponins in the rumen can be enhanced by chemical modifications in their structure. Frontiers in microbiology 8.

Reynolds, C.K., Humphries, D.J., Kirton, P., Kindermann, M., Duval, S., Steinberg, W., 2014. Effects of 3-nitrooxypropanol on methane emission, digestion, and energy and nitrogen balance of lactating dairy cows. Journal of Dairy Science 97, 3777-3789.

Ricci, P., Rooke, J.A., Nevison, I., Waterhouse, A., 2013. Methane emissions from beef and dairy cattle: quantifying the effect of physiological stage and diet characteristics. Journal of animal science 91, 5379-5389.

Ripple, W.J., Smith, P., Haberl, H., Montzka, S.A., McAlpine, C., Boucher, D.H., 2014. Ruminants, climate change and climate policy. Nature Climate Change 4, 2-5.

Roberts, C., Ingham, S., 2008. Using ancient DNA analysis in palaeopathology: a critical analysis of published papers, with recommendations for future work. Int J Osteoarchaeol 18.

Roehe, R., Dewhurst, R.J., Duthie, C.A., Rooke, J.A., McKain, N., Ross, D.W., Hyslop, J.J., Waterhouse, A., Freeman, T.C., Watson, M., 2016. Bovine host genetic variation influences rumen microbial methane production with best selection criterion for low methane emitting and efficiently feed converting hosts based on metagenomic gene abundance. PLoS Genet 12, e1005846.

Rooke, J.A., Wallace, R.J., Duthie, C.A., McKain, N., de Souza, S.M., Hyslop, J.J., Ross, D.W., Waterhouse, T., Roehe, R., 2014. Hydrogen and methane emissions from beef cattle and their rumen microbial community vary with diet, time after feeding and genotype. British journal of nutrition, 1-10.

Rooke JA, Miller G, Flockhart J, McDowell M and MacLeod M 2016. Nutritional strategies to reduce enteric methane emissions. climateXchang (accessed June 2017) from http://www.climatexchange.org.uk/reducing-emissions/emissions-livestock-production/.

Ross, E.M., Moate, P.J., Marett, L., Cocks, B.G., Hayes, B.J., 2013. Investigating the effect of two methane-mitigating diets on the rumen microbiome using massively parallel sequencing. Journal of Dairy Science 96, 6030-6046.

Rymer, C., Huntington, J.A., Williams, B.A., Givens, D.I., 2005. *In vitro* cumulative gas production techniques: History, methodological considerations and challenges. Animal Feed Science and Technology 123ΓÇô124, Part 1, 9-30.

Sauvant, D., Giger-Reverdin, S., 2007. Empirical modelling by meta-analysis of digestive interactions and CH4 production in ruminants. PUBLICATION-EUROPEAN ASSOCIATION FOR ANIMAL PRODUCTION 124, 561.

Sayers, E.W., Barrett, T., Benson, D.A., Bolton, E., Bryant, S.H., Canese, K., Chetvernin, V., Church, D.M., DiCuccio, M., Federhen, S., 2011. Database resources of the national center for biotechnology information. Nucleic Acids Research 39, D38-D51.

Schiere, J.B., Ibrahim, M.N.M., Sewalt, V.J.H., Zemmelink, G., 1989. Response of growing cattle given rice straw to lickblocks containing urea and molasses. Animal Feed Science and Technology 26, 179-189.

Schloss, P.D., Westcott, S.L., 2011. Assessing and improving methods used in operational taxonomic unit-based approaches for 16S rRNA gene sequence analysis. Applied and Environmental Microbiology 77, 3219-3226.

Seedorf, H., Kittelmann, S., Henderson, G., Janssen, P. H. 2014. RIM-DB: a taxonomic framework for community structure analysis of methanogenic archaea from the rumen and other intestinal environments. PeerJ 2:e494.

Shabat, S.K.B., Sasson, G., Doron-Faigenboim, A., Durman, T., Yaacoby, S., Miller, M.E.B., White, B.A., Shterzer, N., Mizrahi, I., 2016. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. The ISME journal.

Shackley, S., Sohi, S., Haszeldine, S., Manning, D., Malek, O., 2009. Biochar, reducing and removing CO2 while improving soils: A significant and sustainable response to climate change. UK Biochar Research Centre, School of GeoSciences, University of Edinburgh.

Shain, D.H., Stock, R.A., Klopfenstein, T.J., Herold, D.W., 1998. Effect of degradable intake protein level on finishing cattle performance and ruminal metabolism. Journal of animal science 76, 242-248.

Shi, W., Moon, C., Leahy, S., Kang, D., Froula, J., Kittelmann, S., Fan, C., Deutsch, S., Gagic, D., Seedorf, H., 2014. Methane yield phenotypes linked to differential gene expression in the sheep rumen microbiome. Genome Research, gr-168245.

Silva, C.J.d., Leonel, F.d.P., Pereira, J.C., Costa, M.G., Moreira, L.M., Oliveira, T.S.d., Abreu, C.L.d., 2014. Sulfur sources in protein supplements for ruminants. Revista Brasileira de Zootecnia 43, 537-543.

Silva, J., Leite, D., Fernandes, M., Mena, C., Gibbs, P.A., Teixeira, P., 2011. Campylobacter spp. as a foodborne pathogen: a review. Frontiers in microbiology 2, 200.

Sirohi, S., Singh, N., Dagar, S., Puniya, A., 2012. Molecular tools for deciphering the microbial community structure and diversity in rumen ecosystem. Appl Microbiol Biotechnol 95, 1135-1154.

Slobodkina, G.B., Mardanov, A.V., Ravin, N.V., Frolova, A.A., Chernyh, N.A., Bonch-Osmolovskaya, E.A., Slobodkin, A.I., 2017. Respiratory Ammonification of Nitrate Coupled to Anaerobic Oxidation of Elemental Sulfur in Deep-Sea Autotrophic Thermophilic Bacteria. Frontiers in microbiology 8, 87.

Sohi, S.P., Krull, E., Lopez-Capel, E., Bol, R., 2010. Chapter 2 - A Review of Biochar and Its Use and Function in Soil. In: Donald, L.S. (Ed.), Advances in Agronomy, Academic Press, pp. 47-82.

Soissan, V., Li, L., Godwin, I.R., Barnett, M.C., Perdok, H.B., Hegarty, R.S., 2014. Use of nitrate and *Propionibacterium acidipropionici* to reduce methane emissions and increase wool growth of Merino sheep. Anim. Prod. Sci. 54, 1860-1866.

Sokolowski, J.H., Hatfield, E.E., Garrigus, U.S., 1969. Effects of inorganic sulfur on potassium nitrate utilization by lambs. Journal of animal science 28, 391-396.

Soliva, C.R., Amelchanka, S.L., Duval, S.p.M., Kreuzer, M., 2011. Ruminal methane inhibition potential of various pure compounds in comparison with garlic oil as

determined with a rumen simulation technique (Rusitec). British journal of nutrition 106, 114-122.

Soliva, C.R., Meile, L., Cielak, A., Kreuzer, M., Machmuller, A., 2004. Rumen simulation technique study on the interactions of dietary lauric and myristic acid supplementation in suppressing ruminal methanogenesis. British journal of nutrition 92, 689-700.

Sophal, C., Khang, D.N., Preston, T.R., Leng, R.A., 2013. Nitrate replacing urea as a fermentable N source decreases enteric methane production and increases the efficiency of feed utilization in Yellow cattle. Livestock Research for Rural Development 25.

Stams, A.J.M., Plugge, C.M., 2009. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nat Rev Micro 7, 568-577.

Steinfeld, H., Gerber, P., Wassenaar, T.D., Castel, V., de Haan, C., 2006. Livestock's long shadow: environmental issues and options. Food & Agriculture Org.

Stetter, K.O., Gaag, G., 1983. Reduction of molecular sulphur by methanogenic bacteria. Nature 305, 309-311.

Sun, X.Z., Waghorn, G.C., Hoskin, S.O., Harrison, S.J., Muetzel, S., Pacheco, D., 2012. Methane emissions from sheep fed fresh brassicas (Brassica spp.) compared to perennial ryegrass (Lolium perenne). Animal Feed Science and Technology 176, 107-116.

Takahashi, J., 1989. Control of ruminal nitrate reduction by sulphur compounds. Asian-Aust. J. Anim. Sci. 2, 530-532.

Takahashi, J., Ikeda, M., Matsuoka, S., Fujita, H., 1998. Prophylactic effect of L-cysteine to acute and subclinical nitrate toxicity in sheep. Animal Feed Science and Technology 74, 273-280.

Tang, J., Zhu, W., Kookana, R., Katayama, A., 2013. Characteristics of biochar and its application in remediation of contaminated soil. Journal of Bioscience and Bioengineering 116, 653-659.

Tapio, I., Shingfield, K.J., McKain, N., Bonin, A.l., Fischer, D., Bayat, A.R., Vilkki, J., Taberlet, P., Snelling, T.J., Wallace, R.J., 2016. Oral samples as non-invasive proxies for assessing the composition of the rumen microbial community. PloS one 11, e0151220.

Tapio, I., Snelling, T.J., Strozzi, F., Wallace, R.J., 2017. The ruminal microbiome associated with methane emissions from ruminant livestock. Journal of animal science and biotechnology 8, 7.

Terre, M., Castells, L., Fabregas, F., Bach, A., 2013. Short communication: Comparison of pH, volatile fatty acids, and microbiome of rumen samples from

preweaned calves obtained via cannula or stomach tube. Journal of Dairy Science 96, 5290-5294.

Thompson, L.H., Wise, M.B., Harvey, R.W., Barrick, E.R., 1972. Starea, urea and sulfur in beef cattle rations. Journal of animal science 35, 474-480.

Tillman, A.D., Sheriha, G.M., Sirny, R.J., 1965. Nitrate reduction studies with sheep. Journal of animal science 24, 1140-1146.

Tomkins, N.W., Colegate, S.M., Hunter, R.A., 2009. A bromochloromethane formulation reduces enteric methanogenesis in cattle fed grain-based diets. Anim. Prod. Sci. 49, 1053-1058.

Toral, P.G., Hervas, G., Bichi, E., Belenguer., Frutos, P., 2011. Tannins as feed additives to modulate ruminal biohydrogenation: Effects on animal performance, milk fatty acid composition and ruminal fermentation in dairy ewes fed a diet containing sunflower oil. Animal Feed Science and Technology 164, 199-206.

Troy, S.M., Duthie, C.A., Hyslop, J.J., Roehe, R., Ross, D.W., Wallace, R.J., Waterhouse, A., Rooke, J.A., 2015. Effectiveness of nitrate addition and increased oil content as methane mitigation strategies for beef cattle fed two contrasting basal diets. Journal of animal science 93, 1815-1823.

Ungerfeld, E.M., Kohn, R.A., 2006. The role of thermodynamics in the control of ruminal fermentation. Ruminant Physiology: Digestion, Metabolism and Impact of Nutrition on Gene Expression, Immunology and Stress, 55-85.

Ungerfeld, E.M., 2013. A theoretical comparison between two ruminal electron sinks. Frontiers in microbiology 4, 319.

Valadares Filho, S.d., Marcondes, M.I., Chizzotti, M.L., Paulino, P.V.R., 2010. Exigencias nutricionais de zebuinos puros e cruzados BR-CORTE. Visosa, MG, Brazil.

Van Kessel, J.A., Russell, J.B., 1996. The effect of pH on ruminal methanogenesis. FEMS Microbiology Ecology 20, 205-210.

Van Soest, P.v., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. Journal of Dairy Science 74, 3583-3597.

van Zijderveld, S.M., 2011. Dietary strategies to reduce methane emissions from ruminants.

van Zijderveld, S.M., Gerrits, W.J.J., Apajalahti, J.A., Newbold, J.R., Dijkstra, J., Leng, R.A., Perdok, H.B., 2010. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. Journal of Dairy Science 93, 5856-5866.

van Zijderveld, S.M., Gerrits, W.J.J., Dijkstra, J., Newbold, J.R., Hulshof, R.B.A., Perdok, H.B., 2011. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. Journal of Dairy Science 94, 4028-4038.

Velazco, J.I., Cottle, D.J., Hegarty, R.S., 2014. Methane emissions and feeding behaviour of feedlot cattle supplemented with nitrate or urea. Anim. Prod. Sci. 54, 1737-1740.

Veneman, J.B., Muetzel, S., Hart, K.J., Faulkner, C.L., Moorby, J.M., Perdok, H.B., Newbold, C.J., 2015. Does Dietary Mitigation of Enteric Methane Production Affect Rumen Function and Animal Productivity in Dairy Cows? PloS one 10, e0140282.

Vlaming, J.B., Brookes, I.M., Hoskin, S.O., Pinares-Patino, C.S., Clark, H., 2007. The possible influence of intra-ruminal sulphur hexafluoride release rates on calculated methane emissions from cattle. Canadian Journal of Animal Science 87, 269.

Waghorn, G., Tavendale, M.H., Woodfield, D.R., 2002. Methanogenisis from forages fed to sheep. In: pp. 167-172.

Wallace, R.J., Arthaud, L., Newbold, C.J., 1994. Influence of Yucca shidigera extract on ruminal ammonia concentrations and ruminal microorganisms. Applied and Environmental Microbiology 60, 1762-1767.

Wallace, R.J., Wood, T.A., Rowe, A., Price, J., Yanez, D.R., Williams, S.P., Newbold, C.J., 2006. Encapsulated fumaric acid as a means of decreasing ruminal methane emissions. In: Elsevier, pp. 148-151.

Wallace, R.J., Rooke, J.A., Duthie, C.A., Hyslop, J.J., Ross, D.W., McKain, N., de Souza, S.M., Snelling, T.J., Waterhouse, A., Roehe, R., 2014. Archaeal abundance in post-mortem ruminal digesta may help predict methane emissions from beef cattle. Scientific reports 4, 5892.

Wallace, R.J., Rooke, J.A., McKain, N., Duthie, C.A., Hyslop, J.J., Ross, D.W., Waterhouse, A., Watson, M., Roehe, R., 2015. The rumen microbial metagenome associated with high methane production in cattle. BMC Genomics 16, 839.

Wallace, R.J., Snelling, T.J., McCartney, C.A., Tapio, I., Strozzi, F., 2017. Application of meta-omics techniques to understand greenhouse gas emissions originating from ruminal metabolism. Genetics Selection Evolution 49, 9.

Welkie, D.G., Stevenson, D.M., Weimer, P.J., 2010. ARISA analysis of ruminal bacterial community dynamics in lactating dairy cows during the feeding cycle. Anaerobe 16, 94-100.

Wilkinson, J.M., 2011. Re-defining efficiency of feed use by livestock. animal 5, 1014-1022.

Williams, B.A., 2000. Cumulative gas-production techniques for forage evaluation. Forage evaluation in ruminant nutrition, 189-213.

Williams, Y.J., Popovski, S., Rea, S.M., Skillman, L.C., Toovey, A.F., Northwood, K.S., Wright, A.D., 2009. A vaccine against rumen methanogens can alter the composition of archaeal populations. Applied and Environmental Microbiology 75, 1860-1866.

Wina, E., Muetzel, S., Becker, K., 2005. The Impact of Saponins or Saponin-Containing Plant Materials on Ruminant Production A Review. Journal of Agricultural and Food Chemistry 53, 8093-8105.

Winning, N., Cloy, J.M., Rees, R.M., Sohi, S., 2014. Evaluation of the effect of biochar on greenhouse gas emissions from slurry storage and slurry amended arable soil.

Wolin, M.J., Miller, T.L., Stewart, C.S., 1997. Microbe-microbe interactions. In: The rumen microbial ecosystem, Springer, pp. 467-491.

Wright, A.D.G., Kennedy, P., ONeill, C.J., Toovey, A.F., Popovski, S., Rea, S.M., Pimm, C.L., Klein, L., 2004. Reducing methane emissions in sheep by immunization against rumen methanogens. Vaccine 22, 3976-3985.

Wright, M.J., Davison, K.L., 1964. Nitrate accumulation in crops and nitrate poisoning in animals. Advances in agronomy 16, 197-247.

XB Chen, E.Ø., 2004. Research on urinary excretion of purine derivatives in ruminants: past, present and future. In: Estimation of microbial protein supply in ruminants using urinary purine derivatives, Springer, pp. 180-210.

Yanez-Ruiz, D.R., Bannink, A., Dijkstra, J., Kebreab, E., Morgavi, D.P., OKiely, P., Reynolds, C.K., Schwarm, A., Shingfield, K.J., Yu, Z., 2016. Design, implementation and interpretation of *in vitro* batch culture experiments to assess enteric methane mitigation in ruminants–a review. Animal Feed Science and Technology 216, 1-18.

Yanez-Ruiz, D.R., Martin-Garcia, A.I., Hart, K.J., Molina-Alcaide, E., Newbold, C.J., 2009. Cellulolytic activity in the rumen of lambs fed a high concentrate diet is not affected by the removal of protozoa. Options Mediterraneennes 85, 251-254.

Yang, C., Rooke, J.A., Cabeza, I., Wallace, R.J., 2016. Nitrate and Inhibition of Ruminal Methanogenesis: Microbial Ecology, Obstacles, and Opportunities for Lowering Methane Emissions from Ruminant Livestock. Frontiers in microbiology 7.

Yang, W., Oba, M., McAllister, T., 2012. Precision processing maximizes the feed value of barley grain for feedlot cattle. Prairie Post Bull Breeders February (12-13).

Yoshii, T., Asanuma, N., Hino, T., 2003. Number of nitrate and nitrite reducing Selenomonas ruminantium in the rumen, and possible factors affecting its growth. Animal Science Journal 74, 483-491.

Young, E.G., Conway, C.F., 1942. On the estimation of allantoin by the Rimini-Schryver reaction. Journal of Biological Chemistry 142, 839-853.

Yu, Z., Morrison, M., 2004. Improved extraction of PCR-quality community DNA from digesta and fecal samples. Biotechniques 36, 808-813.

Zhao, L., Meng, Q., Ren, L., Liu, W., Zhang, X., Huo, Y., Zhou, Z., 2015. Effects of nitrate addition on rumen fermentation, bacterial biodiversity and abundance. Asian-Australasian Journal of Animal Sciences (AJAS) 28, 1433-1441.

Zhao, S., Zhao, J., Bu, D., Sun, P., Wang, J., Dong, Z., 2014. Metabolomics analysis reveals large effect of roughage types on rumen microbial metabolic profile in dairy cows. Letters in Applied Microbiology 59, 79-85.

Zhou, M., Chung, Y., Beauchemin, K.A., Holtshausen, L., Oba, M., McAllister, T.A., Guan, L.L., 2011. Relationship between rumen methanogens and methane production in dairy cows fed diets supplemented with a feed enzyme additive. Journal of applied microbiology 111, 1148-1158.

Zhou, M.I., Hernandez-Sanabria, E., 2009. Assessment of the microbial ecology of ruminal methanogens in cattle with different feed efficiencies. Applied and Environmental Microbiology 75, 6524-6533.

Zhou, Z., Yu, Z., Meng, Q., 2012. Effects of nitrate on methane production, fermentation, and microbial populations in *in vitro* ruminal cultures. Bioresource Technology 103, 173-179.

Zhuang, G.C., Elling, F.J., Nigro, L.M., Samarkin, V., Joye, S.B., Teske, A., Hinrichs, K.U., 2016. Multiple evidence for methylotrophic methanogenesis as the dominant methanogenic pathway in hypersaline sediments from the Orca Basin, Gulf of Mexico. Geochimica et Cosmochimica Acta 187, 1-20.

Zinn, R.A., Alvarez, E., Mendez, M., Montano, M., Ramirez, E., Shen, Y., 1997. Influence of dietary sulfur level on growth performance and digestive function in feedlot cattle. Journal of animal science 75, 1723-1728.

Appendices

Appendix 4.1 Reverse primer sequences. Excel file. Electronic format

Appendix 4.2 Sequencing and indexing primers details. Txt file. Electronic format

Appendix 4.3 Sequencing analysis report (Edinburgh Genomics). Pdf file. Electronic format

Appendix 4.4 List of differentially abundant OTUs between mixed and concentrate diets (Experiment 1). Excel file. Electronic format

Appendix 4.5. List of differentially abundant OTUs between mixed control and mixed nitrate and between concentrate control and concentrate mixed diets (Experiment 1). Excel file. Electronic format

Appendix 4.6 Spearman correlation between bacterial family and genus populations and CH_4 production across all samples and within concentrate and mixed basal diets (Experiment 1). Pag 254

Appendix 4.7 Spearman correlation between OTUs present at >0.1% of relative abundance and CH_4 production across all samples. Pag 255

Appendix 4.8. List of differentially abundant OTUs between mixed control and mixed nitrate diets (Experiment 2)

Appendix 4.9 Pearson correlation between all OTUs and CH_4 production in mixed diet samples (Experiment 2). Excel file. Electronic format

Appendix 4. 6. Spearman correlation between bacterial family and genus populations and CH_4 production across all samples and within concentrate and mixed basal diets (Experiment 1)

		All samples		Concentrate		Mixed	
Families	Genus	R	P-value	R	P-value	R	P-value
Prevotellaceae						-0.441	0.001
	Prevotella			0.521	0.022	-0.514	0.020
Veillonellaceae						-0.326	0.017
	Succiniclasticum						
Succinivibrionaceae		-0.445	0.000				
	Unknown	-0.540	0.000				
	Ruminobacter	0.232	0.049				
Ruminococcaceae		0.503	0.000				
	Ruminococcus	0.471	0.000				
	Unknown	0.440	0.000				
Lachnospiraceae		0.384	0.001				
Paraprevotellaceae		0.330	0.005	-0.513	0.021		
	YRC22	0.325	0.005				
	CF231	0.244	0.039				
Unknown (o Clostridiales)		0.530	0.000				
Clostridiaceae		0.517	0.000				
S24-7		0.233	0.049			0.338	0.013
RFP12		0.611	0.000			0.360	0.008
Mogibacteriaceae		0.604	0.000			0.319	0.020
Erysipelotrichaceae						-0.408	0.002

Appendix 4.7.Spearman correlation between OTUs present at >0.1% of relative abundance and CH_4 production across all samples

Phylum	Family	Genus	Taxonomy	R	P-value	Mean abundance
Archaea		*Methanobrevibacter*	OTU_9	0.41	0.000	2.65
			OTU_7	0.42	0.000	1.83
Bacteroidetes		*Prevotella*	OTU_1521	0.26	0.007	0.68
			OTU_11	0.23	0.016	1.78
			OTU_23	-0.21	0.029	0.94
			OTU_24	-0.24	0.011	0.84
			OTU_32	-0.35	0.000	0.51
			OTU_104	-0.35	0.000	0.18
			OTU_109	-0.35	0.000	0.16
Proteobacteria	*Succinivibrionaceae*		OTU_1	-0.54	0.000	8.62
Firmicutes	*Veillonellaceae*		OTU_36	-0.28	0.004	0.50
			OTU_34	0.33	0.001	0.67
		Succiniclasticum	OTU_6	0.32	0.001	1.93
			OTU_21	-0.29	0.002	0.39
		Dialister	OTU_75	-0.28	0.003	0.15
	Unknown (O Clostridiales)		OTU_28	0.42	0.000	1.47
	Clostridiaceae	*02d06*	OTU_41	0.33	0.001	1.38
	Ruminococcaceae	*Ruminococcus*	OTU_73	0.41	0.000	0.72
			OTU_2802	0.48	0.000	0.70
	Lachnospiraceae	*Butyrivibrio*	OTU_126	-0.23	0.016	0.16
			OTU_30	0.39	0.000	0.98