Methane production from ruminant livestock

Mitigation through dietary manipulation and application of the CO^2 method

Haque, Md Najmul

Publication date:
2014

Document version
Early version, also known as pre-print

Citation for published version (APA):
Haque, M. N. (2014). *Methane production from ruminant livestock: Mitigation through dietary manipulation and application of the CO^2 method*. SL Grafik.

FACULTY OF HEALTH AND MEDICAL SCIENCES
UNIVERSITY OF COPENHAGEN

Methane production from ruminant livestock

Mitigation through dietary manipulation and application of the CO_2 method

PhD Thesis - 2014

Md Najmul Haque

Methane production from ruminant livestock

Mitigation through dietary manipulation and application of the CO_2 method

PhD Thesis by
Md Najmul Haque
2014

Department of Large Animal Sciences
Faculty of Health and Medical Sciences
University of Copenhagen
Denmark

Methane production from ruminant livestock

Mitigation through dietary manipulation and application of the CO_2 method

PhD thesis by Md Najmul Haque
Submitted August 29, 2014

Supervisor:
Professor Jørgen Madsen, PhD, DSc
Department of Large Animal Sciences, Faculty of Health and Medical Sciences
University of Copenhagen, Denmark

Co- supervisor:
Professor Mette Olaf Nielsen, PhD
Department of Veterinary Clinical and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

Assessment Committee:
Peder Nørgaard, PhD (Chairman)
Associate professor
Department of Veterinary Clinical and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark

Harald Volden, PhD, DSc
Professor
Department of Animal and Aquacultural Sciences, Norwegian University of Life Sciences, Norway.

Martin Riis Weisbjerg, PhD
Senior Researcher
Department of Animal Science - Animal nutrition and environmental impact, University of Aarhus, Denmark

Preface

This PhD thesis presents the results of my three years of PhD education at the Faculty of Health and Medical Sciences (former, the Faculty of Life Sciences) at the University of Copenhagen, Denmark. This PhD project was funded by the former Faculty of Life Sciences as a part of the research programme "mitigation of climate change". The experiments were conducted on a private farm (Lynge), on a commercial and research farm (Assendrup), in a faculty research facility under the RAN (Research school of animal nutrition, Rørrendegard) in Denmark and in a private dairy farm in the Netherlands.

August 2014, Copenhagen Md Najmul Haque

Acknowledgements

This PhD is part of a deeper learning process about ruminant digestive physiology, fermentation, metabolism, nutrient utilisation and greenhouse gas emission from livestock. The entire process was intensively supervised by Professor **Jørgen Madsen**, a wonderful human being. He let me grow independently as a researcher but extended his helping hand whenever necessary. His scholastic guidance made this work much easier. It was also a privilege to work with Professor **Mette Olaf Nielsen**. Her constructive, keen and quick feedback helped me a lot during the writing process. I would like to express my honest and sincere gratitude to Jørgen Madsen and Mette Olaf Nielsen.

I would like to thank all of the members of the IPH research group. It was a pleasure working with you. Special thanks to Cecile for her unlimited support in learning R. Thanks to my fellow PhD friends for sharing knowledge, ideas, problems and fears, and helps to solve puzzles. I must admit, Jeanne Talchow Oakman deserves special thanks. I express my sincere appreciation for her endless support regarding all of the administrative issues during my PhD. I am thankful to all of my friends at home and abroad for being with me and supporting and inspiring throughout my PhD study. I thank to the farm personnel who actively supported me during the experiments in Denmark and the Netherlands.

Heartfelt gratitude and deepest appreciation to my beloved parents and younger brother for their blessing, never-ending support, prayer, inspiration and great sacrifice throughout my academic career, especially to my mum, who was dedicated and expected this PhD more than I did. Finally, I would like to thank to my lovely wife, who tolerated my absence during my PhD. I appreciate her patience and promise that life is not only science.

August 2014, Copenhagen Md Najmul Haque

List of scientific papers and manuscripts included in the thesis

Paper I: M. N. Haque, M. Roggenbuck, P. Khanal, M. O. Nielsen, J. Madsen. 2014. Development of methane emission from lambs fed milk replacer and cream for a prolonged period. (*Accepted in Animal Feed Science and Technology; article in press*).

Paper II: M. N. Haque, C. Cornou, J. Madsen. 2014. Estimation of methane emission using the CO_2 method from dairy cows fed concentrate with different carbohydrate compositions in automatic milking system. *Livestock Science* 164, 57-66.

Paper III: M. N. Haque, I. M. L. D. Storm, H. H. Hansen, J. Madsen. 2014. Method based comparative methane estimation from cattle fed three different diets. (*Manuscript ready to submit*).

Paper IV: M. N. Haque, C. Cornou, J. Madsen. 2014. Individual variation and repeatability of methane production from dairy cows measured in automatic milking system. (*Submitted to Animal: An International Journal of Animal Bioscience*).

List of other publications during the PhD period, not included in the thesis

Roggenbuck, M., Haque, M. N., Madsen, J., Sørensen, S. J. 2014. The microbiome assigned to the artificial inhibited rumen development. (*Manuscript ready to submit).*

M. N. Haque, J. Madsen. 2014. Factors affecting methane estimation from ruminants using the CO_2 method. (*Manuscript Under preparation).*

Haque, M. N., Storm, I. M. L. D., Hansen, H. H., Madsen, J. 2012. Methane production from dexter cattle fed three different diets and measured by CO_2 method. *Emissions of gas and dust from livestock (EmiLi)*. Hassouna, M. & Gulngand, N. (edt). p. 316-320.

Storm, I. M. L. D., Haque, M. N., Madsen, J., Hansen, H. H. 2012. Comparison of methane emissions from cattle assessed by three different methods: open-circuit respiration chambers, in vitro gas production and the CO_2-method. *Emissions of gas and dust from livestock (EmiLi)*. Hassouna, M. & Gulngand, N. (edt). p. 346-349.

M. N. Haque, P. Khanal, M. O. Nielsen, J. Madsen. 2014. Methane emission from artificially reared lambs and response to the fibrous diet. *In abstract book of Livestock, Climate change and food security conference*. 19-20 May, 2014. Madrid, Spain. p. 29.

M. N. Haque, C. Cornou, J. Madsen. 2014. Variation and repeatability of methane emission from dairy cows measured in two subsequent years. *In proceedings of 65th EAAP, Copenhagen*. p. 340

Abbreviations

AMS	automatic milking system
C	carbon
CH_4	methane
CH_4:CO_2	methane and carbon dioxide ratio
CO_2	carbon dioxide
CV	coefficient of variation
DM	dry matter
DMI	dry matter intake
eq	equivalent
ECM	energy corrected milk
F:C	forage and concentrate ratio
FADH	flavin adenine dinucleotide
FPCM	fat and protein corrected milk
FTIR	fourier transformed infrared radiation
GHG	greenhouse gas
H_2	hydrogen
N_2O	nitrous oxide
NADH	nicotinamide adenine dinucleotide
NADPH	nicotinamide adenine dinucleotide phosphate
NDF	neutral detergent fibre
NFC	non-fibre carbohydrate
PSM	plant secondary metabolites
SF_6	sulphur hexafluoride
TMR	total mixed ration
VFA	volatile fatty acid
WG	weight gain

CONTENTS

SUMMARY

Methane emission from the enteric fermentation of ruminant livestock is a main source of greenhouse gas (GHG) emission, which is a major concern for global warming. Dietary modification is directly linked to changes in the rumen fermentation pattern and types of end products. Research has shown that some dietary modifications can reduce CH_4 production. To develop dietary strategies and eventually select animals for lower CH_4 production, it is necessary to measure the CH_4 production individually or in a group of many animals with reasonable accuracy and precision. A number of methods for methane estimation are in practice. However, very few methods measure the CH_4 on individual animals in a herd by maintaining them in their natural state. The overall objectives of this PhD thesis were i) to evaluate the nutritional strategy of methane reduction through dietary modification and ii) to validate the CO_2 method for methane estimation from ruminants.

Paper I investigated the development of methane emission from the young and growing lambs that were artificially reared with milk replacer and cream compared to conventionally hay-fed lambs. Feeding milk replacer and cream for a prolonged period nearly prevented CH_4 production in the lambs. The dry matter intake (DMI) was significantly lower in the cream-fed lambs compared to that of the hay fed group. However, the digestible energy intake was similar in both of the groups. A dramatic change in methane production (as reflected by the CH_4:CO_2 ratio) was observed within 4 days in the cream-fed lambs when the cream diet was changed to a conventional hay diet. The CH_4:CO_2 ratio remained lower, however, for 50 days after the diet alteration.

Paper II investigated the effects of the supplementation of starch and sugar through the concentrate that was fed by the automatic milking system (AMS) on daily CH_4 production in cows under commercial conditions. Two groups of cows (n=36) were fed either a starch-based (MELK) or a sugar-based (VEM) concentrate in the AMS. The results indicate that the there was no difference in the milk production or DMI between the groups. No significant difference was found in CH_4 production (g/d, g/kg DMI and g/kg ECM) between the two groups. The calculated CO_2 and CH_4 production and the CH_4:CO_2 ratios were very similar in the two groups. A linear positive correlation was observed between the CH_4 (g/d) and energy-corrected milk (ECM) throughout the entire experimental period.

Paper III focused on the CH_4 emissions in Dexter cattle with starch and sugar supplementation through the total mixed ratio (TMR). This paper also highlighted the precision of the methane estimates using the CO_2 method compared to that using a respiration chamber [with Dexter cattle (RC_1) and Holstein cows (RC_2)] and an in vitro gas production technique. The DMI was significantly ($P<0.05$) lower in the respiration chamber (RC_1) compared to that in the metabolic cage. The starch-based diet significantly ($P<0.05$) reduced methane production (L/d and L/kg DMI) compared to that of the sugar-based diet. The absolute methane (L/d) that was estimated by the CO_2 method was strongly correlated ($r=0.83$) with the amount that was calculated using the measured

CO_2 in the RC_1 and the CH_4:CO_2 ratio. The estimated CH_4 (L/d) using the CO_2 method was also positively correlated with the predicted amount by IPCC, ARC and NRC. The calculated total CO_2 production of the animals using the CO_2 method was strongly correlated with the measured CO_2 in RC_1.

Paper IV investigated the variation and repeatability of CH_4 production from dairy cows as measured in two different years. The average DMI and ECM of the individual cows were different between the two years. The herd average methane production was significantly ($P<0.05$) lower during the 1st year compared to that of the 2nd year. The CH_4 emission from the individual cows was fairly correlated ($r=0.54$) between the years. However, a strong correlation appeared ($r=0.70$) when individual CH_4 emissions were expressed at a standardised ECM production. The diurnal variation of CH_4 showed a significantly ($P<0.05$) lower emission at night (0:00 to 08:00 h). The range of between-cow variation (CV= 8.8-9.1 and 5.9-6.1) of CH_4 emission was lower than the within-cow variation (CV = 8.6-16.3 and 8.6-9.1) during the 1st and 2nd year, respectively. The repeatability of the CH_4 production (L/d) was 0.36 and 0.41 in the 1st and 2nd year.

Based on the four papers, it can be concluded that the artificial feeding of growing lambs nearly prevented CH_4 emission. The lambs responded very quickly when switching from the milk replacer and cream to a hay diet. The residual effect of artificial feeding remained for 50 days after the diet alteration. Starch is more efficient in CH_4 mitigation than is sugar. The supplementation of starch via concentrate in the AMS is not enough for CH_4 reduction. Feeding starch through TMR is an efficient method of CH_4 abatement. Both CH_4 and CO_2 as calculated according to the CO_2 method are strongly correlated with the value that was measured using the respiration chamber. The resulting animal variation in CH_4 production obtained using the CO_2 method is within the acceptable range of the respiration chamber. The repeatability of the CH_4 emission in this study is comparable with that of other methods. Therefore, the CO_2 method can estimate CH_4 emission from ruminants with reasonable accuracy and precision.

SAMMENDRAG

Metan emission fra drøvtyggende husdyrs fordøjelseskanal er en hovedkilde til drivhusgas (GHG) emission, som er en stor bekymring i forbindelse med global opvarmning. Foderændringer er direkte knyttet til ændringer i vommen gæringsmønster og typer af slutprodukter. Forskning har vist, at nogle foderændringer kan reducere CH_4 produktionen. For at kunne udvikle foderstrategier og eventuelt udvælge dyr med lavere CH_4 produktion, er det nødvendigt at kunne måle CH_4 produktionen individuelt eller på en gruppe af mange dyr med en rimelig nøjagtighed og præcision. Et antal metoder til metan estimering anvendes i praksis. Meget få metoder måler imidlertid CH_4 på de enkelte dyr i en besætning i deres naturlige tilstand. De overordnede mål med denne phd afhandling var, i) at vurdere mulighederne for, gennem foderændringer at reducere udledningen af metan og ii) at validere CO_2 metoden til måling af drøvtyggeres metanproduktion.

Artikel I undersøgte udviklingen af metan produktionen fra unge og voksende lam, der blev kunstigt opdrættet med mælkeerstatning og fløde i forhold til metan produktionen hos konventionelt høfodrede lam. Fodring med mælkeerstatning og fløde i en længere periode næsten forhindrede CH_4 produktion fra lammene. Tørstofoptagelsen (DMI) var signifikant lavere hos de flødefodrede lam sammenlignet med den hø fodrede gruppe. Optagelsen af fordøjeligt energi var dog ens i de to grupper. En dramatisk ændring i produktionen af metan (som afspejlet i CH4:CO2-forholdet) blev observeret inden for 4 dage i de flødefodrede lam når fløde rationen blev ændret til en konventionel høration. CH4:CO2 forholdet forblev dog lavere i mindst 50 dage efter foderændringen.

Artikel II undersøgte virkningerne på malkekøers daglig CH_4 produktion af indhold af stivelse eller sukker i kraftfoderet. Forsøget blev udført i en privat kvægbesætning og kraftfoderet blev udfodret under malkning i et automatisk malkesystem (AMS). To grupper af køer (n=36) fik enten et stivelsesbaseret (MELK) eller et sukker baserede (VEM) at kraftfoder. Resultaterne indikerer, at der ikke var nogen forskel i mælkeproduktionen eller DMI mellem grupperne. Ingen signifikant forskel blev fundet i CH_4 produktion (g/d, g/kg DMI eller g/kg energikorrigeret mælk) mellem de to grupper. Den beregnede CO_2 og CH_4 produktion og CH_4:CO_2-forholdet var meget ens i de to grupper. En lineær positiv korrelation observeredes mellem CH4 (g/d) og ydelsen af energikorrigeret mælk i hele forsøgsperioden.

Artikel III fokuserede på CH_4-emissionen hos voksende Dexter kvæg fodret med total mixed ration (TMR) med forskelligt stivelse og sukker indhold. Denne artikel belyste også præcisionen af metan målingerne ved hjælp af CO_2-metoden i forhold til måling i respirationskammer med Dexter kvæg (RC1) eller Holstein-køer (RC2) og en in vitro gasproduktion teknik. DMI var signifikant ($P < 0,05$) lavere i respirationskammeret (RC1) sammenlignet med optagelsen i fordøjelsesstalden. Den stivelsesbaserede ration reducerede produktion af metan (L/d og L/kg DMI) signifikant ($p < 0,05$) sammenlignet med den sukkerbaserede ration. Den absolutte metan produktion (L/d), som blev målt med CO_2-metoden var stærkt korreleret ($r = 0,83$) med den mængde der blev beregnet ved hjælp af den målte CO_2 i RC1 og CH_4:CO_2-forholdet. Metan produktionen (L/d) der blev målt ved hjælp af CO_2-metoden var også positivt korreleret med de af IPCC, ARC og NRC forudsagte værdi. Den

beregnede samlede CO_2-produktion af dyrene ved hjælp af CO_2-metoden var stærkt korreleret med den målte CO_2 i RC1.

Artikel IV undersøgte variationen og gentageligheden af CH_4 produktion hos individuelle malkekøer målt i to forskellige år. Den gennemsnitlige DMI og ECM af de enkelte køer var forskellig i de to år. Besætningens gennemsnitlige metan produktion var signifikant ($p<0,05$) lavere i første år sammenlignet med andet år. Metan produktionen fra de enkelte køer var rimelig korreleret ($r=0,54$) mellem de to år. En stærk korrelation viste sig imidlertid ($r=0,70$), når de enkelte køers CH_4-produktion blev korrigeret til en standardiseret ECM produktion. Døgnvariationen i CH_4 produktion viste en signifikant ($p<0,05$) lavere emission om natten (0:00 til 08:00 h). Størrelsen af mellem-ko variation (CV = 8,8 til 9,1 og fra 5,9 til 6,1) i CH4-emissionen var lavere end indenfor-ko variationen (CV = 8,6 til 16,3 og fra 8,6 til 9,1) i henholdsvis første og andet år. Gentageligheden af CH_4 produktionen (L/d) var henholdsvis 0,36 og 0,41 i første og andet år.

På grundlag af de fire artikler kan det konkluderes, at den kunstige fodring af voksende lam næsten forhindrede CH_4-emission. Lammene reagerede meget hurtigt, når der blev skiftet fra mælkeerstatning-fløde rationen til hø fodring. Den efterfølgende virkning af kunstig fodring forblev i 50 dage efter foderændringen. Stivelse er mere effektiv til CH_4 reduktion end sukker. Tilskud af stivelse via kraftfoder i AMS er ikke nok til at reducere CH_4 produktionen. Fodring af stivelse gennem TMR er en effektiv metode til CH_4 reduktion. Både CH_4 og CO_2 som beregnet i henhold til CO_2-metoden er stærkt korreleret med den værdi, der blev målt ved hjælp respirationskammer. Det målte variationsområde i CH_4 produktionen opnået ved anvendelse af CO_2-metoden er inden for det acceptable område af respirationskammerværdier. Gentageligheden af CH_4 emissionen i denne undersøgelse er sammenlignelig med andre metoder. Baseret på dette kan CO_2-metoden estimere CH_4 emissionen fra drøvtyggere med en rimelig nøjagtighed og præcision.

Chapter 1
Introduction

Overview

This chapter provides a brief overview of the environmental impacts of livestock production, ruminant's digestion and methane production, mitigation strategies of methane emissions and different methods for methane measurements. The chapter also presents the hypothesis and objectives of this thesis.

1. INTRODUCTION

1.1. Livestock production and environmental impact

Livestock contribute to global climate change by emitting GHG either directly (from enteric fermentation and manure management) or indirectly (from feed production and the processing and converting of forest into pasture). The major GHGs from the livestock sector are carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) throughout the production process (Figure 1.1). The CO_2 that is emitted from livestock is not considered a net contributor to climate change because the animals consume plants that use CO_2 during photosynthesis (Steinfeld et al., 2006). Consequently, CH_4 and N_2O are the most important GHGs from the animal production system and have very high global warming potentials (GWP) of 25 and 298 CO_2 equivalent (eq), respectively (Solomon et al., 2007). The first comprehensive analysis of the environmental impact of livestock production (Steinfeld et al., 2006) reported that approximately 18% of the global anthropogenic GHG is contributed by livestock production. The global anthropogenic GHG emissions from agriculture were 5.1 to 6.1 Gigatonnes CO_2-eq in 2005, of which livestock shared approximately 9% (IPCC, 2007). Within livestock, ruminant supply chains are the main contributors to the GHG, estimating approximately 80% of the total sector's emissions (Opio et al., 2013), while non-ruminants, e.g., pigs and poultry, contribute only approximately 9% and 8%, respectively, to the sector's emissions (Gerber et al., 2013). The emissions from beef and milk production, respectively, represent 35 and 30% of the livestock sector emissions. Buffalos and small ruminant supply chains have a much lower contribution, representing, respectively, 8.7 percent and 6.7 percent of sector emissions (Opio et al., 2013). Another report (Gerber et al., 2013) that reported GHG emissions along livestock supply chains estimated approximately 14.5% of all human-induced emissions. Enteric-fermentation- and feed-production-related activities in ruminant production are the primary sources of GHG emissions, representing approximately 39 and 45% of the GHG of the total sector's emissions. The largest source of GHG emissions from ruminant production, i.e., CH_4 derive from enteric fermentation, which accounts for approximately 47%, greater than 90% of the total CH_4 emissions (Opio et al., 2013). According to the US Environmental Protection Agency in 2009, CH_4 emissions from enteric fermentation represented approximately 20% of total CH_4 emissions from anthropogenic sources (EPA, 2011). The rate of emission in terms of carbon footprint at the product levels is 2.8, 3.4 and 6.5 kg CO_2-eq/kg FPCM for milk production from dairy cattle, buffalo and small ruminants, respectively. However, with regard to meat from ruminants, the carbon footprint for beef, buffalo meat and small ruminant meat is 46.2, 53.4 and 23.8 kg CO_2-eq/kg meat, respectively (Opio et al., 2013). According to the values that were projected by EPA (2006), the direct non-CO_2 emissions from livestock would be approximately 7.3 to 7.5% of the global GHG emissions between 2010 and 2020, respectively. Ruminant production faces difficult challenges and must reduce GHG emission while responding to the significant demand of livestock products (projected +70% by 2050 for a world-projected population of 9.6 billion) (Gerber et al., 2013).

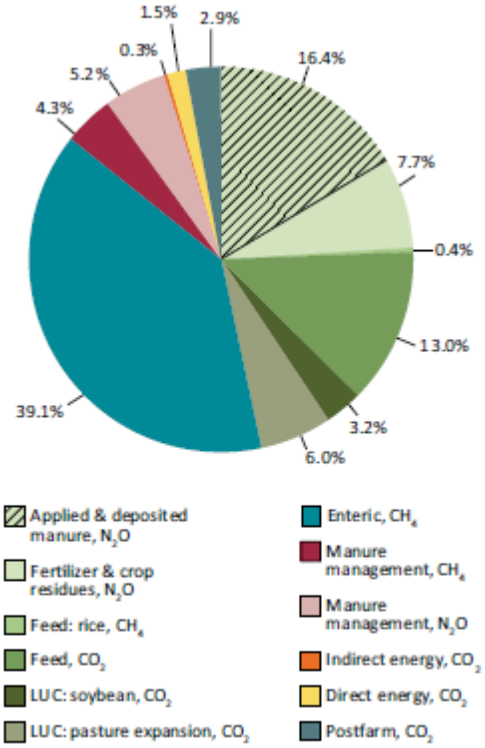


Figure 1.1. Global emission from livestock supply chains by category of emissions (Gerber et al., 2013)

The global food demand will also increase with the rapidly increasing global population. Consequently, the demand for animal products will also increase. Therefore, the environmental impact per unit of animal products will obviously be increased. Thus, the sector will be vulnerable in terms of environmental sustainability. Therefore, sustainable and immediate mitigation strategies are in high demand. This thesis will focus on CH_4 mitigation from ruminants through dietary manipulation.

1.2. Digestive system of ruminants and methane production

1.2.1. Anatomy and physiology of ruminant's digestive system

Ruminants are classified as herbivorous mammals (wild + domesticated) that possess a complex stomach. Unlike monogastric animals, ruminants have a more complex digestive system. The stomach of the ruminants is separated into four distinct chambers, i.e., reticulum, rumen, omasum and abomasum. The reticulum and the rumen are the first two chambers of the ruminant digestive system and are commonly referred as the reticulo-rumen, are well connected and continually exchange contents. The rumen is the largest compartment of the digestive system in ruminants and is where main fermentation takes place with the help of rumen microbes. The ingested materials are initially broken down by chewing, diluted with saliva during eating and rumination (saliva production is approximately 150 L/d in cattle and 10 L/d in sheep) and stored in the rumen. The rumen maintains an anaerobic condition with a temperature of approximately 37-40°C and a stable pH from 5.5 to 7.0 (McDonald et al., 2002) and harbours a full consortium of

rumen microorganisms (elaborated in section 1.2.4) (Bergman, 1990). Rhythmic contractions and relaxation of the rumen wall ensure the proper mixing of the rumen contents with the rumen fluid (McDonald et al., 2002). The density of the rumen mat increases gradually towards the ventral part of the rumen as the particle size reduces through rumination and microbial digestion. The rumen mat is stored in the rumen in three layers depending on the size of the fibre (the top layer consists of >1 cm with a low fluid-to-fibre ratio; the middle layer consists of less than 1 cm with an increased rumen fluid-to-fibre ratio; and the bottom layer consists of rumen fluid and fibre at only a few millimetres) (Welch, 1986). The reticulum stores the rumen contents with the largest particle size that are returned to the mouth for additional breakdown into smaller particles through rumination. In addition, at the end of microbial fermentation, VFA and NH_3 are absorbed via the rumen wall (Emery et al., 1960; Allen, 1997). Omasum contains many folds of leaf-like structures that absorb liquids to pass into the blood stream. Abomasum is the last compartment of the ruminant stomach and is termed the true stomach because it uses acids and enzymes to further break down the particles so that they can be absorbed into the portal system. The absorption of fatty acids and amino acids take place in the small intestine. Very negligible fermentation takes place in the large intestine, helps absorb the remaining liquids and forms the faeces.

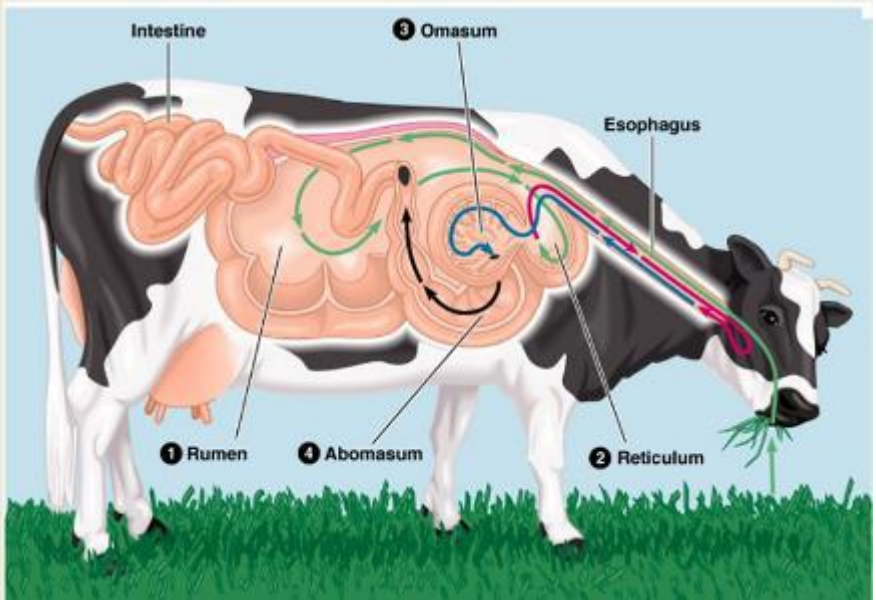
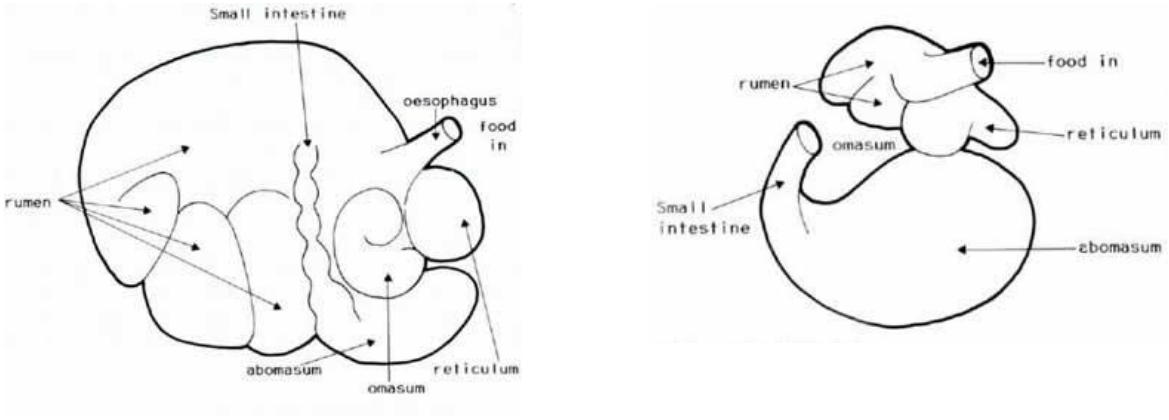


Figure 1.2. The ruminant digestive system. (downloaded from http://www.lelylife.com/2012/04/importance-of-fresh-and-frequent-feeding/, original publication unknown).

1.2.2. Rumen development and methane production

The digestive system in adult ruminants is a result of a series of pre- and postnatal developmental processes. In young ruminants (up to 3 weeks of age), the abomasum is the major functional compartment of the stomach, constituting up to 80% of the total stomach. In young animals during the milk-feeding period, milk flows directly to the abomasum via the oesophageal groove, thereby bypassing the rumen, reticulum and omasum. Only 5% of milk by chance enters the rumen during the milk-feeding period (Smith, 1959). The other compartments, especially the

rumen, grow very quickly once the young animals begin eating fibrous food. The metabolic and physical development of the rumen during the growing phase depends on solid feed consumption (Baldwin et al., 2004). An increased supply of milk to dairy calves produces higher daily weight gain and slower rumen development (Khan et al., 2007). Similarly, Smith (1959) reported that rumen development does not occur in milk-fed calves for an abnormally prolonged time (up to 32 weeks). The initiation of fibrous feed consumption and fermentation processes are needed to inoculate and establishment of the anaerobic rumen microbial ecosystem and development rumen function in young ruminants (Baldwin et al., 2004).



Adult ruminant stomach Young ruminant stomach

Figure 1.3. Anatomy of the ruminant stomach (external view) at young and mature stages. Retrieve from, http://www.daff.qld.gov.au/animal-industries/dairy/improving-your-herd/from-birth-to-weaning.

The digestion process is very different in young milk-fed ruminants from that of adult ruminants because of the diet. At the young stage, milk in liquid form and nearly no roughage leads to an enzymatic digestion of only the milk proteins, fats and simple sugars, producing very different end products than those that are produced in adult ruminants. No microbial fermentation occurs at the early ages, and a fully developed rumen system is required for microbial fermentation in ruminants (Wardrop, 1960). Due to the non-functional rumen and lack of microbial fermentation in newborn ruminants, CH_4 production and energy loss through CH_4 is absent (Eadie, 1962). The establishment of rumen microorganisms and fermentation in neonatal lambs begins at the age of 3-4 weeks (Wardrop and Coombe, 1960), and the type of diet is the primary factor that affects the relative growth of the digestive organs (Wardrop, 1960). Moreover, the metabolic and physical development of the rumen during the growing phase depends on solid feed consumption (Baldwin et al., 2004). An increased supply of milk to dairy calves produces a higher daily weight gain and slower rumen development (Khan et al., 2007). The initiation of fibrous feed consumption and fermentation processes are needed to inoculate and establish the anaerobic rumen microbial ecosystem and development rumen function in young ruminants (Baldwin et al., 2004).

1.2.3. Brief overview of digestion in the rumen

Structural carbohydrates, mainly plant fibre, represent the major feed resource for ruminants. The fibrous portion consists of β-linked polysaccharides, such as cellulose, hemicellulose and lignin. The breakdown of carbohydrates in the rumen can be divided into two stages (Figure 1.4). In the first step, complex carbohydrates are converted into simple sugars. Cellulose is decomposed by β -1-.3-glucosidase to cellobiose, which is later converted into either glucose or glucose-1-phosphate through phosphorylation. Starch and dextrins are first converted by amylase into maltose and isomaltose and subsequently by maltase into maltose phosphorylase or 1,6-glucosidase to glucose or glucose-1-phosphate. Fructans are hydrolysed by the enzyme attacking the 2,1 and 2,6 linkages to produce fructose, which also produces glucose via the digestion of sucrose. Pentose is the major product of hemicellulose breakdown, using the enzyme β-1,4 linkages to produce xylose and uronic acids. Uronic acids are also produced from pectins. This compound attacks by the enzyme polygalacturonidase to produce galactouronic acids, which later yields xylose. The simple sugars that are produced in the first step of carbohydrate digestion are rarely detectable because they are immediately taken and metabolised intracellularly by microbes.

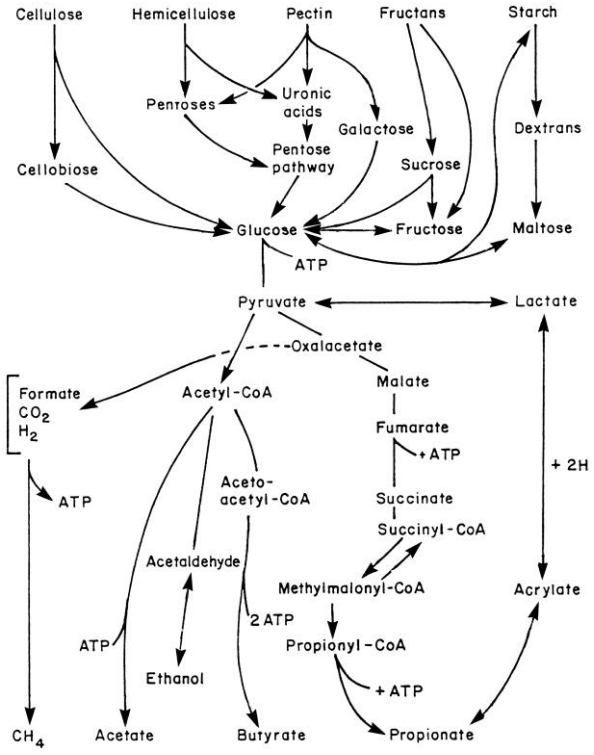


Figure 1.4. Schematic diagram of carbohydrate degradation and fermentation in ruminants (Stevens and Hume, 1998).

In the second step, the key intermediate is pyruvate, which is subsequently converted into volatile fatty acids (VFA), mainly acetate, propionate and butyrate (>90% of the VFA produced;

McDonald et al 2002), the major end products of carbohydrate digestion along with CO_2 and CH_4. The molar proportion of VFAs largely depends on the diet composition (Hassanat et al., 2013). However, in general, the molar proportions VFAs that are derived from hexose are 0.65 acetate, 0.21 propionate and 0.14 butyrate (McDonald et al., 2002). The majority of the VFAs are absorbed into the blood stream through the rumen wall.

Degraded feed and hydrolysed microbial biomass reaches the small intestine, where it is mixed with bile secreted from the liver (an alkaline medium) and enzymes (such as glycogen, α-amylase, trypsin and chymotrypsin) that are secreted from the pancreas. In addition, the liver also secretes the enzyme "lipase" to emulsify fat into triglycerol and monoglycerol. Microbial cell components are further digested and absorbed into the small intestine [approximately 200 g/kg feed ingested and digested (McDonald et al., 2002)], contributing a higher proportion of amino acids reaching the small intestine. The undigested materials pass into the large intestine, where a secondary microbial fermentation occurs, and water minerals and amino acids are absorbed before the materials are finally expelled as faeces through anus (McDonald et al., 2002).

The digestion of protein and fat in ruminants is described by McDonald et al. (2002). The degradable part of the proteins is hydrolysed into peptides and amino acids by rumen microorganisms. The amino acids are used by the microbes to produce microbial protein. The un-degradable part of the protein directly reaches the small intestine. Both the microbial proteins and un-degradable proteins undergo the enzymatic digestion to yield amino acids, which are absorbed by the small intestine. The most common NPN rapidly hydrolyses into ammonia in the rumen by bacterial urease. This ammonia helps form microbial proteins. The excess ammonia is directly absorbed by the rumen wall.

The capacity of rumen microorganisms to digest fat is strictly limited to when the dietary lipids content is greater than 100 g/kg of the DMI. Dietary fat in the form of triacylglycerol is hydrolysed in the rumen by bacterial lipase. Once released from the ester combination, unsaturated fatty acids undergo hydrogenation by the rumen bacteria, yielding monoenoic acid and consequently stearic acid. The short-chain fatty acids are absorbed directly through the rumen wall. The long-chain fatty acids reach the small intestine mostly in saturated and unesterified forms and are hydrolysed and absorbed into the small intestine.

1.2.4. Rumen microbial ecosystem

The reticulo-rumen of ruminants harbours microorganisms degrade fibrous plant materials. Rumen microorganisms ferment a portion of the ingested materials, while the other portions pass into the omasum and subsequently into the abomasum (France and Kebreab, 2008). The microbial ecosystem in the rumen consists of predominantly bacteria (10^9-10^{11} bacteria ml^{-1}), protozoa (10^4-10^6 protozoa ml^{-1}) and fungi (10^3-10^5 fungi ml^{-1}) that are classified as obligated anaerobes (Hungate, 1966; Russell and Hespell, 1981; France and Kebreab, 2008). The species diversity and activity of the microorganisms varies according to the change in the dietary composition, and diverse interactions have been noted (Russell and Hespell, 1981). The rumen bacteria are the

predominant microbes that constitute most of the microbial biomass (Dijkstra et al., 2005), the majority of which are gram-negative and obligate anaerobes. These microbes are divided in to three functional fractions based on their location in the rumen: the fluid-associated group, the particle-associated group and the rumen epithelium-colonising group. The rumen bacteria mainly degrade cellulose, hemicellulose and other resistant polysaccharides to volatile fatty acids (McDonald et al., 2002). These bacteria are also involved in nitrogen and sulphur cycling in the rumen (Kamra, 2005).

The archaeal community in the rumen is strictly composed of anaerobic CH_4 producers belonging to the kingdom Euryarchaeota (Janssen and Kirs, 2008). This kingdom is phylogenetically diverse and physiologically restricted, being categorised into five established orders: Methanobacteriales, Methanococcales, Methanomicrobiales, Methanosarcinales and Methanopyrales, which are further distributed into 10 families and 31 genera (Liu and Whitman, 2008). The diversity of methanogens is considerably less than that of bacteria. Methanogens grow in a gas mixture of H_2/CO_2 (Ma et al., 2005) and have a greater nitrate reductase activity (Rapheal et al., 2003). Methanogens are freely suspended in the rumen fluid and attached to particulate matter, rumen epithelium and even rumen protozoa as endosymbionts within the protozoa. There are various strains of methanogens, but a few specific strains are more dominant in the bovine rumen. The majority of rumen archaea are within the genera *Methanobrevibacter* and *Methanomicrobium* (Janssen and Kirs, 2008). However, two-thirds of the rumen archaea belong to *Methanobrevibacter spp.* while the remaining one-third are roughly *Methanomicrobium* (Morgavi et al., 2010).

Protozoa are the second most common microbes, constituting approximately 40% of the rumen microbial biomass (Dijkstra et al., 2005), especially ciliated protozoa, which are dominated by the species of *Isotricha* and *Entodinium* (Williams, 1986). The specific common protozoa include *Entodiniumexiguum*, *Eudiplodiniummaggii*, and *Isotrichaintestinalis.* The composition of rumen protozoa is highly correlated with the diet of the ruminants (Eadie et al., 1970). The protozoan community contributes to the reduction of the feed particle size by shredding and feeding, and some of the protozoa even appear digest cellulose (Hungate, 1966). Protozoa might play an important role in CH_4 emission. A defaunation study in calves showed a positive correlation between CH_4 production and the presence of protozoa in the rumen (Schonhusen et al., 2003).

Fungi in the rumen microbial ecosystem constitute approximately 8% of the total microbial biomass (Dijkstra et al., 2005), mostly obligated anaerobes that ferment most of carbohydrates and soluble sugars (McDonald et al., 2002). Rumen fungi colonise in the fibrous plant materials of the rumen and have potential cellulolytic activity (Bauchop, 1981). Rumen fungi are significant degraders of fibre by attacking lignocellulosic tissues, which are resistant to rumen bacteria (Akin et al., 1983). Recent research has indicated the high diversity of genes that are involved in fibre degradation, among which the genome of *Neocallimastix partriciarum* is predominant (Wang and McAllister, 2002).

1.2.5. Methanogenesis and methane production in the rumen

Methanogenesis is a process of CH_4 production in the rumen where H_2 reduced the CO_2 with the help of methanogenic archaea. This is a dynamic process, in which methanogens strongly influence the metabolism of fermentative and acetogenic bacteria via interspecies hydrogen transfer (Stams and Plugge, 2009). The carbohydrate fraction of the feed constitutes structural plant fibre that has been degraded by a consortium of rumen microbes under anaerobic conditions with the production of volatile fatty acids (VFA), CO_2 and H_2 (summarised in Table 1.1). During fermentation, hydrogen (H_2) is released into the rumen via the re-oxidation of the reduced cofactors (NADH, NADPH and FADH). The produced H_2 and CO_2 are the major substrates that are used by methanogens, which is considered being the predominant pathway of CH_4 production in the rumen (Ellis et al., 2008). Methane production from H_2 and CO_2 reduces the partial pressure of H_2, thereby favouring continued fermentation (Ellis et al., 2008). Without the removal of H_2, the further re-oxidation of reduced cofactors (NADH, NADPH and FADH) would be inhibited by the accumulation of H_2, consequently inhibiting the production of VFA (Wolin, 1975).

Table 1.1. Volatile fatty acids production (VFA) and reductive process in the rumen adopted from (Kohn and Boston, 2000; Ungerfeld, 2013).

Substrate		Products	ΔG (KJ)[1]	Reactions
VFA production				
$C_6H_{12}O_6+2H_2O$	→	$2\ C_2H_4O_2 + 2\ CO_2 + 8H^+$		Acetate production
$C_6H_{12}O_6 + 4H^+$	→	$2\ C_3H_6O_3 + 2\ H_2O$		Propionate production
$C_6H_{12}O_6$	→	$C_4H_8O_4 + 2\ CO_2 + 4H^+$		Butyrate production
Reductive process				
$CO_2 + 4H_2$	→	$CH_4 + 2\ H_2O$	-67.4	Methane production
$2\ CO_2 + 4H_2$	→	$C_2H_4O_2 + 2\ H_2O$	-8.8	Reductive acetogenesis
$SO_4^{2-} + 4H_2 + H^+$	→	$HS^- + 4\ H_2O$	-84.4	Sulfate reduction
$NO_3^- + 4H_2 + 2H^+$	→	$NH_4 + 3\ H_2O$	-371	Nitrate reduction

[1]under following rumen conditions: H_2 = 162 pa; pH = 6.5; [H_2O] = 50 M; [$succinate^{2-}$] = 4 x10^{-6} M; [$malate^{2-}$] = [β-hydroxybutyryl-CoA] = [butyryl-CoA] = 10^{-6} M; [$acetate^-$] = 70 mM; [$propionate^-$] = 25 mM; [$butyrate^-$] = 15 mM; [$lactate^-$] = 1 mM; [NH_4^+] = 11 mM (20 mg/dL); [HS^-] = 0.14 mM. ΔG = free energy change indicates how energetically favourable it is i.e. the higher ΔG, the more energy utilization and negative ΔG indicates the energy release.

In addition, the functional group of methanogens also uses formate, acetate, methanol, methylamines (mono-, di- and trimethylamine) and alcohol (Ellis et al., 2008) as presented in Figure 1.5. Formate is used by many of hydrogenotrophic rumen methanogens as an alternative to H_2 (Carroll and Hungate, 1955), accounting for up to 18% of the total CH_4 production in the rumen (Hungate, 1970). Acetate is highly available in the rumen environment, but acetoclastic methanogenesis bears very limited importance in the rumen system (Liu and Whitman, 2008) because the acetate-utilising methanogen *Methanosarcinales* has a very low growth rate and is consequently flushed from the ruminants digestive system (Thauer et al., 2008). Furthermore, acetogens have a lower affinity to H_2 (Morgavi et al., 2010). Other substrates, including methylamine and methanol, have been investigated for CH_4 production in the rumen. The methyl group is rapid converted by the rumen microorganisms to trimethylamine via di- and

monomethylamine and is possibly used for CH_4 production (Neill et al., 1978). However, only *Methylotrophic* methanogens within the order *Methanosphaera spp.* use methanol for CH_4 production (Liu and Whitman, 2008).

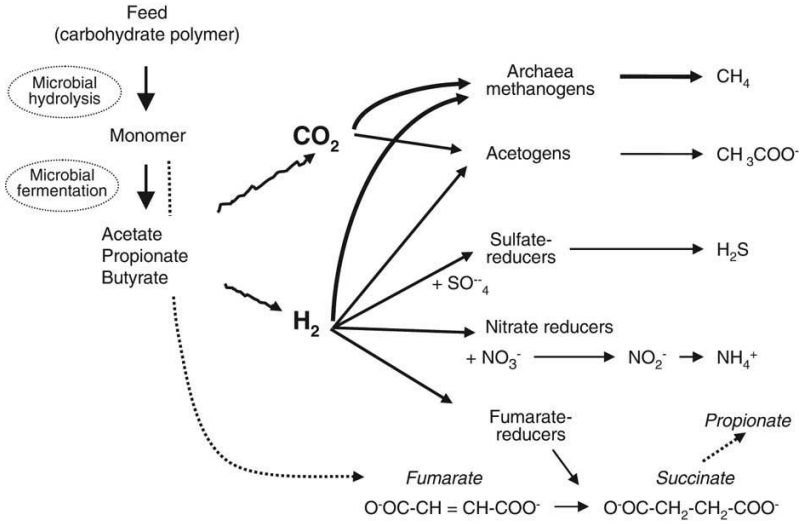


Figure 1.5. Schematic microbial fermentation and the H_2 reduction pathway in the rumen (Morgavi et al., 2010).

Because neither of these microbes are abundant in the rumen (Janssen and Kirs, 2008), the contribution of these substrates to total CH_4 production is expected to be lower (Morgavi et al., 2010). Consequently, the most favourable CH_4 production pathway in ruminants is the product of H_2 oxidation using CO_2 as an external electron acceptor (Ellis et al., 2008; Hook et al., 2010).

Methanogens can grow with H_2, CO_2 and encode most of the required enzymes and cofactors to convert the substrates into CH_4 (Smith and Hungate, 1958) (Figure 1.6). During methanogenesis, the fermentation byproduct CO_2 is reduced successively to CH_4 through the formyl, methylene and methyl levels. The first step is aided by the special coenzymes methanofuran (MFR), tetrahydromethanopterin (H_4MPT) and coenzyme M (HS-CoM). Initially, CO_2 binds with MFR and is reduced to the formyl level. Thereafter, ferredoxin (Fd) donates an electron and reduces H_2. The anion gradient is induced by the formation of formyl-MFR through endergonic conversion. The formyl group is then transferred to H_4MPT, resulting in formyl-H_4MPT, and is dehydrated to a methenyl group, which is subsequently reduced to methylene-H_4MPT and subsequently methyl-H_4MPT. In these two reduction steps, the reducing factor F_{420} ($F_{420}H_2$) acts as an electron donor. In the final step, methyl-CoM is reduced to CH_4 by the methyl coenzyme M reductase.

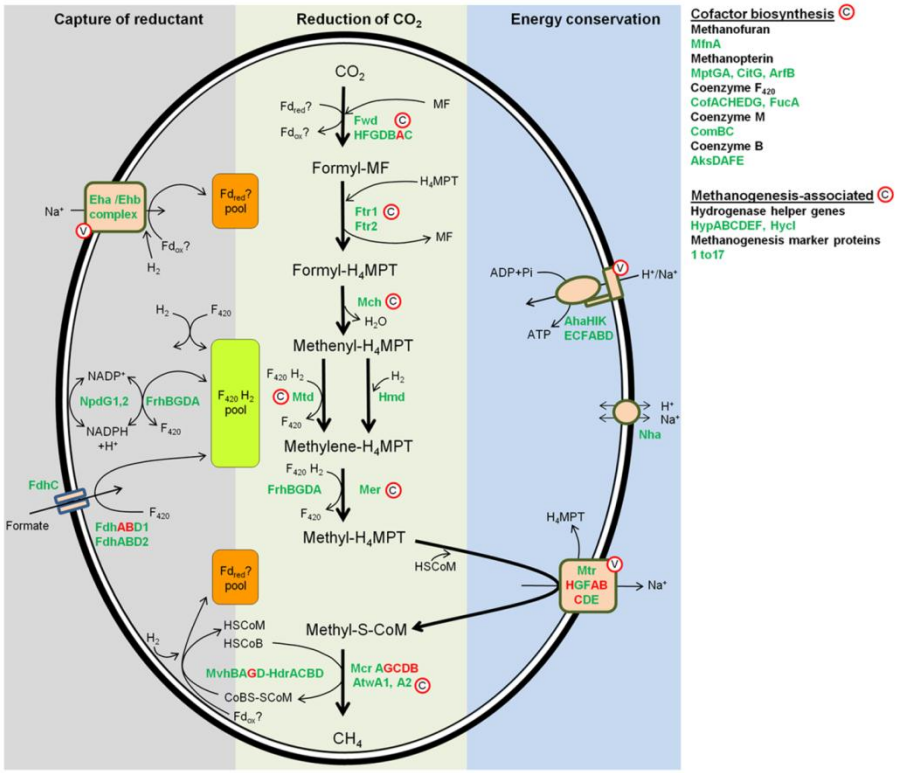


Figure 1.6. Reduction of CO_2 into CH_4; adopted from (Leahy et al., 2010).

Methyl-CoM is the key enzyme in methanogenesis. Coenzyme B (HS-CoB) is the electron donor in this reaction after the oxidation of heterodisulphide with HS-CoM (CoM-S-S-CoB). Heterodisulphide is then reduced to HS-CoB and HS-CoM. The transfer of the methyl group from H_4MPT to HS-CoM and the reduction of CoM-S-S-CoB are the steps during which energy conversion occurs (Liu and Whitman, 2008; Thauer et al., 2008).

1.3. Methane mitigation strategies

The atmospheric content of CH_4 has been increasing at a rate of 0.8-1.0% per year (Gurijala and Suflita, 1993). Methane is expected to contribute approximately 18% of the total expected global warming within the next 50 years (Milich, 1999), of which the contribution of livestock to the total global emission is approximately 9% (IPCC, 2007). Domestic animals account approximately 94% of the total global emissions of animals (Milich, 1999). Although emissions have decreased per unit of animal product, the total emission has increased from a vast animal population around the globe (Opio et al., 2013). By 2050, the total CH_4 emission from ruminant livestock is expected to increase significantly due to the growing demand of milk and meat for a rapidly growing world population (Gerber et al., 2013). Therefore, it is of utmost importance to mitigate CH_4 emission from the livestock industry. There are several strategies for CH_4 mitigation from ruminants that have recently been reviewed (Beauchemin et al., 2008; Eckard et al., 2010; Martin et al., 2010). A brief description of the mitigation strategies is presented in the following sections.

1.3.1. Dietary manipulation

Among the nutritional strategies of CH_4 mitigation, dietary manipulation is a simplistic and pragmatic approach that can ensure better animal productivity as well as a lower CH_4 emission. The schematic diagram of dietary manipulation, which alters the pathway of fermentation to reduce CH_4, is summarised in Figure 1.7.

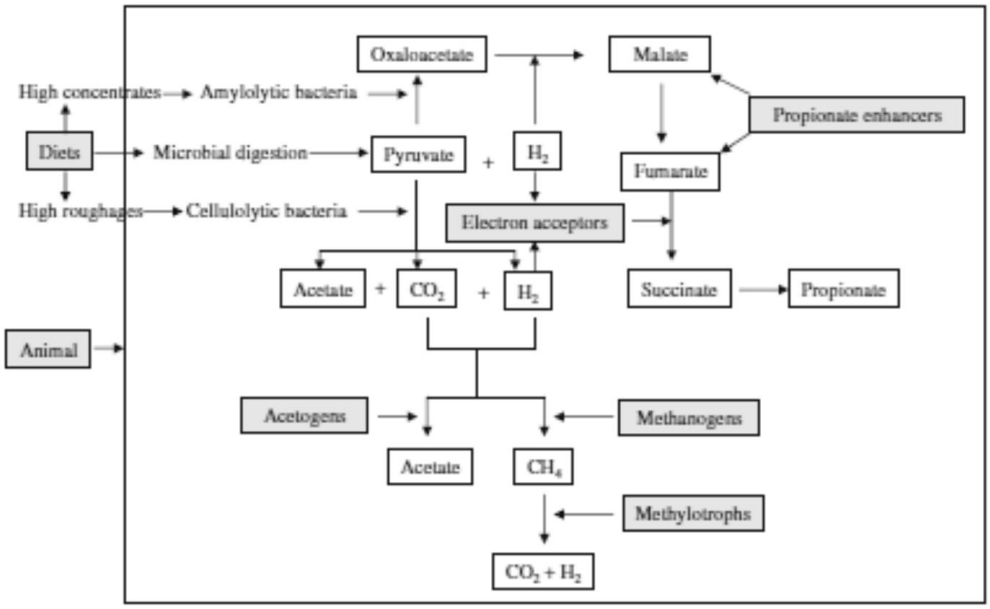


Figure 1.7. Target points (marked grey) at which dietary manipulation alters the fermentation pathway to reduce CH_4 in the rumen (Patra, 2012).

Dietary manipulation can reduce CH_4 emission up to 40% depending the degree of change and the nature of the intervention (Benchaar et al., 2001). Another study also indicated that CH_4 emissions can possibly be reduced up to 75% through better nutrition (Mosier et al., 1998). However, dietary manipulation is the most commonly practiced approach. Dietary strategies can be divided into two main categories: i) improving the forage quality and changing the proportion of the diet and ii) dietary supplementation of feed additives that either directly inhibit methanogens or altering the metabolic pathways leading to a reduction of the substrate for methanogenesis.

1.3.1.1. Forage

Forage quality has influences CH_4 production in the rumen (Boadi and Wittenberg, 2002). High-quality forage, e.g., young plants, can reduce CH_4 production by altering the fermentation pathway because this forage contains higher amounts of easily fermentable carbohydrates and less NDF, leading to a higher digestibility and passage rate (Beever et al., 1986). In contrast, more mature forage induces a higher CH_4 yield mainly due to an increased C:N ratio, which decreases the digestibility (Milich, 1999). Different types of forage can also affect CH_4 emission due to the differences in their chemical composition (Benchaar et al., 2001). However, Hammond et al. (2013)

found an inconsistent effect of the chemical composition of white clover and ryegrass on CH_4 production. Legume forage has a lower CH_4 yield, which is explained by the presence of condensed tannins, a low fibre content, a high dry matter intake and a fast passage rate (Beauchemin et al., 2008). Generally, C4 grasses yield more CH_4 than the C3 plants (Archimède et al., 2011). Forage processing and preservation also affect CH_4 emission (Martin et al., 2010). For instance chopping or pelleting forages can reduce the CH_4 emission per kg of DMI, as smaller particles require less degradation in the rumen (Benchaar et al., 2001; Boadi et al., 2004b). Methanogenesis tends to be lower in the ensiled forages (Boadi et al., 2004b), presumably because the ensiled forages are already partially fermented during the ensiling process. Feeding improves the forage quality by feeding young forage with a lower fibre content and a higher soluble carbohydrate content; supplementing a small amount of grain with forage is a promising mitigation approach.

1.3.1.2. Replacement of grass silage by maize silage

Grass silage is usually harvested at a later stage of maturity, resulting in a lower content of digestible organic matter, lower sugar and nitrogen contents and a fraction of lactate as a result of the ensiling process (Tamminga et al., 2007). Consequently, the CH_4 emission from animals that are fed grass silage is likely to be higher. In contrast, maize silage or other whole-crop small-grain silage typically provides higher contents of dry matter with readily digestible carbohydrates, e.g., starch, increasing the DMI and animal performance (Tamminga et al., 2007; Beauchemin et al., 2008) and ultimately resulting in a lower CH_4 yield from animals. There are three possible ways by which maize silage or whole-crop silage can reduce CH_4 production in the rumen. First, the higher starch content favours propionate production rather than acetate. Second, the increased total DMI and passage rate reduce the ruminal residence time, thereby reducing ruminal fermentation and promoting post-ruminal digestion. Third, replacing grass silage with maize silage improves animal performance, resulting in fewer CH_4 emissions per unit of animal product (O'Mara et al., 1998). Several recent studies have indicated the positive effects of replacing grass silage with maize silage. Hassanat et al. (2013) reported lower CH_4 emission when alfalfa silage is replaced by 100% corn silage. Maize silage that is harvested during the later stage of maturity has also claimed to reduce CH_4 (Tamminga et al., 2007).

1.3.1.3. Concentrates

High-producing dairy cows have a higher requirement that exceeds their capacity to ingest nutrients from forage only. Therefore, forages must be supplemented with concentrates with a higher density of nutrients and less fibre. Due to less cell walls and readily fermentable carbohydrates (starch and sugar), concentrates favour propionic acid production, decreasing CH_4 emission (Martin et al., 2010). The CH_4 reduction effect of concentrates can be described in two ways as below.

1.3.1.4. Proportion of concentrate

The increased dietary level of concentrate reduces CH_4 production as the energy proportion is mostly utilised by the animal products, such as milk and meat (Martin et al., 2010). This effect is independent of genetic merit (Ferris et al., 1999). Decreased CH_4 emission was observed at 80 and 90% concentrate supplementation, whereas no effect was found at 35 or 60% concentrate supplementation (Lovett et al., 2003). Most energy-rich concentrates are associated with increased DMI, rate of rumen fermentation and feed-turnover rate, causing a greater change in the rumen environment and microbial composition (Martin et al., 2010). An extremely low CH_4 loss of 2-3% of the gross energy intake was reported for feedlot cattle that were fed diet a 90% concentrate (Johnson and Johnson, 1995). However, high-concentrate diets are low in structural fibre and in the long term disturb rumen function by leading to subacute or acute acidosis; therefore, these diets are not sustainable for ruminant production. Feeding concentrate with a suitable F:C ration would obviously be effective in methane mitigation as well as animal productivity.

1.3.1.5. Concentrate composition

Concentrates that are composed of different ingredients have variable carbohydrate compositions, ranging from structural (cellulose and hemicellulose) to non-structural (starch and sugar) carbohydrates. The degradable rate of both of these types of carbohydrates also varies widely according to the volatile fatty acid profile and CH_4 loss. In beef cattle (Johnson and Johnson, 1995), the digestion of the cell wall leads to a higher acetate:propionate ratio and CH_4 loss compared to other carbohydrate fraction; within non-structural components, sugar is more methanogenic than starch. All of the carbohydrate fractions contribute to CH_4 loss, of which the least contribution is that from starch, probably due to the maintenance of a propionate-dominating VFA profile (Tamminga et al., 2007). Feeding more starch to ruminants reduces enteric CH_4 energy losses compared to feeding a forage diet (Johnson and Johnson, 1995; Benchaar et al., 2001; Beauchemin et al., 2009). Starch fermentation promotes propionate production in the rumen by creating an alternative H_2 sink (Murphy et al., 1982), a lower rumen pH, inhibiting the growth of methanogens (van Kessel and Russell, 1996), decreasing the rumen protozoan numbers and limiting the interspecies H_2 transfer between methanogens and protozoa (Finlay et al., 1994). In addition, feeding starch, which can escape rumen fermentation, could potentially supply energy to the host animals while avoiding methanogenesis in the rumen. Up to 30% of the starch from corn can escape rumen fermentation and be digested in the small intestine (Ørskov, 1986). However, the bypass starch has limited digestibility (up to 60%) in the small intestine (Harmon et al., 2004). Very limited results are available on the effects of bypass starch on methane mitigation. Further investigation is required for detailed information.

In contrast, sugar as a water-soluble carbohydrate is rapidly and completely degradable in the rumen, enhancing butyrate production at the expense of propionate, thereby making sugar concentrates more methanogenic than starch (Hindrichsen et al., 2005). Sugars enhance butyrate production at a higher H_2 partial pressure and higher rumen pH, as confirmed by Hindrichsen and

Kreuzer (2009), who reported a 40% higher CH_4 production with sucrose at a high pH compared to starch, while the opposite result was observed at a low pH with a significantly lower pH for sucrose.

1.3.1.6. Fat Supplementation

The addition of fat to the diet has traditionally been used to increase the dietary energy content to meet the energy demand of high-producing dairy cows. More recently, fat has been used for CH_4 mitigation. If the energy supplementation in a ruminant's diet is changed from carbohydrate to fat, then less fermentation and CH_4 production will occur. The CH_4-suppressing mechanism of fat is induced by reducing organic matter fermentation, fibre digestibility and consequently the methanogenic pathway and by the direct inhibition of methanogens in the rumen via the hydrogenation of unsaturated fatty acids (Johnson and Johnson, 1995). The greatest reduction comes from the unsaturated fatty acids, which act as an H_2 sink in the rumen through dehydrogenation (Boadi et al., 2004a; Hook et al., 2010), although other studies have reported that hydrogenation contributes only 1% of the H_2 in the rumen (Giger-Reverdin et al., 2003). Among fatty acids, the medium-chain C_8:C_{14} from coconut or palm oil is the most effective in CH_4 mitigation. Furthermore, fats are not metabolised in the rumen (Jenkins, 1993) and therefore do not contribute to methanogenesis (Johnson and Johnson, 1995). Martin et al. (2010) and Grainger and Beauchemin (2011) also reported that fat supplementation often reduces carbohydrate fermentation due to the toxic effects of fat on cellulolytic bacteria and protozoa, while starch fermentation remains unaffected. Consequently, fat depresses CH_4 emission (Doreau and Chilliard, 1997). However, fat supplementation to the ruminant diet is a persistent mitigation strategy (Grainger and Beauchemin, 2011).

1.3.1.7. Organic acids

The addition of organic acids, the intermediates of carbohydrate degradation, to the rumen has been suggested as potential feed additives for CH_4 mitigation. Organic acids probably stimulate propionic acid production in the rumen by acting as an H_2 sink, thereby reducing the amount of CH_4 (Castillo et al., 2004). Newbold et al. (2005) tested 15 propionate precursors in vitro and concluded that the structure appears to be more effective as an H_2 sink that can reduce CH_4 up to 17%. Fumarate and acrylate produce the most consistent reductions in CH_4 formation in batch cultures, while fumarate is more effective than acrylate in artificial rumens (McAllister and Newbold, 2008). Furthermore, fumarate (3.5 g/L) reduces the CH_4 output by 38% in continuous fermenters using forage as a substrate (Kolver Es, 2004). However, a meta-analysis (Ungerfeld et al., 2007) reported a lower CH_4 reduction effect in a continuous batch culture. Including multiple forms of propionate precursors in the diet yielded an additive inhibition of CH_4 emissions as the reductive pathways differ among organic acid sources (McAllister and Newbold, 2008). In contrast, an in vivo study with growing beef cattle reported a potential beneficial change in rumen fermentation by fumarate, although CH_4 reduction was unaffected (Beauchemin and McGinn, 2006). Organic acid supplementation has mostly been tested for CH_4 production in vitro, producing inconsistent results. Therefore, there is the potential to invest more research in farm animals.

1.3.1.8. Essential oils

Essential oils are plant secondary metabolites, volatile components (Tamminga et al., 2007) and aromatic lipophilic compounds (Greathead, 2003) with very strong antimicrobial properties (Burt, 2004), which inhibit the growth and survival of most of microorganisms in rumen (Benchaar et al., 2008). The mode of action varies in individual essential oils (Benchaar and Greathead, 2011). However, all essential oils contain chemical constituents and functional groups, such as terpenoids, phenolic and phenols, which have strong antimicrobial properties. Because of their lipophilic nature, essential oils have a high affinity for microbial cell membranes, and functional groups interact with the microbial cell membrane (Jouany and Morgavi, 2007). Methanogenesis decreases with the application of essential oil, especially by reducing microbial populations. However, no effect has been observed so far on the major aspects of rumen fermentation (Newbold et al., 2004). Limited studies have investigated the effect on CH_4 reduction in vivo. However, methanogenesis is inhibited by altering protein degradation and amino acid determination (Newbold et al., 2004). Further research needs to investigate the potential use of essential oils in mainstream livestock farming.

1.3.1.9. Ionophores

Antibiotics, such as monensin, are antimicrobial compounds that are typically used in beef and dairy cattle production to modulate feed intake and improve feed efficiency and animal productivity (McGuffey et al., 2001). Monensin increases the acetate:propionate ratio in rumen fermentation by increasing reducing equivalents that help to form propionate (Beauchemin et al., 2008). Monensin may also decrease ruminal protozoa. This antibiotic is typically added to the diet as premix or via a slow-releasing capsule and has an anti-methanogenic effect (Beauchemin et al., 2008). Ionophores do not alter the diversity of methanogens (Hook et al., 2009) but change the bacterial population from Gram-positive to Gram-negative with a consequent change in the fermentation from acetate to propionate, thereby reducing CH_4 (Patra, 2012). A high dose of monensin reduces CH_4 production (g/d) by 4-10% in dairy and beef cattle (McGinn et al., 2004; Odongo et al., 2007). Furthermore, Guan et al. (2006) reported a 30% CH_4 reduction in beef cattle that were fed monensin (33 mg/kg), which was related to the number of ciliated protozoa. The inhibitory effects of ionophores on CH_4 production may not persist over time, and microorganisms adapt to ionophores (Johnson and Johnson, 1995; Guan et al., 2006; Beauchemin et al., 2008). However, the possible transient effect of ionophores and increasing public pressure to reduce the use of antimicrobial feed additives in agricultural production will obviously limit the scope for a long-term solution to CH_4 mitigation (Beauchemin et al., 2008).

1.3.1.10. Probiotics

The use of probiotics for CH_4 mitigation has recently been described (Moss et al., 2000);(Boadi et al., 2004a). The specific CH_4 reduction potential of probiotics has not been well documented due to the unsuccessful introduction of acetogens to the rumen as competitors of methanogens (Lopez et al., 1999). Probiotics, such as lactic acid producers (*Lactobacillus*

plantarum, L. casei, L. acidophilus and Enterococcus faecium), acetate and propionate producers (*Selenomonas ruminantium and Megasphaera elsdenii*) and yeast (*Saccharomyces cerevisiae and Aspergillus oryzae*) are widely used for the health of both human and animals (McAllister et al., 2011). Probiotics based on *Saccharomyces cerevisiae* are increasingly used in ruminant diets to improve rumen fermentation, dry matter intake and milk yield (Beauchemin et al., 2008). The underlying mechanism is probably the alteration of H_2 production by the increased number of bacteria due to the partitioning of degraded carbohydrates between the microbial cells and fermented products (Newbold and Rode, 2006). Due to their modest price and wide use in ruminant production, the acceptance of CH_4-reducing probiotics has a high probability in CH_4 abatement. However, further research is needed to investigate the best possible products (Beauchemin et al., 2008).

1.3.1.11. Exogenous enzymes

Enzymes, such as cellulase and hemicellulase, are currently being used in ruminant diets. When properly formulated, enzymes can improve fibre digestibility and animal productivity (Beauchemin et al., 2003). Enzymes that improve fibre digestibility typically lower the acetate:propionate ratio in the rumen, ultimately reducing CH_4 production (Eun and Beauchemin, 2007). Subsequently, in a recent review, Beauchemin et al. (2008) suggested the possibility of developing a commercial enzyme additive to reduce CH_4. However, searching for potential enzymes for methane abatement warrants future research.

1.3.1.12. Alternative H_2 sink

Alternative H_2 sinks, for example, nitrate and sulphate, are used at lower concentrations in the basic diets of ruminants. As alternative electron acceptors, nitrate and sulphate have a greater reduction potential and are thermodynamically highly favourable for some rumen microbes (Kristjansson et al., 1982). Regarding methane mitigation, Leng (2008) described the potential of nitrate supplementation in the ruminant diet. Furthermore, van Zijderveld et al. (2010) demonstrated that the reduction effect of nitrate and sulphate is electronically more favourable than is CH_4 production, which can potentially change the competitiveness of H_2 scavengers. In recent years, nitrate and sulphate have been increasingly tested for CH_4 abatement. A 32% methane reduction was reported for nitrate, 16% for sulphate and 47% for a combination of nitrate and sulphate fed to lambs (van Zijderveld et al., 2010). The same author in a subsequent study indicated an approximately 16% CH_4 (g/d and g/kg DMI) reduction in dairy cows (van Zijderveld et al., 2011b). However, nitrate supplementation has not been established in many countries (e.g., in Denmark) due to toxic effects that could lead to animal death. One potential toxic effect occurs via the reduction of nitrate to nitrite, which causes methemoglobinemia, a condition in which blood haemoglobin cannot carry oxygen (van Zijderveld et al., 2010). Because a lower amount of nitrate in the diet is safe for the animal (Bruning-Fann and Kaneene, 1993), nitrate supplementation can be an effective CH_4 mitigation measure. However, more research is needed to determine the inclusion levels for different ruminant species.

1.3.1.13. Plant secondary metabolites

The potential effect of plant secondary metabolites (PSM) in CH_4 reduction has been recently recognised (Beauchemin et al., 2008). The CH_4-suppressing effect of PSM is mainly associated with antimicrobial properties that kill the bacteria (Bodas et al., 2012), protozoa (Hristov et al., 2003) and fungi (Patra and Saxena, 2009a) in the rumen. Plant secondary metabolites contain phenolic compounds the main active components that have antimicrobial activity (Dorman and Deans, 2000). Plants produce a variety of secondary compounds, among which condensed tannins (Ramirez-Restrepo and Barry, 2005) and saponins (Wallace, 2004) have received much attention.

1.3.1.14. Condensed tannins

An interesting development in CH_4 mitigation research is the development of forages with higher levels of tannins, such as clover and other legumes, including trefoil, vetch, sulla and chicory (Tamminga et al., 2007). The anti-methanogenic activity of tannins has recently been investigated in vitro and in vivo (Hess et al., 2003; Goel and Makkar, 2012). The CH_4-suppressing mechanism of tannins has not been described clearly; however, this mechanism may inhibit ruminal microorganisms (Bodas et al., 2012). Tannins may inhibit, through bactericidal or bacteriostatic activities, the growth or activity of rumen methanogens and protozoa (Liu et al., 2011; Tan et al., 2011). Methane production was reduced (up to 55%) when ruminants were fed tannin-rich forages, such as lucerne, sulla, red clover, chicory and lotus (Ramirez-Restrepo and Barry, 2005). Although tannins appear promising for CH_4 mitigation, these impede forage digestibility and animal productivity when fed at a higher concentration, limiting their future wide-scale use in CH_4 abatement (Beauchemin et al., 2008). However, more research may identify the balance between CH_4 reduction and possible anti-nutritional side effects as associated with tannin supplementation.

1.3.1.15. Saponins

Saponins are naturally occurring surface-active glycosides that are found in a wide variety of cultivated and wild plant species that reduce CH_4 production in the rumen (Tamminga et al., 2007; Patra and Saxena, 2009a). Saponins have a potent antiprotozoal activity by forming complex sterols in protozoan cell membranes (Goel and Makkar, 2012) and, to some extent, exhibit bacteriolytic activity in the rumen (Moss et al., 2000). Saponins are antiprotozoal at lower concentrations (Newbold et al., 1997), whereas higher concentrations can suppress methanogens (Bodas et al., 2012). Saponins inhibit ruminal bacterial and fungal species (Patra and Saxena, 2009a) and limit the H_2 availability for methanogenesis in the rumen, thereby reducing CH_4 production (Bodas et al., 2012). Methane reduction of up to 50% has been reported with the addition of saponins (Patra and Saxena, 2009b). However, a wider range of CH_4 reduction (14-96% depending on the plant and the solvent that was used for extraction) has been reported (Patra, 2012).

1.3.2. Rumen manipulation

Manipulating the microbial diversity in the rumen through chemical means (e.g., halogenated compounds and chloroform) by introducing competitive or predatory microbes or through direct immunisation can reduce methanogenesis in ruminants (Eckard et al., 2010). A preliminary study suggested that vaccination against methanogens can reduce CH_4 emission up to 8% (Wright et al., 2004). However, the long-term effect of vaccination on CH_4 reduction is still uncertain (Williams et al., 2009). Furthermore, methanogen populations in the rumen are influenced by diet and geographic location (Wright et al., 2007); therefore, it is challenging to develop a broad-spectrum vaccine against all methanogens. Instead, the development of a vaccine against the cell-surface proteins of methanogens may improve the efficacy of vaccination for CH_4 mitigation (McAllister and Newbold, 2008). Biological control bacteriophages or bacteriocins could be effective in the direct inhibition of methanogens and in redirecting H_2 to other reductive rumen microbes, such as propionate producers or acetogens (McAllister and Newbold, 2008). However, most of these options are still conceptual, and significant research is required.

Halogenated compounds, such as bromochloromethane and chloroform, are potent inhibitors of CH_4 production in ruminants. Methane reduction has been reported with bromochloromethane mainly due to the reduction of methanogen abundance (Goel et al., 2009). An approximately 26% CH_4 reduction was reported by McAllister and Newbold (2008) through the chemical inhibition of protozoa because the methanogens are often attached to the surface or endosymbionts within ciliated protozoa (McAllister and Newbold, 2008).

Defaunation also reduces CH_4 emission. Two major advantages of defaunation are that it increases nutrient utilisation by animals and limits H_2 transfer between protozoa and methanogens. The methanogens that are attached to ciliated protozoa contribute approximately 9-37% of the methanogenesis in the rumen (Finlay et al., 1994; Newbold et al., 1995). Protozoa-free lambs and sheep exhibit 26 and 20% CH_4 reduction, respectively (McAllister and Newbold, 2008; Morgavi et al., 2008). The elimination of the protozoan population in CH_4 mitigation is interesting, but the absence of protozoa in the rumen can hinder digestibility and animal performance.

Reductive acetogenesis, in which H_2 and CO_2 form acetate rather than CH_4 as a source of energy, has been suggested as an alternative to methanogenesis (Joblin, 1999). The production of acetate instead of CH_4 can increase the energy supply to the animals. Joblin (1999) suggested that if the CH_4 emissions in ruminant were fully replaced by acetate, this could represent an energetic gain of 4-15%. However, acetogenesis in CH_4 reduction has not been successful due to the failure in acetogens competing for H_2 in the rumen. Research in acetogenesis as a CH_4 mitigation measure is still in the initial phase and warrant more research.

1.3.3. Animal manipulation

Several options, such as culling low-producing animals, increasing animal productivity and breeding animals with lower CH_4, have been suggested for CH_4 mitigation through animal

manipulation. Methane emission is directly proportional to the number of animals in a herd. The replacement of non-productive and low-producing animals would cut the total CH_4 budget from the herd. Maintaining high-producing animals will increase the total production, but the CH_4 emission per unit of animal product will decrease (Patra, 2012; Weisbjerg et al., 2012). Therefore, proper nutrition management to improve productivity is an option to reduce the CH_4 emission per unit of animal product.

Several studies have demonstrated a substantial variation in CH_4 production in sheep and cows (Pinares-Patio et al., 2003; Clark et al., 2005; Madsen et al., 2010b), which may be linked to phenotypic traits and heritability. This animal variation in CH_4 production suggests a possibility of breeding animals with low CH_4 emission. However, Eckard et al. (2010) suggested that breeding for reduce CH_4 production is unlikely to be compatible with other breeding objectives.

1.4. Methane measurement methods

Methane emission from ruminants was probably attempted for the first time by Møllgaard and Andersen (1917). To date, several methods for CH_4 measurement and estimation have been developed, none of which is perfect. A brief description of these methods is presented in this section.

1.4.1. Respiration chamber

This method was the first to estimate CH_4 production in animals. There are two types of respiration chambers, closed-circuit and open-circuit, the latter of which is dominant. The principle of the chamber technique is to collect and measure the concentration of CH_4 emission from all sources of enteric fermentation. This technique has complete control of gas exchange and CH_4 recovery and has good accuracy and precision in measuring the daily CH_4 production from the animals. However, the results that are generated from the respiration chamber cannot be exploited to animals in loose housing systems and animals in pastures. The chamber technique is also limited with regards to the number of animals (Storm et al., 2012b). Building a respiration chamber is a very expensive and labour-intensive operation and requires animal training. Moreover, the limited movement ability of the animals causes stress, which may compromise animal behaviour and reduce the daily intake and high laborious input (Pinares-Patino and Clark, 2008).

1.4.2. SF_6 tracer technique

The SF_6 tracer technique was described in 1993-1994 (Johnson et al., 1994). The principle of this technique is that the CH_4 emission from the animals can be measured if the tracer gas from the rumen is known. This technique is widely used in Australia and New Zealand and can be applied to free-range and grazing animals. This technique can be used to study nearly all aspects of feeding and nutrition. However, this technique generates more variable results (Pinares-Patiño et al., 2011) and requires animal training, as the animals need to carry the equipment. Moreover, SF_6 is a greenhouse gas; therefore, mitigating GHG using another GHG is not logical.

1.4.3. The CO_2 method

The newly developed CO_2 method is described by Madsen et al. (2010a). This method uses CO_2 production as a marker to estimate CH_4 emission from ruminants and is similar to the SF_6 technique; the only difference is that instead of the externally added tracer gas SF_6 in the SF_6 technique, the CO_2 method uses naturally emitted CO_2 from the animals. The CH_4:CO_2 ratios from breath samples are used with the total CO_2 to quantify CH_4 production from the animals. This method can be applied under various circumstances, for example, total CH_4 emission from a whole stable with dairy cows (Bjerg et al., 2012) and measurement from individual animals (Lassen et al., 2012). This method is a simple, easy and cheap technique that can collect measurements from large number of animals in a short period (Madsen et al., 2010a). One aim of this thesis is to introduce this method, and a detailed elaboration is presented in sections [2.5,4.2, and 4.3].

1.4.4. GreenFeed technique

A newly described technique for CH_4 emission called GreenFeed (C-lock Inc., USA), was first described by Zimmerman et al. (2013). GreenFeed is an automatic feeding system that is fitted with CH_4 and CO_2 measurement sensors. Air is contentiously pumped through the feeder to quantify the flow of CH_4 and CO_2 during feeding. The system can be used in the automatic milking system under commercial dairy production and even under grazing conditions. However, GreenFeed only measures CH_4 when the animals have their head down in the feeder to eat (Waghorn et al., 2013). Therefore, a day-long emission pattern must be tested.

1.4.5. Ventilation hood technique

The ventilation hood technique uses an airtight box that encloses the head of the animals. This box is large enough to allow the animal to move its head freely and allows access to feed and water. Outside air is circulated around the animal's head, mouth and nose through the sleeve, expired air is collected (McLean and Tobin, 1987), and gas exchange is quantified. Ventilated head-hoods or head-boxes, which are less expensive, can also be used to quantify gaseous exchange using the same principle as the respiration chamber. However, the major disadvantage is that it this technique is not applicable to grazing animals, does not consider all emissions, compromises animal behaviour and lowers feed intake.

1.4.6. In vitro gas production technique for methane measurement

With the increasing interest in GHG emission from agriculture, traditional in vitro gas production techniques have been modified to estimate CH_4 production from animals (Pellikaan et al., 2011). The basic principle of these in vitro techniques is to ferment feed under controlled laboratory conditions using rumen liquid from fistulated animals. The in vitro technique provides the first approach to test potential feedstuffs and additives (Storm et al., 2012b). Although gas production using an in vitro technique shows a good correlation with in vivo results, this technique can only simulate feed fermentation and not CH_4 emission or diet digestibility by the entire animal

(Storm et al., 2012b). Furthermore, the in vitro technique does not include the long-term microbial adaption to the tested feed, which is a significant disadvantage when considering the total emission from an animal.

1.4.7. Micrometeorological techniques

Micrometeorological techniques have been defined as measuring fluxes of gas in the free atmosphere and relating these fluxes to animal emissions. This technique is based on the measurement of wind velocity and CH_4 concentration. A number of measurement points are used to estimate the emission rate. A number of different micrometeorological techniques exist, such as the mass balance technique, vertical flux technique, inverse dispersion analysis and boundary layer budgeting (Harper et al., 2011). Each of these techniques has different way of calculating the emission rate. The penitential advantage of this technique is that it does not require animal handling, thus measuring the emission rate in the animals' natural state. Moreover, measurements can be collected from a large number of animals. However, measuring CH_4 emissions in naturally ventilated housing systems is difficult because of the difficulties in measuring the air exchange rate.

1.4.8. Proxy methods

Proxy methods are being developed with the aim of examining a large number of animals at once without an experimental set-up or invasive intervention. These methods measure the emission rate with parameters that are easy to obtain from biological samples such as milk or faeces (Dehareng et al., 2012). The milk fatty acid profile is correlated with CH_4 emission (Chilliard et al., 2009); the link is that certain type of milk fatty acids are correlated with feed intake (Chilliard et al., 2009) or with the amount of methanogenic archaea in the rumen (Vlaeminck et al., 2006). This technique still requires validation.

1.4.9. Modelling approaches

Modelling approaches are used to estimate CH_4 on national and international bases. These models are used in CH_4 estimation from livestock, can be classified as empirical models that use statistical information of nutrient intake and are dynamic mechanistic models that predict CH_4 emission via a mathematical description of rumen fermentation biochemistry. IPCC (2006) reported guidelines for estimating enteric CH_4 emission. There are three different levels, all of which are based on the proportion of cow's gross energy intake that is excreted via CH_4. Several CH_4 models that are based on the respiration chamber were recently reviewed by Storm et al. (2012b). Ellis et al. (2009) describe a simulation model to predict CH_4 emission using feed intake and nutrient composition data from several chamber and ventilation hood experiments. An empirical model to estimate CH_4 production from US cattle was developed using the intake of carbohydrate fractions (Moe and Tyrrell, 1979). Another model known as the rumen model is considered a mechanistic model based on a series of dynamic, deterministic, and nonlinear differential equations (Dijkstra et al., 1992). Very recently, a dynamic mechanistic model has been described that attempts to simulate CH_4 emission based on a mathematical description of rumen fermentation biochemistry (Kebreab et

al., 2008). These modelling approaches predict CH_4 without an experimental set-up, and the results are often accurate. Although several statistical models have successfully predicted CH_4 production, many use some inputs that are not commonly measured. Moreover, data insufficiency is another issue that can limit the applicability of some models.

1.4.10. Other methods

Methane estimation based on the sampling of air that is released by eructation during milking has been described by Garnsworthy et al. (2012a). Similar to the CO_2 method, this method follows the methane measurement from the sampled air during milking in the AMS. This method only uses the peak emission during eructation. This method is a non-invasive technique and can potentially be applied to a large number of animals. However, further confirmation is needed regarding the precision and accuracy of the measurements and the reliability of the technique.

Hypothesis and objectives

Nutritional strategies through dietary manipulation appear to be the most sustainable approaches in CH_4 mitigation from ruminants. Several studies have investigated the effects of different feeds, supplements or additives on CH_4 emission from ruminants. Most of these studies used either the respiration chamber or SF_6 techniques to estimate CH_4 emission, which are limited by the number of animals that are used. Very few studies have investigated the dietary effects on CH_4 emission using techniques that are non-invasive, non-interfering with animal behaviour and, more importantly, consider a large number of animals. We hypothesised that in addition to choosing a sustainable mitigation strategy, such as dietary manipulation, it is equally important to use a technique that is simple and easy to apply, non-invasive, does not interfere with animal behaviour, is relatively precise, can consider larger number of animals at once and is applicable to different housing systems, even grazing conditions. The CO_2 method meets all of these criteria as previously mentioned. The overall aims of this PhD project were as follows:

- To investigate the nutritional strategies of CH_4 mitigation from ruminants.
- To validate the CO_2 method to quantify the CH_4 that is emitted from ruminants.

The specific objectives were

- To investigate the development of CH_4 emissions in growing lambs that were reared artificially.
- To study the effect of carbohydrate composition (mainly starch and sugar) on CH_4 emission from commercial dairy herd.
- To study the individual variation of cows in CH_4 production.
- To study the accuracy and precision of the CO_2 method.

Chapter 2
Project Description and Methodology

Overview

This chapter represents a short description of the PhD project and the experimental procedure of the studies included in the thesis.

2. PROJECT DESCRIPTION AND METHODOLOGY

This PhD project was designed to investigate CH_4 emissions from ruminant livestock. Among the ruminants, growing lambs, heifers and dairy cows were used in the different studies. In the first two studies, the effects of dietary manipulation on CH_4 mitigation from growing lambs and dairy cows were investigated. The third study examined methane estimation using two different techniques and mitigation through supplementing different carbohydrate sources. The fourth study highlighted the variation in the CH_4 emissions of individual cows, which were measured during two different years. The general methodology for each study is described below.

2.1. Study I

2.1.1. Animal housing and feeding

This study was part of a larger study with 70 growing lambs, 18 of which (average age: 90 days; body weight: 21±3.6 kg (mean ± standard deviation, SD) were used to investigate the development of CH_4 emission. The lambs were housed individually in a large barn, which was maintained with adequate ventilation at a temperature of 18-22°C. Initially, the lambs were placed in smaller pens (1.5 × 0.75 m) up to 2 months old, with sawdust as bedding material, and then the lambs were transferred to larger pens (1.5 × 1.5 m). From 3 days until 8 weeks of age, group 1, which was named the "hay" group, was fed milk replacer (180 g milk powder L^{-1}) from a suckling bucket and received grass hay from 2 weeks old. From 9 weeks old, the hay group was only fed the hay diet without any milk replacer (Figure 2.1). Daily allowances for the hay group were adjusted on a weekly basis to achieve moderate daily live weight gains of approx. 225 g/d.

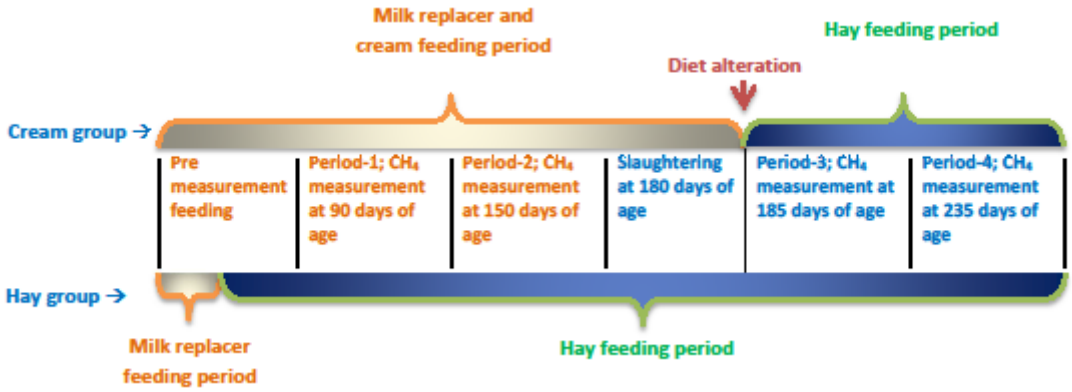


Figure 2.1. Experimental feeding periods

The lambs in the second group, which was named the "cream" group, received 50% milk replacer and 50% dairy cream *ad libitum* (until the lambs reached the daily predefined maximum of 2.5 (L/d) up to 180 days old). In addition, rolled maize was fed *ad libitum* (until the lambs reached

the predefined maximum allowance of 1 kg/d). The milk replacer-dairy cream mixture was fed from a suckling bucket. From 3 to 7 days old, both groups of lambs were fed four times a day and twice daily thereafter at approximately 07:00 h and 16:00 h, respectively. To prevent disorders of rumen function in cream-fed lambs, a small amount of barley straw (approx. 10 g/d) was provided to both groups. Water was available *ad libitum* at all times, and a vitamin-mineral mix was provided based on National Research Council (NRC) nutrient requirements (NRC, 2007). At 180 days old, 26 lambs, 4 of which were from the CH_4 measurement groups (two from each group), were slaughtered for the collection and analysis of rumen contents for rumen microbial diversity. The remaining lambs were managed together and fed a hay diet. The CH_4 measurements were conducted in four periods at approximately 90, 150, 185 and 235 days old, respectively. The 3rd and 4th periods of measurements were performed at 4 and 50 days after the cream-fed lambs were transferred to a normal hay diet.

2.2. Study II

2.2.1. Experimental design, animals, housing and feeding

In total, 36 Holstein-Friesian dairy cows with an average body weight of 660± 75.13 (mean±SD) kg and with average milk production of 31.7±8.98 (mean±SD) kg/d were selected for this study. The cows were primarily selected in 18 pairs according to their age, parity, and milk yield. Then, cows from each pair were randomly assigned in two groups named “MELK” and “VEM”. During the first period, both of the groups received 50% of each concentrate. From day 18, the MELK group received MELK concentrate, and the VEM group received VEM concentrate in the automatic milking system (AMS). The animals were housed in a closed housing system with adequate ventilation and fitted with one AMS. Both groups were allocated total mixed rations (TMRs) on an *ad libitum* basis. Cows were fed TMRs two times a day, whereas concentrates were automatically supplied during the milking in AMS. The amount of concentrates for each cow was fixed based on their daily milk production. Methane measurements were performed in three periods, which each lasted for 5 days, with a 14 day waiting time in between these periods.

2.3. Study III

2.3.1. Experimental design, animals, housing and feeding

This study was conducted with a 3×3 Latin square design, where three Dexter heifers with an average body weight of 226±11 kg (mean±SD) were sequentially allocated to balance cages and to the respiration chamber in 3 periods. The three periods were divided into two parts, with the first two weeks considered an adaptation period, followed by one week of measurements. Twice daily, the heifers were fed a total mixed ration (TMR) consisting (on DM basis) of 49% grass-clover silage, 14% soybean meal, and 35% of one of three supplements: wheat (W), molasses (M), and molasses + sodium bicarbonate (Mbic).

2.4. Study IV

2.4.1. Experimental design, animals, housing and feeding

In total, 21 dairy cows with average body weights of 621±14.7 and 640±8.0 kg (mean±SD) and milk production of 30.0±7.71 and 33.0±6.04 kg/d (mean±SD) were used during the 1st and 2nd year, respectively. The cows were housed in a closed housing system with adequate ventilation and fitted with an automatic milking system (AMS). The study was conducted at the farm without interfering with the planned feeding and management. During both years, the measurements were taken from the same cows in the same AMS. The cows were offered a total mixed ration (TMR) *ad libitum* during both years. In addition to the TMR, cows were offered concentrate in the AMS based on their milk production.

2.5. Gas measurement

Methane and CO_2 emissions from the cows were analysed using a Gasmet DX-4030 continuous gas analyser (Gasmet™, 2010), which is based on the Fourier transform infrared radiation. In the case of the 1st study: before gas measurement, the lamb pens were covered by Plexiglas to restrict air movement as much as possible. However, the pens were not completely airtight. The glass was transparent to avoid blocking views of the lambs from each other. For both periods 1 and 2, the measurements were performed every 15 seconds for 6 days. Each of the 18 lambs was measured individually for 8 hours, which were equally distributed during 3 periods over the 24 hour day. During periods 3 and 4, on each of the 18 lambs were measured for 30 minutes consecutively during the day, without considering potential diurnal variations. During each experimental day, background CH_4 and CO_2 concentrations were also measured.

For the 2nd study: The inlet filter of the Gasmet analyser was fitted on the feeding pen of the AMS to receive the concentrated breath samples from individual cows. The CH_4 measurements were obtained from the cows during milking while the cows were visiting the AMS. The breath sample passes through the filter and then through the Gasmet analyser to determine the CH_4 and CO_2 concentrations. The measurements were performed every 20 seconds over 24 hours for 15 days, which were divided into 3 periods (with 5 days each). Before the first measurement in the AMS, the Gasmet analyser was calibrated with known standard gases to verify the accuracy of the measurements. Each measurement day, the inlet filter of the Gasmet analyser was disconnected for 10 minutes to obtain the barn concentration of CH_4 and CO_2. Later, these stable concentrations were used as a correction factor for breath concentrations of CH_4 and CO_2.

During the 3rd study: Breath samples from the heifers were continuously analysed every 20 seconds in the metabolic cage to determine the CH_4 and CO_2 concentrations. The measurements were performed for 24 hours, and then the heifers were moved to the respiration chambers (RC_1) to measure the CH_4 and CO_2 emissions. However, the CH_4 measurement in the RC_1 was not recorded due to instrumental drift. Therefore, these data were excluded from the analysis.

The gas measurement protocol used for the 4th study was similar to that of the 2nd study. However, the only difference is that the measurements were obtained from 21 cows every 15 seconds over a 7 day period during two subsequent years.

2.6. Estimation of daily methane emission

In all of the studies, the calculations for CH_4 estimation were performed according to the CO_2 method (Madsen et al., 2010a). First, the barn concentrations of CH_4 and CO_2 were subtracted from the individual breath concentrations of CH_4 and CO_2. Then, the ratio of CH_4 to CO_2 was determined. In the next step the total heat production values for the animals were determined according to CIGR (2002). The CO_2 (L/d) excretion was calculated according to Pedersen et al. (2008), and subsequently, the amount of CH_4 (L/d or g/d) was calculated as described by Madsen et al. (2010a)

In the case of Paper III, the CH_4 emissions of the heifers were further calculated using the measured CO_2 in the respiration chamber (RC_1) and the CH_4:CO_2 ratio from the breath sample analysis using Gasmet equipment. The CH_4 emission (L/kg DMI) data from another respiration chamber (RC_2) study with Holstein cows (Hellwing et al., 2012) and from an in vitro gas production technique (IVGT) study with Dexter heifers (Storm et al., 2012a) were used to compare the precision of the CH_4 estimation methods. The RC_2 and IVGT study was conducted using the same diet as that used in study III.

Chapter 3
Summary of the Included Papers

Overview

This chapter presents the summary of the included papers labelled as I to IV.

3. SUMMARY OF THE INCLUDED PAPERS

3.1. Paper I.

Development of methane emissions from lambs fed milk replacer and cream for a prolonged period

M. N. Haque, M. Roggenbuck, P. Khanal, M. O. Nielsen, J. Madsen

The objective of this study was to investigate the development of CH_4 emissions from artificially reared growing lambs fed milk replacer and cream compared with lambs reared on a conventional grass-hay diet. This study was part of a larger study with 70 lambs, 18 of which with average body weights of 21±3.6 kg (mean±SD) were included. The lambs were housed in individual pens (1.5×1.5 m). From 3 to 180 days old, the lambs were fed either a restricted grass hay diet or a "cream" diet consisting of 50% milk replacer and 50% cream *ad libitum* until reaching a daily maximum allocation of 2.5 L/d. In addition, rolled maize was fed *ad libitum* (maximum allowance 1 kg/d) in the cream-fed group. The milk replacer-dairy cream mix was fed from a suckling bucket. From 3 to 7 days old, the lambs in both of the groups were fed four times a day and twice daily thereafter. At 180 days old, 26 lambs, 4 of which were from the CH_4 measurement groups (two from each group), were slaughtered to collect rumen samples for microbiological studies. After collecting rumen samples, the remaining lambs in the two groups were placed together and supplied a hay diet. Methane and CO_2 were measured in periods 1 to 4 (approx. 90, 150, 185 and 235 days old, respectively). For periods 1 and 2, the measurements were performed every 15 seconds for 6 days. Each of the 18 lambs was measured individually for 8 hours, which were equally distributed during 3 periods over the 24 hour day. During periods 3 and 4, the CH_4 measurements were obtained from each of the 18 lambs for 30 minutes consecutively during the day without considering the potential diurnal variation. Background concentrations of CH_4 and CO_2 were measured during each experimental day. The dry matter intake (DMI) g/d was significantly lower ($P<0.001$) in the "cream"-fed group. The weight gain (WG) was higher in the "cream"-fed group ($P=0.02$) during the first two periods. Methane production (g/d) was 84 and 87% lower in the cream group compared with the hay group during periods 1 and 2, respectively. The same group displayed lower emissions when the amount of CH_4 was expressed in terms of DMI and DEI ($P<0.001$). Within 4 days after changing the diet (period 3), the CH_4:CO_2 ratio of the ex-cream-fed lambs became 0.035, which was noticeably higher compared with the CH_4:CO_2 ratio during period 1 at 90 days old ($P<0.001$). Fifty days after the diet alteration (period 4), the ratio had increased further 0.039 ($P<0.001$) compared with that during period 1. A significantly lower CH_4:CO_2 ratio ($P<0.001$) was observed in the cream group compared with the hay group during periods 3 and 4, respectively. The abundance of rumen methanogens was lower in the fluid portion of the cream group, and the rumen archaea primarily adapted in the solid phase of the rumen content. In conclusion, artificial rearing of lambs with milk replacer and cream nearly prevented CH_4 release. Switching from milk replacer and cream to a fibrous hay diet dramatically changed the fermentation pattern and, consequently, the CH_4:CO_2 ratio in the cream group within 4 days. The CH_4:CO_2 ratio remained lower for 50 days after the diet alteration. Feeding milk replacer and cream to the lambs for up to 180 days reduced the CH_4 emissions for an extended period. However, further studies are required to determine whether this effect is long-lasting.

3.2. Paper II

Estimation of methane emission using the CO_2 method from dairy cows fed concentrate with different carbohydrate compositions in automatic milking system

M. N. Haque, C. Cornou, J. Madsen

The objectives of this study were i) to explore the effect of concentrate supplementation in an automatic milking system (AMS) on dairy cow CH_4 emissions and ii) to investigate the precision of the CO_2 method while measuring individual animals in an AMS. Thirty-six Holstein cows with mean body weights of 660±75.13 kg (mean±SD) and average milk yields of 31.7±8.98 kg (mean±SD) were used. The cows were divided into two groups called MELK and VEM and were fed two different concentrates with the same name, MELK (rich in starch) and VEM (rich in sugar). These concentrates were supplemented during milking in an automatic milking system (AMS). After a 5 day adaptation period (period 1), each group of cows received only MELK or VEM during periods 2 and 3. In addition, both of the groups were fed a total mixed ration (TMR) *ad libitum* in the barn. The CH_4 concentrations of breath samples were analysed every 20 seconds using the Gasmet equipment in the AMS through an inlet point placed near the feeding pen. The CH_4 measuring equipment constantly ran for 15 days over the 3 periods of measurement (5 days each, including the adaptation period) with a 14 day waiting time in between the periods. The records for the CH_4 and CO_2 concentrations from the breath samples were calculated after correcting for the background concentrations of CH_4 and CO_2. To obtain the quantitative CH_4 production, the ratio between CH_4 and CO_2 (CH_4:CO_2) was multiplied by the calculated CO_2 production of the individual animals. The milk yield and dry matter intake (DMI, kg/d) were similar between the two groups. The concentrate allocation was based on individual milk production. The concentrate intake in the AMS ranged from 1.60 to 7.30 kg/d in the MELK group and from 2.06 to 7.20 kg/d in the VEM group. The CH_4 production in MELK and VEM groups was not significantly different ($P>0.05$) over the three measurement periods. A linear positive relation was observed for the entire period between the CH_4 (g/d) production and the energy-corrected milk (ECM, kg/d) production and the DMI (kg/d). The precision analysis of the CO_2 method indicated that a 5 or 15 day measuring period was required to show a 9 or 5 percent difference in CH_4 production, respectively, under the present experimental conditions. In conclusion, the present study found no significant effect of a limited change in starch and sugar on CH_4 production through feeding concentrates in an AMS. For a change the carbohydrate composition of a diet to affect CH_4 production, a greater change in the diet is likely required. This change can be efficiently performed by changing the TMR portion of the diet.

3.3. Paper III

Method-based comparative methane estimation from cattle fed three different diets

M. N. Haque, I. M. L. D. Storm, H. H. Hansen, J. Madsen

The objective of this study was to estimate the effects of different sources of carbohydrate supplementation on CH_4 emissions and to compare the precision of the CO_2 method with a respiration chamber in vitro gas production technique. The present study used a 3×3 Latin square design, where three Dexter heifers were sequentially allocated to balance cages and subsequently to a respiration chamber during 3 periods, which consisted of a two week adaptation period, followed by one week of measurements. The average body weight (BW) of the heifers was 226±11 kg (mean±SD), and the average dry matter intake DMI was 5.1±0.3 kg/d (mean±SD). The heifers were fed *ad libitum* a total mixed ration (TMR) that consisted (on DM basis) of 49% grass-clover silage, 14% soybean meal and 35% of one of three supplements: wheat (W), molasses (M), and molasses + sodium bicarbonate (Mbic). Breath samples from the heifers were continuously analysed in a metabolic cage every 20 seconds to determine the CH_4 and CO_2 concentrations. The CH_4 and CO_2 emission calculations from heifers were performed using the CO_2 method. The dry matter intake (DMI, kg/d) was significantly higher ($P<0.001$) in the metabolic cage compared with the intake in the respiration chamber. The absolute CH_4 (L/d) production estimated using the CO_2 method was significantly different ($P<0.05$) between the three diets. The wheat-based diet "W" produced significantly less CH_4 compared with the molasses-based "M" and "Mbic" diets. The ranking of the diets based on the absolute CH_4 (L/d) production was W < M < Mbic. The CH_4 (L/kg DMI) emissions followed the same ranking ($P<0.05$). The absolute CH_4 (L/d) emission values between the CO_2 method and the respiration chamber was strongly correlated ($r = 0.83$). A strong correlation was also found between the estimated CH_4 determined using the CO_2 method and that of other recommended prediction models, such as IPCC, ARC and NRC. The daily CH_4 (L/kg DMI) emission value was lower using the CO_2 method compared with that determined using the respiration chamber technique with Holstein cows. A substantial animal variation of daily average CH_4 production was observed within the diet. The between-animal variation (CV_b) was 7.4-8.0 higher than the within-animal variation (CV_w) (17.3-17.4) of the CO_2 method. The measured CO_2 in the respiration chamber and calculated CO_2 according to CO_2 method was strongly ($r=0.85$) correlated. In conclusion, the DMI was lower in the respiration chamber. The wheat-based diet showed significantly lower CH_4 emissions (L/d and L/kg DMI) compared with the molasses-based diets. All three diets displayed a numerically lower CH_4 (L/kg DMI), as estimated by the CO_2 method, than those values in the study using a respiration chamber. The level of variations in CH_4 production estimated by the CO_2 method was within the acceptable range. The CO_2 method can predict CH_4 emissions with reasonable accuracy and precision compared with the chamber technique. This precision can be improved by using either more animals or a longer measurement period.

3.4. Paper IV

Individual variation and repeatability of methane production from dairy cows measured in automatic milking system

M. N. Haque, C. Cornou, J. Madsen

The objective of this study was to investigate the individual variation, repeatability and phenotypic correlation of methane (CH_4) production from dairy cows measured during two different years. In total, 21 cows were used, with average body weights of 621±14.0 and 640±8.0 kg (mean±SD) and milk yields of 29.1±6.54 and 33.4±6.00 kg/d (mean ± SD) during the 1st and 2nd years, respectively. The cows were housed in a loose housing system fitted with an automatic milking system (AMS). A total mixed ration (TMR) diet was fed to the cows *ad libitum* during both years. In addition, the cows were offered concentrate in the AMS based on their milk yield. The cow's CH_4 and CO_2 production were analysed using a continuous gas analyser, "Gasmet DX-4030". The dry matter intake (DMI) values were 19.8±0.96 and 23.1±0.78 kg/d (mean±SD), and the energy-corrected milk (ECM) production values were 30.8±8.03 and 33.7±5.25 kg/d (mean±SD) during the 1st and 2nd years, respectively. The DMI and ECM had a significant influence ($P<0.001$) on the CH_4 (L/d) yield during both years. The CH_4 emissions were significantly different between these two years ($P<0.05$). The CH_4 production of individual cows showed a fair correlation ($r=0.54$) between these two years. A strong positive phenotypic correlation ($r=0.70$) was found in the CH_4 emissions between these two years when standardized using ECM production (30 L/d). The diurnal variation of CH_4 (L/h) output displayed significantly lower ($P<0.05$) emissions during the night (0:00 to 08:00 h). The between-cow variation of the CH_4 measurements (L/d, L/kg DMI and L/kg ECM) were always lower (CV_{bc} = 8.8 – 9.1 and 5.9 – 6.1) compared with the within-cow variation (CV_{wc} = 8.6 – 16.3 and 8.6 – 9.1) during the 1st and 2nd years, respectively. The repeatability (R) values of the CH_4 yield (L/d) were 0.36 and 0.41 for the 1st and 2nd years, respectively. In conclusion, the daily CH_4 emissions were significantly higher the second year due to higher DM intake. The DMI appeared to be the key factor of the variation in CH_4 release, which was described by the ECM production. The between-cow variation in CH_4 (L/d) emission values was lower than the within-cow variation. The repeatability of the daily CH_4 output (L/d) was lower for the 1st year compared with that for the 2nd year. The CH_4 emissions at a standardized ECM production displayed a strong positive phenotypic relation between two years.

Chapter 4
General Discussion

Overview

The discussion is based on the results that were presented in four papers. This is focused on the effects of dietary manipulation on methane mitigation, application of the CO_2 method and its accuracy and precision.

4. GENERAL DISCUSSION

This section focuses on the results highlighted in the papers regarding CH_4 estimation using the CO_2 method and mitigation through dietary manipulation. The papers included in this thesis are labelled using the numbers I to IV.

4.1. Dietary manipulation and methane mitigation

4.1.1. Fat supplementation

Dietary manipulation is one of the most promising strategies in the mitigation of CH_4 emissions from ruminants (Martin et al., 2010; Patra, 2012, 2013). The addition of fats in animal diets has recently been highlighted as one of the mitigation approaches for enteric CH_4 emissions, although the reduction effects are not always consistent (Beauchemin et al., 2008). The inhibitory effect of fats on CH_4 production depends on the concentration, type, and fatty acid composition of fats and on the nutrient composition of diets (Beauchemin et al., 2008). Higher concentrations of fats result a substantially decrease in CH_4 production but often exert detrimental effects on feed digestibility, rumen fermentation, and animal performance. Fat supplementation often reduces the DMI (Grainger and Beauchemin, 2011), organic matter fermentation and fibre digestibility by either inhibiting methanogens or limiting the activity of the cellulolytic bacteria in the rumen (Johnson and Johnson, 1995), thus inducing CH_4 reduction. The results of the present study (Paper I) indicated a drastically reduced DMI in the lambs that were fed milk replacer and cream compared with the hay-fed lambs due to the higher energy concentration in the diet, as revealed by similar digestible energy intake values for both groups. Similar results were described by Haddad and Younis (2004) where DMI was reduced in lambs with 5% fat added to a high concentrate fattening diet. Supplementing with 25 to 75 g (per kg of concentrate) of coconut oil from 15 to 180 days reduced the daily intake in lambs (Bhatt et al., 2011). Moreover, Machmuller and Kreuzer (1999) found that the DMI was reduced by feeding adult sheep 70 g/kg of coconut oil. Fat concentrations to a maximum 6% of the diet DM may improve milk production and decrease enteric CH_4 emissions by 15% in cattle compared with 2% fats that are generally present in diets (Patra, 2013). In a recent review, Grainger and Beauchemin (2011) reported no adverse effect of fat in the form of salt (calcium formate) while reducing CH_4 in dairy and in beef without affecting production. A combination of coconut oil and fish oil at a low dose may additively reduce methanogenesis in the rumen without any adverse effect on rumen fermentation (Patra and Yu, 2013). Supplementation with refined coconut oil in a beef diet at a level of 250 g/d reduced CH_4 emissions by 18%, with normal feed intake and animal performance. In a recent study, Brask et al. (2013) reported that the physical form of fat had no influence on CH_4 reduction when fed rapeseed as a source of fat. Dietary fatty acid composition also affects the levels of CH_4 reduction. In general, poly-unsaturated fatty acids have a higher potential for CH_4 reduction than saturated fatty acids (Patra, 2013). In contrast, Beauchemin et al. (2007) found that saturated and unsaturated fatty acids had an equal effect when fed at a rate of 3% lipid to high forage diet.

In the present study (Paper I), a completely different fat source was used at an extremely high concentration in the diet of growing lambs raised artificially up to 6 months old. The lambs were fed a mixture of milk replacer and dairy cream at 50% each. This extreme diet makes this experiment extremely exceptional when the total fat intake of the lambs was > 200 g/d in the supplemented group. Feeding milk replacer and cream resulted in 84-87% CH_4 reduction, and consistent results were reported by Machmuller and Kreuzer (1999), who reported a 73% reduction by the addition of 3.5 to 7% coconut oil to the diet. In another study, the same author reported a 43-57% CH_4 reduction by supplementing with 3 and 6% coconut oil (Machmuller, 2006). Although a different fat source was used in this study, cream is also characterised by high levels of medium-chain fatty acids, similar to coconut oil products. In fact, coconut oil is one of the only other fat sources, except for milk fat from ruminants, that contains many medium-chain (C_{12}:C_{16}) fatty acids. Similarly, Patra (2013) reported that fatty acids with longer chain lengths (C_{12}-C_{14}) and particularly poly-unsaturated fatty acids (C18:3) had a greater effect on CH_4 reduction.

The degree of CH_4 reduction presented in Paper I was the most extreme, which was expected because of the amount of total fat in the diet and because this study was not a typical supplementation study. This study focused on the development of CH_4 emissions by feeding an extremely high fat diet. Therefore, this study is not directly comparable with those previously mentioned studies; however, this study may still open a new window regarding mitigation research. The persistency of the effects of an extreme diet was not confirmed by the results presented in Paper I. Further studies are required to confirm the persistency of fats on methane mitigation.

Despite the greater reduction in CH_4 emissions (Paper I), artificial rearing of ruminants is not feasible from both economic and ethical considerations. This type of feeding strategy is neither practical nor economically viable for global ruminant production. Therefore, adding fat to the basal diet at a reasonable concentration could be an effective mitigation measure (Beauchemin et al., 2008). Although identifying the source of the fats that can potentially be added to the diet to reduce CH_4 emissions is challenging (Grainger and Beauchemin, 2011), the use of by-products of feeds from agricultural/food processing industries, which contain fat, could be a useful approach to reducing enteric CH_4 emissions and global GHG emissions.

4.1.2. Starch and sugar

The inclusion of non-fibrous carbohydrates (NFC) in the range of 35 to 42% of the dietary DM has been chosen as an effective means to increase energy concentration and milk production in dairy cows (Lykos et al., 1997; Cherney et al., 2003). However, the global increase in milk production is causing a greater increase in CH_4 gas emissions, contributing to climate change (Opio et al., 2013). Therefore, several efforts have recently attempted to mitigate CH_4 emissions. Supplementing with readily soluble carbohydrates, such as starch and sugar, was tested in several studies regarding dairy cow CH_4 emissions (Aguerre et al., 2011; Benchaar et al., 2013; Hassanat et al., 2013). Clearly, supplementation with readily degradable carbohydrates changes the fermentation patter, volatile fatty acid proportion and, consequently, CH_4 production. However, the

CH_4 reduction effect varies depending on the type of carbohydrate. Fahey and Berger (1988) reported that starch is the most propionate producer in rumen fermentation than any other carbohydrates, which most likely lowers rumen pH and increases the proportion of propionate instead of acetate and butyrate (Hassanat et al., 2013), thus reducing CH_4 release from the rumen. Starch supplementation through whole crop silage reduced CH_4 emissions per unit of DMI relative to grass silage (Mc Geough et al., 2010). Hassanat et al. (2013) reported that CH_4 emissions were reduced by supplementing 30% starch through 100% corn silage. These authors reported that the observed CH_4 reduction was due to changes in the rumenal environment, digestibility, a proportional increase in propionate and lower NDF digestibility. Similarly, Lechartier and Peyraud (2011) reported that long-term feeding with starch reduced fibrolytic activity and changed the VFA proportion, which will most likely lead to a lower CH_4 emission.

Unlike starch, sugar, which is another important fraction of readily fermentable carbohydrates, has more methanogenic potential (Hindrichsen et al., 2005; Hindrichsen and Kreuzer, 2009). A recent simulation study reported a higher CH_4 emission rate with water-soluble carbohydrates by feeding high sugar grasses (Ellis et al., 2012); methane emissions were higher per unit of OM using sucrose instead of starch. The higher methanogenic potential of sugar is most likely related to higher butyrate production (Hindrichsen et al., 2004).

The results presented in Paper II were based on supplementing the diet with starch-rich (termed the MELK group) and sugar-rich (termed the VEM group) concentrates that were supplied in the AMS. The effectiveness of starch at reducing CH_4 production is well documented. These results (Paper II) showed a tendency of lower CH_4 (g/d) for those cows that received the highest amount of starch-rich concentrate. However, no difference in CH_4 production was found between the two concentrate-supplemented groups most likely due to the limited increase in the amount of starch content in the concentrate supplied in the AMS. As shown in Table 4.1, which was previously presented in Paper II, only slight changes in the starch content (+5.8%) of the MELK group occurred based on the total diet, which appeared to be insufficient to observe any effect on CH_4 emissions. Mc Geough et al. (2010) observed lower CH_4 (g/kg DMI) production in beef cows with increasing amounts of starch fed through whole-crop wheat silages. Likewise, Hassanat et al. (2013) reported that dairy cow CH_4 emissions were reduced by supplying 30% starch through corn silage as the only diet. These authors further observed no effect on dairy cow CH_4 emissions when feeding starch at the rate of 17 to 27% through corn silage. In the current study (Paper II), only the 5.8% increase in the starch content resulted in no effect on CH_4 (g/d) reduction, which is consistent with the findings by Hassanat et al. (2013). In contrast, Aguerre et al. (2011) found that dairy cow CH_4 emissions increased when the dietary starch content was decreased with increasing fibre concentrations in the diet.

The ingested amount of starch (Paper II) through the concentrate DM in the AMS was clearly insufficient for decreasing CH_4 production compared with the DM consumed through the TMR portion of the diet. Additionally, the concentrate intake was extremely different between the cows, depending on their milk production. Supplying diets containing high quantities of starch via grain or

cereal forages has been proposed as a means of CH_4 reduction (Beauchemin et al., 2008). The current study made a parallel effort to mitigate CH_4 release by increasing the starch supply in the diet. However, evidently, an inappropriate supplementation method was chosen to supply starch through concentrate in the AMS. Feeding starch through TMR could have been more effective for visualizing the CH_4 reduction effect. This recommendation was followed in the subsequent study (Paper III), where starch- and sugar-based diets were supplemented through the TMR. In this case (Paper III), a significant CH_4 reduction was observed for the starch-based diet compared with the sugar-based diet.

Table 4.1 Average non-fiber and fibrous carbohydrate intake of the cows in two groups (kg DM/d)

	MELK			VEM			Change in nutrient (%), VEM to MELK	
	Total	TMR	Concentrate	Total	TMR	Concentrate	Total	Concentrate
Sugar	2.8	2.4	0.4	3.1	2.4	0.7	-10	-43
Starch	6.4	4.7	1.7	5.1	4.7	0.4	+25	+325
NDF	19.8	18.6	1.2	20.5	18.6	1.9	-3	-37
Total DM	23.2			23.5				
% Starch in DM	27.5			21.7			+5.8	

TMR = total mixed ration; NDF = neutral detergent fiber

Changing carbohydrate composition through diet supplementation should always be performed while maintaining a proper forage and concentrate (F:C) ratio because the F:C ratio has an effect on rumen fermentation, which changes the VFA proportion and, consequently, CH_4 production (Moss et al., 2000). Although Johnson and Johnson (1995) reported a CH_4 energy loss ranging from 2 to 12%, the loss decreased to 2-3% when the animals were fed a high concentrate diet (>90%). Recently, Hassanat et al. (2013) reported reduced CH_4 emissions with a total corn silage diet, whereas no effect was found when feeding corn and alfalfa silage at the same proportions. In addition, (Aguerre et al. (2011)) reported that CH_4 emissions increased when the forage concentrate ratio increased from 47:53 to 68:32.

In the current PhD study, no direct investigation regarding the effect of F:C ratio on CH_4 production was performed. However, Papers II and III investigated the effect of the carbohydrate composition on CH_4 emissions. The results (Paper II) indicate that although starch displayed a potential tendency to reduce CH_4, the actual amount ingested by the cows through the concentrate in the AMS was scarce compared with the TMR portion of the diet. Therefore, no CH_4 reduction effect was apparent presumably due to the inappropriate F:C ratio. From these results (Paper II), choosing an appropriate method of supplementation, in addition to the type and composition of the concentrate, is equally important so that the F:C ratio can be effectively changed and maintained. This hypothesis was confirmed by the significant CH_4 reduction observed in Paper III with starch feeding through the TMR.

4.2. Choice of method for methane measurements

Over the last several decades, different methods have been developed for measuring and estimating ruminant CH_4 emissions. All of these methods have a variety of scopes for application, merits and demerits (Storm et al., 2012b). Ruminants methane production is a biological process derived from rumen fermentation, which can easily be influenced by changes in the physiological states of the animals. Specifically, several factors, such as animal handling and confinement, cause stress. Theses external factors can easily influence feed intake, movement and rumen fermentation. Therefore, to estimate the actual emissions from the animals, the animals must be kept in their natural state while measuring CH_4. Extremely few methods have considered these issues. The gold standard chamber method requires total confinement of the animals for a certain time. Therefore, the animals' CH_4 release measured in a respiration chamber will not be exactly similar to measurements in a barn, where the animals are undisturbed. Another example is the SF_6 technique, which demands that the animals must carry the equipment, which might interfere with normal behaviour. Moreover, to obtain a snapshot of the global CH_4 emissions from the ruminants, CH_4 emissions must be estimated from many animals. Considering all of these facts, the CO_2 method was developed, which is a non-invasive technique that does not interfere with animal behaviour and can consider many animals. Additionally, from the method comparison presented in Table 4.2 (Storm et al., 2012b), the CO_2 method has a wide range of applicability compared with other methods. Therefore, the CO_2 method can be used as an extensive tool for ruminant CH_4 estimation.

4.3. Primary considerations for the CO_2 method

The use of total CO_2 emissions from animals as marker is based on knowledge compiled from more than 100 years of feeding and metabolism experiments. In practice, CO_2 excretion can be calculated using animal production and nutritional data (Madsen et al., 2014) or using the animal data suggested by (CIGR, 2002), as shown in Papers I to IV. The nutritional consideration of CO_2 production is that CO_2 production is closely related to the energy metabolism of the animals. The heat production per L of CO_2 is different, for example, fat = 27.8 kJ/L CO_2, protein = 23.1 kJ/L CO_2 and carbohydrate = 21.1 kJ/L CO_2. This information can be used to estimate the total CO_2 emissions under different feeding regimes and production stages. When the feed intake of the animal is known, then the amount of CO_2 can be calculated using the metabolizable energy values used for maintenance and production (Madsen et al., 2014). The data from the air analysis of livestock buildings indicates a close relation between CO_2 production and the amount of heat producing units (HPU) in a barn. Again, this situation indicates the feasibility of using CO_2 as a potential marker for quantitative CH_4 estimation (Madsen et al., 2010a). The CH_4:CO_2 ratio, which is measured at regular intervals, can be multiplied by the total amount of CO_2 to obtain the amount of CH_4 emitted. Therefore, the most important consideration should be the accurate estimation of heat and CO_2 production while estimating CH_4 production using the CO_2 method.

4.4. Key factors that may influence the accuracy of CH_4 estimates using the CO_2 method

4.4.1. Breath sampling for methane estimation

Breath sample analysis has been shown as a potential diagnostic tool in humans (Turner et al., 2012). Sampling breath from animals and humans has been used for identifying metabolic end products, such as hydrogen, CH_4, volatile fatty acids (VFAs) and other volatile organic compounds (Spinhirne et al., 2003). One of the major advantages of breath sampling is that this method is non-invasive and eliminates handling stress. Non-invasive breath sampling and its chemical analysis could provide valuable information related to health, well-being and, more importantly, metabolic indicators of the end products (Spinhirne et al., 2003). Sampling bovine breath has increasingly become interesting to researchers for estimating greenhouse gas emissions and energy loss from animals. This PhD project used breath samples for estimating ruminant CH_4 gas emissions. The primary focus was to analyse CH_4 and CO_2 concentrations in the breath samples. The average CO_2 concentration in breath samples typically ranges between 30,000 and 50,000 ppm (Smith et al., 2009). The concentration of the sampled air can vary depending on the position of the nose when measuring in the AMS and depending on the ambient exposure of the exhaled breaths. The breath sampling was performed in the AMS during milking in Papers II and IV. However, the breath sampling method must be modified when measurements are taken from small ruminants (Paper I) and/or when using an AMS is not an option (Paper III). Another important aspect of breath sampling is the length of the measurements. In a large-scale study with lactating dairy cows, (Lassen et al., 2012) mentioned that taking 2-3 days of measurements in the AMS can provide reasonable CH_4 estimates. In this connection, (Madsen et al., 2010a) mentioned that approximately 2-3% breath samples were sufficient for CH_4 estimation using the CO_2 method. In the current studies, Paper I considered 8 hours and 30 minutes in different measurement periods from lamb placed in individual pens covered by transparent Plexiglas. Papers II and IV measured breath samples in an AMS for 5 and 7 days, respectively, whereas Paper III considered a 24 hour continuous measurement in a metabolic cage. Because the concentration primarily varies between the samples, several measurements were considered in all of the studies to obtain a reliable mean concentration of the gases. Moreover, a certain correction factor (background concentration) for the standard ambient concentrations of CH_4 and CO_2 was followed in all of the studies to obtain an actual breath concentration from the animals.

4.4.2. Determination of total CO_2 production of the animals

The accuracy of the CH_4 estimate determined by the CO_2 method in a given situation will depend on the accuracy of the determination heat and, consequently, CO_2 production. Moreover, the diurnal variation of the CH_4:CO_2 ratio, followed by the total CO_2 production and the sampling situation, will influence the calculated daily airflow (Bjerg et al., 2012). However, this problem was avoided in the present study by performing several measurements over a day [details in section 4.4.1]. The CH_4 estimation from individual cows requires all of the information related to animal production and intake. When the information is available (Papers I and III), the CH_4 estimation can be performed with higher precision. However, the precision of the CH_4 estimation could be

influenced when the individual animal information is lacking. Under commercial farming conditions, recording individual animal information, particularly the feed intake, is rare. Obtaining typical breath concentrations from the animals is difficult; however, as little as a 2-5% breath concentration is sufficient because the concentration of CO_2 in the sampled air is much higher than the atmospheric CO_2 concentration (Madsen et al., 2014). Abnormal milking and feeding behaviours of certain cows in a herd can also lead to underestimation of the actual CH_4 emissions from that specific cow, resulting in a lower CH_4:CO_2 ratio; this situation was indicated in Paper IV.

4.4.3. Levels of intake

Methane production generally increases with increases in DMI (Kirchgessner et al., 1991). However, the percent of dietary gross energy loss through CH_4 decreases by an average of 1.6% intake levels (Johnson and Ward, 1996). In one study, Allard (2009) reported a gradual decrease in the gross energy loss (10.8, 9.3 and 8.2%) through CH_4 with same diet fed at 0.9, 1.7 and 2.3 times the maintenance requirements. Similarly, Pelchen and Peters (1998) also found that the CH_4 energy loss decreased at a higher feeding levels when increasing the dietary energy density, although the daily CH_4 emission increased. Recently, several studies reported that the DMI is the core determinant of CH_4 release from ruminants (Blaxter and Clapperton, 1965; Johnson and Johnson, 1995; Grainger et al., 2007), which is also determined by the feed digestibility (Blaxter and Clapperton, 1965; Johnson and Johnson, 1995). The results presented in Paper II demonstrated the declining tendency of CH_4 emissions in those cows who received a higher amount of starch-rich concentrate in the AMS because of the higher DMI of the target component. In contrast, Paper III demonstrated a lower DMI in the chamber compared with the intake in the metabolic cage. However, the CH_4 reduction in the chamber was not confirmed by the chamber due to instrumental drift. In addition, Paper IV indicated that the calculated DMI is the prime factor that influences ruminant methane emissions. Similarly, Boadi and Wittenberg (2002) mentioned that approximately 64% variation in CH_4 production is caused by the DMI. A greater range of variation (3 - 34%) in CH_4 production is caused by varying levels of the DMI (Ellis et al., 2010). Furthermore, Grainger et al. (2007) and Garnsworthy et al. (2012b) described similar results where the DMI was mentioned as the primary determinant of CH_4 production. Methane estimation using the CO_2 method is also greatly influenced by the DMI because the breath composition changes with the feeding levels and, consequently, with the CH_4:CO_2 ratio. Therefore, the level of animal intake should be carefully considered when measuring methane.

Table 4.2. Comparison of different methods for measuring and estimation CH_4 emission from ruminants.

Method parameters	Chamber	SF6 Technique	In vitro gas technique	the CO_2-method	IPCC	other models
Prerequisites (except for the instruments used)			Access to rumen fluid	information required to calculate total CO_2	Information regarding animals and feeding	Dependent on the published information
Aspects of dietary factors that can be investigated						
Feeding levels	Y	Y	N	Y	N	Y - Some cases
Physical form of the diet	Y	Y	N	Y	N	N
Chemical composition of diet	Y	Y	Y	Y	N	Y - Some cases
Supplementation of feed additives	Y	Y	Y	Y	N	N
Influence on animals						
Fixation needed	Y	N	*	Depends on the aim	*	*
Animal needs to carry equipment	N	Y	*	N	*	*
Use of automatic milking machine	N	N	*	Y	*	*
Methods estimates						
Individual animals	Y	Y	N	Y	Y	Y
Within animal variation	Y	Y	N	Y	N	N
Between animal variation	Y	Y	N	Y	N	N
Daily variation	Y	Y	N	Y	N	N
Time resolution[1]	Few minutes to hours	8-24 hours	Few minutes to 6 hours	Smaller interval of few minutes	*	*
Output formate						
Relative to animal	Y	Y	N	Y	Y	Y
Relative to DMI	Y	Y	Y	Y	Y	Y
Relative to DE	Y	Y	Y	Y	N	Depends on the model
Relative to NDF	Y	Y	Y	Y	N	Y
Relative to milk yield	Y	Y	N	Y	*	Y
Relative to GEI	Y	Y	N	Y	*	Y

*Not relevant for the method; [1]will depend on the individual settings; Y=yes; N=no; DMI=dry matter intake; DE=digestible energy; NDF=neutral detergent fibre; GEI=gross energy intake; Source: Storm et al. (2012b)

4.5. Accuracy and precision of methane estimates using the CO_2 method

The precision of the estimates is more important than accuracy when comparing the effect of different diets on groups of animals (Madsen et al., 2014). Similarly, McGinn (2006) stated that accuracy is less important but precision is critical when validating mitigation strategies using a particular measurement technique to observe a change in emissions due to a change in management. In this study, the first 3 experiments (Papers I, II and III) were based on comparative diet effects on CH_4 emissions. Paper II discussed the precision of the CH_4 estimates (in terms of standard errors) compared with other studies with dairy cows using different methods. The comparative CH_4 estimates and corresponding standard errors are shown in Table 4.3. The CH_4 estimates presented in Paper II were consistent with Benchaar et al. (2013) and with Hassanat et al. (2013), where the reported standard error using the respiration chamber technique was closer to the SE produced in Paper II. Danielsson et al. (2012) reported a larger variation in CH_4 (g/kg DMI) emissions measured by SF_6 techniques. Likewise, Pinares-Patiño et al. (2011) reported that the SF_6 techniques usually display higher variation, with more than twice as much variation compared with that for chamber measurements. The respiration chamber technique is considered the gold standard (Cassandro et al., 2013), which potentially produces the most precise estimates among the methods in practice. The most problematic issue for this method is the lower feed intake often experienced in the shielded respiration chamber. The CO_2 method for individual animals may not be as precise as the chamber technique; however, the precision can be improved by either increasing the number of measurements or using more animals (Paper II and III). The CO_2 method facilitates both of the options while measuring the animals' CH_4 emissions. The current study includes 18, 36, 3 and 21 animals with measurement durations of 14, 15, 3 and 7 days (total) in experiments I, II, III and IV, respectively. Moreover, the measurements were taken every 15 or 20 seconds, which produced many measurements per animal, hence, helping to increase the number of repeated measurements and improving the precision of CH_4 estimation.

The precision in terms of the co-efficient of variation (CV) within and between cows was discussed in Papers III and IV. Apart from dietary factors, CH_4 production can also vary due to the genetic variation of the animals (Lassen et al., 2012; Pinares-Patino et al., 2013). An earlier study reported a 7% within-animal variation (coefficients of variation, CV) and 7-8% between-animal variation (Blaxter and Clapperton, 1965) in CH_4 production. More recently, several authors reported CV of 4.3% for within-animal and 17.8% for between-animal (Grainger et al., 2007). Between-animal variation values of 26.6% and 25.3% have been reported for dairy and beef heifers with *ad libitum* and restricted feeding, respectively (Boadi and Wittenberg, 2002). A wider range of variation of CH_4 emissions (3 to 34%) was reported by Ellis et al. (2010). All of the papers included in this thesis observed substantial individual variation in CH_4 emissions. The between-animal variation of CH_4 production was CV_b = 7.4 – 8.0%, as shown in Paper III. The observed variation in CH_4 (L/d) emissions (Paper IV) was 5.9 – 8.8% for between-cows during two study years. This variation in CH_4 emissions in both of the studies is lower than those values reported by Grainger et al. (2007) and by Ellis et al. (2010) and is greater than the results reported by Vlaming et al. (2008) and by Garnsworthy et al. (2012b). The observed within-cow variation in CH_4 (L/d) ranged between

17.3 - 17.4 (Paper III) and between 8.6 - 15.5% during the two study years (Paper IV). This range is considerably narrower than that reported previously (Grainger et al., 2007; Garnsworthy et al., 2012b). The slightly higher within-cow variation reported in Paper IV might be due to the varying levels of individual DMI, which is considered the key determinant of CH_4 production. However, animal variation remains even after adjustment for feed intake or for ECM (Pinares-Patino et al., 2013). In a typical feed evaluation study using a respiration chamber, animal variation of CH_4 production is minimized by a fixed amount of feed provided to a limited number of animals. When CH_4 emissions are measured under herd conditions engaging many animals, animal variation appears to be important to consider for obtaining better estimates of CH_4 emissions.

Repeatability, which is another aspect of precision, accounts for the total variation reproducible among the repeated measures of the same subject (Nakagawa and Schielzeth, 2010), as highlighted in Paper IV. The reported repeatability (Paper IV) is consistent with earlier findings for dairy cows and for sheep (Vlaming et al., 2008), with the repeatability of absolute CH_4 emissions (g/d) measurements ranging from 0.55-0.59. The repeatabilities of the CH_4:CO_2 ratio in Holstein and Jersey cows were 0.37 and 0.33, respectively (Lassen et al., 2012), analogous to the repeatability of the CH_4:CO_2 0.34-0.41 in the present study (Paper IV), which is considered to be a better measure of CH_4 production than raw CH_4 measures.

Table 4.3. Comparative methane estimates in different studies using different methods.

Studies	**Methods**	**Cows**	**Length (days)**	**CH_4, g/kg DMI (SE)**
Aguerre et al. (2011)	Chamber	8	4	25.9 (1.21)
van Zijderveld et al. (2011a)	Chamber	2	7	22.1 - 20.5(0.65)
Benchaar et al. (2013)	Chamber	12	3	18.9 - 20.6(0.62)
Hassanat et al. (2013)	Chamber	9	32	20.3 - 22.9(0.82)
Danielsson et al. (2012)	SF_6	5	5	16.9 (2.9)
O'Neill et al. (2011)	SF_6	48	10	20.28 (0.57)
Grainger et al. (2010)	SF_6	30	3	25.7
Mc Geough et al. (2010)	SF_6	90[s]	5	25.9 - 30.1(0.85)
(Eugene et al. (2011))	SF_6	56 [b]	5	32.5 - 50.8(2.08)
This study				
Paper I	CO_2-method	18[l]	14	22.2 – 24.5[*]
Paper II	CO_2-method	36	10	18.9 - 18.2(0.69)
Paper III	CO_2-method	3[h]	7	22.9 – 25.6
Paper IV	CO_2-method	21	28	16.9

[s] = steers; [b] = bulls; [h] = heifers; [l] = lamb; [*] = reported only from hay fed group

Furthermore, in a study by Robinson et al. (2010), a repeatability of 0.32 was reported for 1 h CH_4 measurements, which is lower than the value found in the present study (Paper IV). This repeatability could have been higher if the measurements were performed considering the diurnal variation of the emission, which was ensured in all of the experiments in the current study.

Therefore, based on the above discussion, the CO_2 method can estimate CH_4 emissions with a reasonable accuracy, and the precision of this method is acceptable.

4.6. Expression of methane emission

The choice of the units for the expression of methane (CH_4) emission depends on the objective of the study. The present study used gram or litre as the unit to describe the CH_4 emissions from the animals and expressed it per day, per kg DMI, per kg digestible energy intake (DEI), per kg ECM (Paper I to IV). Generally raw data are produced as parts per million (ppm) of CH_4 volume for volume (v/v) by most of the gas analyser. Therefore, an appropriate conversion is required based on the molecular weight of CH_4 and the volume of one mole of CH_4 at standard temperature and pressure (STP). Eventually the commonly used unit to express CH_4 emissions is gram or Litre of CH_4 per day. More often it is also expressed as the percent of feed GE (MJ). Several reports focused on the global agricultural greenhouse gas emissions were used the unit CO_2-equivalent (CO_2-eq) (Gerber et al., 2013; Opio et al., 2013). The CO_2-eq emission is a standard expression for comparing emissions of different GHGs (IPCC, 2007). When comparing the CH_4 mitigation potential of different diets, the CH_4 emissions can be expressed in gram or Litre per animal, per kg DMI, and per MJ of GE, DE, ME or even NE. At the animal level the CH_4 emission is expressed per unit of DMI or unit of products (e.g. milk and meat). The global CH_4 mitigation strategies are emphasised to assess the CH_4 emissions per unit of animal products (Opio et al., 2013). This is termed as emission intensity (mass of emissions per unit of product).

Chapter 5
Conclusions and Future Perspectives

5. GENERAL CONCLUSIONS

This study demonstrates that artificial rearing of lambs with an extreme diet (milk replacer and cream) prohibited CH_4 release. Switching to a fibrous diet dramatically changed the fermentation pattern and, consequently, the CH_4:CO_2 ratio in the cream group with few days. The CH_4 reduction effects persisted for 50 days, with a lower CH_4:CO_2 ratio. Changing the composition of the concentrate fed in the AMS to higher starch content and to less fibre and sugar had no effect on the CH_4 output. The hypothesised CH_4 reduction was absent most likely due to the small proportion of starch consumed from the allocated concentrate in the AMS, which was insufficient for CH_4 abetment. Changing the composition of the TMR portion of the diet is recommended for CH_4 mitigation. When the starch-based diet was supplemented through the TMR, a significant CH_4 reduction was observed. A substantial variation in CH_4 emissions was observed in individual cows. Diurnal variation in CH_4 emissions was most likely primarily influenced by the feeding behaviour of the cows. A phenotypic correlation of CH_4 emissions was observed at a standardized ECM production between the two study years. The estimation of emissions using the CO_2 method indicates reasonable accuracy and higher precision. The results from the CO_2 method illustrate that higher precision can be obtained by either having more cows in the experiment or measuring for a longer period.

Future perspectives

1. Breath sampling could essentially be an effective tool for identifying the rumen fermentation and metabolic indexes, the occurrences of certain metabolic disorders and other health problems. The analysis of breath concentrations could be a snapshot of healthy rumens and productive cows. Further detailed investigations are required regarding the sampling technique, equipment use and identification of a wide variety of volatile compounds that could represent the metabolic, health and productive statuses of the animals.
2. Similar to the CH_4:CO_2 ratio, an acetone and CO_2 ratio, which could most likely be an index of the levels of acetone production and the identification of sub-acute ketosis in high-yielding cows, could also be established.
3. The CO_2 method can be established as a handy technique for CH_4 estimation in a wide range of circumstances, from feedlot animals to free-range grazing animals.
4. Because the pure breath concentration is relatively constant for the CO_2 concentration (approx. 3-4%); the ratio CO_2 and other substances, such as acetone, can be used without quantifying the total CO_2 emissions from the animals.
5. The CH_4:CO_2 ratio can be used for monitoring sufficient feed intake of the individual animals in a herd as an indicator of heat detection and disease proxy.

References

REFERENCES

Aguerre, M.J., Wattiaux, M.A., Powell, J.M., Broderick, G.A., Arndt, C., 2011. Effect of forage-to-concentrate ratio in dairy cow diets on emission of methane, carbon dioxide, and ammonia, lactation performance, and manure excretion. J. Dairy Sci. 94, 3081-3093.

Akin, D.E., Gordon, G.L.R., Hogan, J.P., 1983. Rumen bacterial and fungal degradation of digitaria-pentzii grown with or without sulfur. Appl. Environ. Microbiol. 46, 738-748.

Allard, H., 2009. Methane emissions from Swedish sheep production. Swedish University of Agricultural Sciences, SLU, Uppsala, Sweden.

Allen, M.S., 1997. Relationship between fermentation acid production in the rumen and the requirement for physically effective fiber. J. Dairy Sci. 80, 1447-1462.

Archimède, H., Eugène, M., Marie Magdeleine, C., Boval, M., Martin, C., Morgavi, D.P., Lecomte, P., Doreau, M., 2011. Comparison of methane production between C3 and C4 grasses and legumes. Anim. Feed Sci. Technol. 166-167, 59-64.

Baldwin, R.L., McLeod, K.R., Klotz, J.L., Heitmann, R.N., 2004. Rumen development, intestinal growth and hepatic metabolism in the pre- and postweaning ruminant. J. Dairy Sci. 87, E55-E65.

Bauchop, T., 1981. The Anaerobic Fungi in Rumen Fiber Digestion. Agr Environ 6, 339-348.

Beauchemin, K., McGinn, S., 2006. Methane emission from beef cattle: effects of fumaric acid, essential oil and canola oil. J. Anim. Sci. 84, 1489 - 1496.

Beauchemin, K.A., Colombatto, D., Morgavi, D.P., Yang, W.Z., 2003. Use of Exogenous Fibrolytic Enzymes to Improve Feed Utilization by Ruminants. J. Anim. Sci. 81, E37-E47.

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., McAllister, T.A., McGinn, S.M., 2009. Dietary mitigation of enteric methane from cattle. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 4, 1-18.

Beauchemin, K.A., McGinn, S.M., Petit, H.V., 2007. Methane abatement strategies for cattle: Lipid supplementation of diets. Can. J. Anim. Sci. 87, 431-440.

Beever, D.E., Dhanoa, M.S., Losada, H.R., Evans, R.T., Cammell, S.B., France, J., 1986. The effect of forage species and stage of harvest on the processes of digestion occurring in the rumen of cattle. The British journal of nutrition 56, 439-454.

Benchaar, C., Greathead, H., 2011. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. Anim. Feed Sci. Technol. 166-67, 338-355.

Benchaar, C., Hassanat, F., Gervais, R., Chouinard, P.Y., 2013. Effects of increasing amounts of corn dried distillers grains with solubles in dairy cow diets on methane production, ruminal fermentation, digestion, N balance, and milk production. J. Dairy Sci. 96, 2413-2427.

Benchaar, C., Pomar, C., Chiquette, J., 2001. Evaluation of dietary strategies to reduce methane production in ruminants: A modelling approach. Can. J. Anim. Sci. 81, 563-574.

Bergman, E.N., 1990. Energy contributions of volatile fatty-acids from the gastrointestinal-tract in various species. Physiol. Rev. 70, 567-590.

Bhatt, R.S., Soren, N.M., Tripathi, M.K., Karim, S.A., 2011. Effects of different levels of coconut oil supplementation on performance, digestibility, rumen fermentation and carcass traits of Malpura lambs. Anim. Feed Sci. Technol. 164, 29-37.

Bjerg, B., Zhang, G., Madsen, J., Rom, H., 2012. Methane emission from naturally ventilated livestock buildings can be determined from gas concentration measurements. Environ. Monit. Assess. 184, 5989-6000.

Blaxter, K.L., Clapperton, J.L., 1965. Prediction of the amount of methane produced by ruminants. The British journal of nutrition 19, 511-522.

Boadi, D., Benchaar, C., Chiquette, J., Masse, D., 2004a. Mitigation strategies to reduce enteric methane emissions from dairy cows: Update review. Can. J. Anim. Sci. 84, 319-335.

Boadi, D.A., Wittenberg, K.M., 2002. Methane production from dairy and beef heifers fed forages differing in nutrient density using the sulphur hexafluoride (SF6) tracer gas technique. Can. J. Anim. Sci. 82, 201-206.

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

Bodas, R., Prieto, N., García-González, R., Andrés, S., Giráldez, F.J., López, S., 2012. Manipulation of rumen fermentation and methane production with plant secondary metabolites. Anim. Feed Sci. Technol. 176, 78-93.

Brask, M., Lund, P., Weisbjerg, M.R., Hellwing, A.L.F., Poulsen, M., Larsen, M.K., Hvelplund, T., 2013. Methane production and digestion of different physical forms of rapeseed as fat supplements in dairy cows. J. Dairy Sci. 96, 2356-2365.

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

Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods - a review. Int. J. Food Microbiol. 94, 223-253.

Carroll, E.J., Hungate, R.E., 1955. Formate dissimilation and methane production in bovine rumen contents. Archives of Biochemistry and Biophysics 56, 525-536.

Cassandro, M., Mele, M., Stefanon, B., 2013. Genetic aspects of enteric methane emission in livestock ruminants. Italian Journal of Animal Science 12, 450-458.

Castillo, C., Benedito, J.L., Mendez, J., Pereira, V., Lopez-Alonso, M., Miranda, M., Hernandez, J., 2004. Organic acids as a substitute for monensin in diets for beef cattle. Anim. Feed Sci. Technol. 115, 101-116.

Cherney, D.J.R., Cherney, J.H., Chase, L.E., 2003. Influence of dietary nonfiber carbohydrate concentration and supplementation of sucrose on lactation performance of cows fed fescue silage. J. Dairy Sci. 86, 3983-3991.

Chilliard, Y., Martin, C., Rouel, J., Doreau, M., 2009. Milk fatty acids in dairy cows fed whole crude linseed, extruded linseed, or linseed oil, and their relationship with methane output. J. Dairy Sci. 92, 5199-5211.

CIGR, 2002. Climatization of animal houses – heat and moisture production at animal and house level. In: Pedersen, S., Sällvik, K. (Eds.), 4Th Report of CIGR Working Group (Research Centre Bygholm, Danish Institute of Agricultural Sciences, P.O Box 536, DK-8700 Horsens, Denmark.

Clark, H., Pinares-Patino, C., deKlein, C., 2005. Methane and nitrous oxide emissions from grazed grasslands. In: McGilloway, D.A. (Ed.), 20th International Grassland Congress. Grassland: A Global Resource. Wageningen Academic Publishers, pp. 279-293.

Danielsson, R., Schnurer, A., Arthurson, V., Bertilsson, J., 2012. Methanogenic population and CH4 production in swedish dairy cows fed different levels of forage. Appl. Environ. Microbiol. 78, 6172-6179.

Dehareng, F., Delfosse, C., Froidmont, E., Soyeurt, H., Martin, C., Gengler, N., Vanlierde, A., Dardenne, P., 2012. Potential use of milk mid-infrared spectra to predict individual methane emission of dairy cows. Animal 6, 1694-1701.

Dijkstra, J., Forbes, J.M., France, J., 2005. Quantitative aspects of ruminant digestion and metabolism (2nd edition). CABI Publishing, Wallingford, Oxfordshire, GBR.

Dijkstra, J., Neal, H.D., Beever, D.E., France, J., 1992. Simulation of nutrient digestion, absorption and outflow in the rumen: model description. J. Nutr. 122, 2239-2256.

Doreau, M., Chilliard, Y., 1997. Digestion and metabolism of dietary fat in farm animals. The British journal of nutrition 78 Suppl 1, S15-35.

Dorman, H.J.D., Deans, S.G., 2000. Antimicrobial agents from plants: antibacterial activity of plant volatile oils. J. Appl. Microbiol. 88, 308-316.

Eadie, J.M., 1962. The Development of Rumen Microbial Populations in Lambs and Calves Under Various Conditions of management. J. Gen. Microbiol 29, 563-578.

Eadie, J.M., Hyldgaard-Jensen, J., Mann, S.O., Reid, R.S., Whitelaw, F.G., 1970. Observations on the microbiology and biochemistry of the rumen in cattle given different quantities of a pelleted barley ration. The British journal of nutrition 24, 157-177.

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. Livest. Sci. 130, 47-56.

Ellis, J.L., Bannink, A., France, J., Kebreab, E., Dijkstra, J., 2010. Evaluation of enteric methane prediction equations for dairy cows used in whole farm models. Global Change Biol. 16, 3246-3256.

Ellis, J.L., Dijkstra, J., France, J., Parsons, A.J., Edwards, G.R., Rasmussen, S., Kebreab, E., Bannink, A., 2012. Effect of high-sugar grasses on methane emissions simulated using a dynamic model. J. Dairy Sci. 95, 272-285.

Ellis, J.L., Dijkstra, J., Kebreab, E., Bannink, A., Odongo, N.E., McBride, B.W., France, J., 2008. Aspects of rumen microbiology central to mechanistic modelling of methane production in cattle. J. Agric. Sci. 146, 213-233.

Ellis, J.L., Kebreab, E., Odongo, N.E., Beauchemin, K., McGinn, S., Nkrumah, J.D., Moore, S.S., Christopherson, R., Murdoch, G.K., McBride, B.W., Okine, E.K., France, J., 2009. Modeling methane production from beef cattle using linear and nonlinear approaches. J. Anim. Sci. 87, 1334-1345.

Emery, R.S., Smith, C.K., Grimes, R.M., Huffman, C.F., Duncan, C.W., 1960. Physical and chemical changes in bovine saliva and rumen liquid with different hay-grain rations. J. Dairy Sci. 43, 76-80.

EPA, 2006. Global mitigation of non-CO2 gases. Environmental Protection Agency (EPA). Washington, DC, USA.

EPA, 2011. Inventory of U.S. greenhouse gas emissions and sinks. Environmental Protection Agency (EPA). Washington, DC, USA.

Eugene, M., Martin, C., Mialon, M.M., Krauss, D., Renand, G., Doreau, M., 2011. Dietary linseed and starch supplementation decreases methane production of fattening bulls. Anim. Feed Sci. Technol. 166-67, 330-337.

Eun, J.S., Beauchemin, K.A., 2007. Assessment of the efficacy of varying experimental exogenous fibrolytic enzymes using in vitro fermentation characteristics. Anim. Feed Sci. Technol. 132, 298-315.

Fahey, G.C., Berger, L.L., 1988. Carbohydrate nutrition of ruminants. In The Ruminant animal : digestive physiology and nutrition. D. C. Church, ed. Prentice Hall Inc., Englewood Cliffs, NJ.

Ferris, C.P., Gordon, F.J., Patterson, D.C., Porter, M.G., Yan, T., 1999. The effect of genetic merit and concentrate proportion in the diet on nutrient utilization by lactating dairy cows. J. Agric. Sci. 132, 483-490.

Finlay, B.J., Esteban, G., Clarke, K.J., Williams, A.G., Embley, T.M., Hirt, R.P., 1994. Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiol. Lett. 117, 157-161.

France, J., Kebreab, E., 2008. Mathematical modelling in animal nutrition. CAB International. Nosworthy Way, Wallingford, Oxfordshire OX10 8DE, UK.

Garnsworthy, P.C., Craigon, J., Hernandez-Medrano, J.H., Saunders, N., 2012a. On-farm methane measurements during milking correlate with total methane production by individual dairy cows. J. Dairy Sci. 95, 3166-3180.

Garnsworthy, P.C., Craigon, J., Hernandez-Medrano, J.H., Saunders, N., 2012b. Variation among individual dairy cows in methane measurements made on farm during milking. J. Dairy Sci. 95, 3181-3189.

Gasmet™, 2010. Gasmet Technologies Oy Pulttitie 8 A, FI-00880 Helsinki, Finland. Accessed April 17, 2013.

http://www.gasmet.com/products/ftir_gas_analyzers/on-site_%28portable%29/gasmet_dx4030.

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), Rome, Italy.

Giger-Reverdin, S., Morand-Fehr, P., Tran, G., 2003. Literature survey of the influence of dietary fat composition on methane production in dairy cattle. Livest. Prod. Sci. 82, 73-79.

Goel, G., Makkar, H.P., 2012. Methane mitigation from ruminants using tannins and saponins. Trop. Anim. Health Prod. 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. The British journal of nutrition 101, 1484-1492.

Grainger, C., Beauchemin, K.A., 2011. Can enteric methane emissions from ruminants be lowered without lowering their production? Anim. Feed Sci. Technol. 166-67, 308-320.

Grainger, C., Clarke, T., McGinn, S.M., Auldist, M.J., Beauchemin, K.A., Hannah, M.C., Waghorn, G.C., Clark, H., Eckard, R.J., 2007. Methane emissions from dairy cows measured using the sulfur hexafluoride (SF6) tracer and chamber techniques. J. Dairy Sci. 90, 2755-2766.

Grainger, C., Williams, R., Clarke, T., Wright, A.D.G., Eckard, R.J., 2010. Supplementation with whole cottonseed causes long-term reduction of methane emissions from lactating dairy cows offered a forage and cereal grain diet. J. Dairy Sci. 93, 2612-2619.

Greathead, H., 2003. Plants and plant extracts for improving animal productivity. Proceedings of the Nutrition Society 62, 279-290.

Guan, H., Wittenberg, K.M., Ominski, K.H., Krause, D.O., 2006. Efficacy of ionophores in cattle diets for mitigation of enteric methane. J. Anim. Sci. 84, 1896-1906.

Gurijala, K.R., Suflita, J.M., 1993. Environmental-factors influencing methanogenesis from refuse in landfill samples. Environ. Sci. Technol. 27, 1176-1181.

Haddad, S.G., Younis, H.M., 2004. The effect of adding ruminally protected fat in fattening diets on nutrient intake, digestibility and growth performance of Awassi lambs. Anim. Feed Sci. Technol. 113, 61-69.

Hammond, K.J., Burke, J.L., Koolaard, J.P., Muetzel, S., Pinares-Patiño, 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. Anim. Feed Sci. Technol. 179, 121-132.

Harmon, D.L., Yamka, R.M., Elam, N.A., 2004. Factors affecting intestinal starch digestion in ruminants: A review. Can. J. Anim. Sci. 84, 309-318.

Harper, L.A., Denmead, O.T., Flesch, T.K., 2011. Micrometeorological techniques for measurement of enteric greenhouse gas emissions. Anim. Feed Sci. Technol. 166-167, 227-239.

Hassanat, F., Gervais, R., Julien, C., Massé, D.I., 2013. Replacing alfalfa silage with corn silage in dairy cow diets: Effects on enteric methane production, ruminal fermentation, digestion, N balance, and milk production. J. Dairy Sci. 96, 4553-4567.

Hellwing, A.L.F., Brask, M., Lund, P., Weisbjerg, M.R., 2012. Effect of carbohydrate source and rumen pH on enteric methane from dairy cows. In: Hassouna, M., Guingand, N. (Eds.), International symposium on Emission of gas and dust from livestock (EMILI 2012), Saint-Malo, France, pp. 206-208.

Hess, H.D., Monsalve, L.M., Lascano, C.E., Carulla, J.E., Diaz, T.E., Kreuzer, M., 2003. Supplementation of a tropical grass diet with forage legumes and Sapindus saponaria fruits: effects on in vitro ruminal nitrogen turnover and methanogenesis. Aust. J. Agric. Res. 54, 703-713.

Hindrichsen, I.K., Kreuzer, M., 2009. High methanogenic potential of sucrose compared with starch at high ruminal pH. J. Anim. Physiol. Anim. Nutr. 93, 61-65.

Hindrichsen, I.K., Wettstein, H.R., Machmuller, A., Jorg, B., Kreuzer, M., 2005. Effect of the carbohydrate composition of feed concentratates on methane emission from dairy cows and their slurry. Environ. Monit. Assess. 107, 329-350.

Hindrichsen, I.K., Wettstein, H.R., Machmuller, A., Soliva, C.R., Knudsen, K.E.B., Madsen, J., Kreuzer, M., 2004. Effects of feed carbohydrates with contrasting properties on rumen fermentation and methane release in vitro. Can. J. Anim. Sci. 84, 265-276.

Hook, S.E., Northwood, K.S., Wright, A.D.G., McBride, B.W., 2009. Long-term monensin supplementation does not significantly affect the quantity or diversity of methanogens in the rumen of the lactating dairy cow. Appl. Environ. Microbiol. 75, 374-380.

Hook, S.E., Wright, A.D.G., McBride, B.W., 2010. Methanogens: Methane producers of the rumen and mitigation strategies. Archaea-an International Microbiological Journal.

Hristov, A.N., Ivan, M., Neill, L., McAllister, T.A., 2003. Evaluation of several potential bioactive agents for reducing protozoal activity in vitro. Anim. Feed Sci. Technol. 105, 163-184.

Hungate, R.E., 1966. The Rumen and Its Microbes. New York: Academic Press.

Hungate, R.E., 1970. Formate as an intermediate in bovine rumen fermentation. J. Bacteriol. 102, 389-&.

IPCC, 2006. 2006 IPCC Guidelines for National Greenhouse Gas Inventories. Intergovermental Panel on Climate Change. Accessed January, 2012, http://www.ipcc-nggip.iges.or.jp/public/2006gl/index.html.

IPCC, 2007. Climate change 2007: mitigation. Contribution of working group III to the fourth assessment report of the intergovernmental panel on climate change. Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA.

Janssen, P.H., Kirs, M., 2008. Structure of the archaeal community of the rumen. Appl. Environ. Microbiol. 74, 3619-3625.

Jenkins, T.C., 1993. Lipid-metabolism in the rumen. J. Dairy Sci. 76, 3851-3863.

Joblin, K.N., 1999. Ruminal acetogens and their potential to lower ruminant methane emissions. Aust. J. Agric. Res. 50, 1307-1313.

Johnson, D., Ward, G., 1996. Estimates of animal methane emissions. Environ. Monit. Assess. 42, 133-141.

Johnson, K., Huyler, M., Westberg, H., Lamb, B., Zimmerman, P., 1994. Measurement of methane emissions from ruminant livestock using a sulfur hexafluoride tracer technique. Environ. Sci. Technol. 28, 359-362.

Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. J. Anim. Sci. 73, 2483-2492.

Jouany, J.P., Morgavi, D.R., 2007. Use of 'natural' products as alternatives to antibiotic feed additives in ruminant production. Animal 1, 1443-1466.

Kamra, D.N., 2005. Rumen microbial ecosystem. Curr. Sci. 89, 124-135.

Kebreab, E., Johnson, K.A., Archibeque, S.L., Pape, D., Wirth, T., 2008. Model for estimating enteric methane emissions from United States dairy and feedlot cattle. J. Anim. Sci. 86, 2738-2748.

Khan, M.A., Lee, H.J., Lee, W.S., Kim, H.S., Ki, K.S., Hur, T.Y., Suh, G.H., Kang, S.J., Choi, Y.J., 2007. Structural growth, rumen development, and metabolic and immune responses of Holstein male calves fed milk through step-down and conventional methods. J. Dairy Sci. 90, 3376-3387.

Kirchgessner, M., Windisch, W., Muller, H.L., Kreuzer, M., 1991. Release of methane and of carbon-dioxide by dairy-cattle. Agribiological Research-Zeitschrift Fur Agrarbiologie Agrikulturchemie Okologie 44, 91-102.

Kohn, R.A., Boston, R.C., 2000. The role of thermodynamics in controlling rumen metabolism. CABI Publishing, Wallingford, Oxon, GBR.

Kolver Es, A.P.W.J.G.N.E.K.M.R.J.R., 2004. Fumarate reduces methane production from pasture fermented in continuous culture. New Zealand Society of Animal Production.

Kristjansson, J., Schönheit, P., Thauer, R., 1982. Different Ks values for hydrogen of methanogenic bacteria and sulfate reducing bacteria: An explanation for the apparent inhibition of methanogenesis by sulfate. Arch. Microbiol. 131, 278-282.

Lassen, J., Lovendahl, P., Madsen, J., 2012. Accuracy of noninvasive breath methane measurements using Fourier transform infrared methods on individual cows. J. Dairy Sci. 95, 890-898.

Leahy, S.C., Kelly, W.J., Altermann, E., Ronimus, R.S., Yeoman, C.J., Pacheco, D.M., Li, D., Kong, Z., McTavish, S., Sang, C., Lambie, S.C., Janssen, P.H., Dey, D., Attwood, G.T., 2010. The genome sequence of the rumen methanogen Methanobrevibacter ruminantium reveals new possibilities for controlling ruminant methane emissions. PLoS One 5, e8926.

Lechartier, C., Peyraud, J.L., 2011. The effects of starch and rapidly degradable dry matter from concentrate on ruminal digestion in dairy cows fed corn silage-based diets with fixed forage proportion. J. Dairy Sci. 94, 2440-2454.

Leng, R.A., 2008. The potential of feeding nitrate to reduce enteric methane production in ruminants. Report,. The Department of Climate Change, Commonwealth Government of Australia Canberra ACT Australia. Available on http://www.penambulbooks.com/Downloads/Leng-Final%20Modified%20%2017-9-2008.pdf

Liu, H., Vaddella, V., Zhou, D., 2011. Effects of chestnut tannins and coconut oil on growth performance, methane emission, ruminal fermentation, and microbial populations in sheep. J. Dairy Sci. 94, 6069-6077.

Liu, Y.C., Whitman, W.B., 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. In: Wiegel, J., Maier, R.J., Adams, M.W.W. (Eds.), Incredible Anaerobes: From Physiology to Genomics to Fuels, pp. 171-189.

Lopez, S., McIntosh, E., Wallace, R.J., Newbold, C.J., 1999. Effect of adding acetogenic bacteria on methane production by mixed rumen microorganisms. Anim. Feed Sci. Technol. 78, 1-9.

Lovett, D., Lovell, S., Stack, L., Callan, J., Finlay, M., Conolly, J., O'Mara, F.P., 2003. Effect of forage/concentrate ratio and dietary coconut oil level on methane output and performance of finishing beef heifers. Livest Prod Sci 84, 135-146.

Lykos, T., Varga, G.A., Casper, D., 1997. Varying degradation rates of total nonstructural carbohydrates: effects on ruminal fermentation, blood metabolites, and milk production and composition in high producing Holstein cows. J. Dairy Sci. 80, 3341-3355.

Ma, K., Liu, X., Dong, X., 2005. Methanobacterium beijingense sp. nov., a novel methanogen isolated from anaerobic digesters. Int. J. Syst. Evol. Microbiol. 55, 325-329.

Machmuller, A., 2006. Medium-chain fatty acids and their potential to reduce methanogenesis in domestic ruminants. Agr Ecosyst Environ 112, 107-114.

Machmuller, A., Kreuzer, M., 1999. Methane suppression by coconut oil and associated effects on nutrient and energy balance in sheep. Can. J. Anim. Sci. 79, 65-72.

Madsen, J., Bjerg, B.S., Hvelplund, T., Weisbjerg, M.R., Lund, P., 2010a. Methane and carbon dioxide ratio in excreted air for quantification of the methane production from ruminants. Livest. Sci. 129, 223-227.

Madsen, J., Chwalibogb, A., Hvelplundc, T., Weisbjergc, M.R., 2014. Use of carbon dioxide as a breath marker. Book of abstracts of 65th Annual Meeting of the European Federation of Animal Science, Copenhagen, Denmark, p. 408.

Madsen, J., Lassen, J., Hvelplund, T., Weisbjerg, M.R., 2010b. A fast, easy, reliable and cheap method to measure the methane production from ruminants. EAAP Publication No. 127. Wageningen Academic Publishers, Wageningen, pp. 121-122.

Martin, C., Morgavi, D.P., Doreau, M., 2010. Methane mitigation in ruminants: from microbe to the farm scale. animal 4, 351-365.

Mc Geough, E.J., O'Kiely, P., Hart, K.J., Moloney, A.P., Boland, T.M., Kenny, D.A., 2010. Methane emissions, feed intake, performance, digestibility, and rumen fermentation of finishing beef cattle offered whole-crop wheat silages differing in grain content. J. Anim. Sci. 88, 2703-2716.

McAllister, T.A., Beauchemin, K.A., Alazzeh, A.Y., Baah, J., Teather, R.M., Stanford, K., 2011. Review: The use of direct fed microbials to mitigate pathogens and enhance production in cattle. Can. J. Anim. Sci. 91, 193-211.

McAllister, T.A., Newbold, C.J., 2008. Redirecting rumen fermentation to reduce methanogenesis. Aust J Exp Agric 48, 7-13.

McDonald, P., Edwards, R.A., Greenhalgh, J.F.D., Morgan, C.A., 2002. Animal Nutrition. Pearson Education, Prentice Hall,Essex Harlow.

McGinn, S.M., 2006. Measuring greenhouse gas emissions from point sources in agriculture. Can. J. Anim. Sci. 86, 355-371.

McGinn, S.M., Beauchemin, K.A., Coates, T., Colombatto, D., 2004. Methane emissions from beef cattle: Effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. J. Anim. Sci. 82, 3346-3356.

McGuffey, R.K., Richardson, L.F., Wilkinson, J.I.D., 2001. Ionophores for dairy cattle: Current status and future outlook. J. Dairy Sci. 84, E194-E203.

Milich, L., 1999. The role of methane in global warming: where might mitigation strategies be focused? Global Environ. Change 9, 179-201.

Moe, P.W., Tyrrell, H.F., 1979. Methane production in dairy cows. J. Dairy Sci. 62, 1583-1586.

Morgavi, D.P., Forano, E., Martin, C., Newbold, C.J., 2010. Microbial ecosystem and methanogenesis in ruminants. animal 4, 1024-1036.

Morgavi, D.P., Jouany, J.P., Martin, C., 2008. Changes in methane emission and rumen fermentation parameters induced by refaunation in sheep. Aust J Exp Agric 48, 69-72.

Mosier, A.R., Duxbury, J.M., Freney, J.R., Heinemeyer, O., Minami, K., Johnson, D.E., 1998. Mitigating Agricultural Emissions of Methane. Clim. Change 40, 39-80.

Moss, A.R., Jouany, J.P., Newbold, J., 2000. Methane production by ruminants: its contribution to global warming. Ann. Zootech. 49, 231-253.

Murphy, M.R., Baldwin, R.L., Koong, L.J., 1982. Estimation of stoichiometric parameters for rumen fermentation of roughage and concentrate diets. J. Anim. Sci. 55, 411-421.

Møllgaard, H., Andersen, A.C., 1917. Respirationsapparatet, dets betydning og anvendelse ved rationelle forsøg over hornkvægets mælkeydelse. 94de Beretning fra Forsøgslaboratoriet. Veterinær- og Landbohøjskoles Laboratorium for landøkonomiske Forsøg, København.

Nakagawa, S., Schielzeth, H., 2010. Repeatability for gaussian and non-gaussian data: A practical guide for biologists. Biol. Rev. Camb. Philos. Soc. 85, 935-956.

Neill, A.R., Grime, D.W., Dawson, R.M., 1978. Conversion of choline methyl groups through trimethylamine into methane in the rumen. Biochem. J. 170, 529-535.

Newbold, C.J., el Hassan, S.M., Wang, J., Ortega, M.E., Wallace, R.J., 1997. Influence of foliage from African multipurpose trees on activity of rumen protozoa and bacteria. The British journal of nutrition 78, 237-249.

Newbold, C.J., Lassalas, B., Jouany, J.P., 1995. The importance of methanogens associated with ciliate protozoa in ruminal methane production in-vitro. Lett. Appl. Microbiol. 21, 230-234.

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. The British journal of nutrition 94, 27-35.

Newbold, C.J., McIntosh, F.M., Williams, P., Losa, R., Wallace, R.J., 2004. Effects of a specific blend of essential oil compounds on rumen fermentation. Anim. Feed Sci. Technol. 114, 105-112.

Newbold, C.J., Rode, L.M., 2006. Dietary additives to control methanogenesis in the rumen. International Congress Series 1293, 138-147.

NRC, 2007. Nutrient requirements for small ruminants. The National Academies Press, Washington, DC.

O'Mara, F.P., Fitzgerald, J.J., Murphy, J.J., Rath, M., 1998. The effect on milk production of replacing grass silage with maize silage in the diet of dairy cows. Livest. Prod. Sci. 55, 79-87.

O'Neill, B.F., Deighton, M.H., O'Loughlin, B.M., Mulligan, F.J., Boland, T.M., O'Donovan, M., Lewis, E., 2011. Effects of a perennial ryegrass diet or total mixed ration diet offered to spring-calving Holstein-Friesian dairy cows on methane emissions, dry matter intake, and milk production. J. Dairy Sci. 94, 1941-1951.

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. J. Dairy Sci. 90, 1781-1788.

Opio, C., Gerber, P., Mottet, A., Falcucci, A., Tempio, G., MacLeod, M., Vellinga, T., Henderson, B., Steinfeld, H., 2013. Greenhouse gas emissions from ruminant supply chains – A global life cycle assessment. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.

Patra, A.K., 2012. Enteric methane mitigation technologies for ruminant livestock: a synthesis of current research and future directions. Environ. Monit. Assess. 184, 1929-1952.

Patra, A.K., 2013. The effect of dietary fats on methane emissions, and its other effects on digestibility, rumen fermentation and lactation performance in cattle: A meta-analysis. Livest. Sci. 155, 244-254.

Patra, A.K., Saxena, J., 2009a. Dietary phytochemicals as rumen modifiers: a review of the effects on microbial populations. Antonie Van Leeuwenhoek 96, 363-375.

Patra, A.K., Saxena, J., 2009b. The effect and mode of action of saponins on the microbial populations and fermentation in the rumen and ruminant production. Nutr Res Rev 22, 204-219.

Patra, A.K., Yu, Z., 2013. Effects of coconut and fish oils on ruminal methanogenesis, fermentation, and abundance and diversity of microbial populations in vitro. J. Dairy Sci. 96, 1782-1792.

Pedersen, S., Blanes-Vidal, V., Joergensen, H., Chwalibog, A., Haeussermann, A., Heetkamp, M.J.W., Aarnink, A.J.A., 2008. Carbon dioxide production in animal houses: A literature review. Agricultural Engineering International: CIGR Ejournal. Retrieved on 07 July 2014, from http://www.cigrjournal.org/index.php/Ejounral/article/viewFile/1205/1132

Pelchen, A., Peters, K.J., 1998. Methane emissions from sheep. Small Ruminant Res. 27, 137-150.

Pellikaan, W.F., Hendriks, W.H., Uwimana, G., Bongers, L.J.G.M., Becker, P.M., Cone, J.W., 2011. A novel method to determine simultaneously methane production during in vitro gas production using fully automated equipment. Anim. Feed Sci. Technol. 168, 196-205.

Pinares-Patino, C.S., Clark, H., 2008. Reliability of the sulfur hexafluoride tracer technique for methane emission measurement from individual animals: an overview. Aust J Exp Agric 48, 223-229.

Pinares-Patino, C.S., Hickey, S.M., Young, E.A., Dodds, K.G., MacLean, S., Molano, G., Sandoval, E., Kjestrup, H., Harland, R., Hunt, C., Pickering, N.K., McEwan, J.C., 2013. Heritability estimates of methane emissions from sheep. Animal 7 Suppl 2, 316-321.

Pinares-Patiño, C.S., Lassey, K.R., Martin, R.J., Molano, G., Fernandez, M., MacLean, S., Sandoval, E., Luo, D., Clark, H., 2011. Assessment of the sulphur hexafluoride (SF6) tracer technique using respiration chambers for estimation of methane emissions from sheep. Anim. Feed Sci. Technol. 166-167, 201-209.

Pinares-Patio, 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. J. Agric. Sci. 140, 227-233.

Ramirez-Restrepo, C.A., Barry, T.N., 2005. Alternative temperate forages containing secondary compounds for improving sustainable productivity in grazing ruminants. Anim. Feed Sci. Technol. 120, 179-201.

Rapheal, S.V., Swaminathan, K.R., Lalitha, K., 2003. Metabolic characteristics of an aerobe isolated from a methylotrophic methanogenic enrichment culture. J. Biosci. (Bangalore) 28, 235-242.

Robinson, D.L., Goopy, J.P., Hegarty, R.S., Vercoe, P.E., 2010. Repeatability, animal and sire variation in 1-hr methane emissions & relationships with rumen volatile fatty acid concentrations. In Book of Abstracts (abstract no. 712). World Congress in genetic Applied to Livestock. .

Russell, J.B., Hespell, R.B., 1981. Microbial rumen fermentation. J. Dairy Sci. 64, 1153-1169.

Schonhusen, U., Zitnan, R., Kuhla, S., Jentsch, W., Derno, M., Voigt, J., 2003. Effects of protozoa on methane production in rumen and hindgut of calves around time of weaning. Arch. Anim. Nutr.-Arch. Tierernahr. 57, 279-295.

Smith, D., Pysanenko, A., Spanel, P., 2009. The quantification of carbon dioxide in humid air and exhaled breath by selected ion flow tube mass spectrometry. Rapid Commun. Mass Spectrom. 23, 1419-1425.

Smith, P.H., Hungate, R.E., 1958. Isolation and characterization of methanobacterium-ruminantium n-sp. J. Bacteriol. 75, 713-718.

Smith, R.H., 1959. The development and function of the rumen in milk-fed calves. J. Agric. Sci. 52, 72-78.

Solomon, S., Qin, D., Manning, M., 2007. Technical Summary. In: Climate Change 2007. The Physical Science Basis. Contribution of Working Group I to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. (Solomon, S. Qin, D. Manning, M. Marquis, M. Averyt, K. Tignor, M. M. B. Miller, H. L. Chen, Z. L. (eds)). Cambridge University Press, Cambridge, United Kingdom and New York, NY, USA, pp. 19-91.

Spinhirne, J.P., Koziel, J.A., Chirase, N.K., 2003. A Device for Non-invasive On-site Sampling of Cattle Breath with Solid-Phase Microextraction. Biosys. Eng. 84, 239-246.

Stams, A.J., Plugge, C.M., 2009. Electron transfer in syntrophic communities of anaerobic bacteria and archaea. Nat. Rev. Microbiol. 7, 568-577.

Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., Rosales, M., Haan, C.d., 2006. Livestock's Long Shadow: Environmental Issues and Options. Food and Agriculture Organization of the United Nations (FAO), Rome, Italy.

Stevens, C.E., Hume, I.D., 1998. Contributions of microbes in vertebrate gastrointestinal tract to production and conservation of nutrients. Physiol. Rev. 78, 393-427.

Storm, I.M.L.D., Haque, M.N., Madsen, J., Hansen, H.H., 2012a. Comparison of methane emissions from cattle assessed by three different methods: open-circuit respiration chambers, in vitro gas production and the CO2-method. In: Hassouna, M., Guingand, N. (Eds.), International

symposium on Emission of gas and dust from livestock (EMILI 2012), Saint-Malo, France, pp. 346-349.

Storm, I.M.L.D., Hellwing, A.L.F., Nielsen, N.I., Madsen, J., 2012b. Methods for measuring and estimating methane emission from ruminants. Animals 2, 160-183.

Tamminga, S., Bannink, A., Dijkstra, J., Zom, R., 2007. Feeding strategies to reduce methane loss in cattle. Report: Animal Nutrition and Animal Sciences Group, Wageningen UR, Lelystad, The Netherlands.

Tan, H.Y., Sieo, C.C., Abdullah, N., Liang, J.B., Huang, X.D., Ho, Y.W., 2011. Effects of condensed tannins from Leucaena on methane production, rumen fermentation and populations of methanogens and protozoa in vitro. Anim. Feed Sci. Technol. 169, 185-193.

Thauer, R.K., Kaster, A.K., Seedorf, H., Buckel, W., Hedderich, R., 2008. Methanogenic archaea: ecologically relevant differences in energy conservation. Nat. Rev. Microbiol. 6, 579-591.

Turner, C., Knobloch, H., Richards, J., Richards, P., Mottram, T.T.F., Marlin, D., Chambers, M.A., 2012. Development of a device for sampling cattle breath. Biosys. Eng. 112, 75-81.

Ungerfeld, E.M., 2013. A theoretical comparison between two ruminal electron sinks. Frontiers in Microbiol 4.

Ungerfeld, E.M., Kohn, R.A., Wallace, R.J., Newbold, C.J., 2007. A meta-analysis of fumarate effects on methane production in ruminal batch cultures. J. Anim. Sci. 85, 2556-2563.

van Kessel, J.A.S., Russell, J.B., 1996. The effect of pH on ruminal methanogenesis. FEMS Microbiol. Ecol. 20, 205-210.

van Zijderveld, S.M., Fonken, B., Dijkstra, J., Gerrits, W.J.J., Perdok, H.B., Fokkink, W., Newbold, J.R., 2011a. Effects of a combination of feed additives on methane production, diet digestibility, and animal performance in lactating dairy cows. J. Dairy Sci. 94, 1445-1454.

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. J. Dairy Sci. 93, 5856-5866.

van Zijderveld, S.M., Gerrits, W.J.J., Dijkstra, J., Newbold, J.R., Hulshof, R.B.A., Perdok, H.B., 2011b. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. J. Dairy Sci. 94, 4028-4038.

Vlaeminck, B., Fievez, V., Cabrita, A.R.J., Fonseca, A.J.M., Dewhurst, R.J., 2006. Factors affecting odd- and branched-chain fatty acids in milk: A review. Anim. Feed Sci. Technol. 131, 389-417.

Vlaming, J.B., Lopez-Villalobos, N., Brookes, I.M., Hoskin, S.O., Clark, H., 2008. Within-and between-animal variance in methane emissions in non-lactating dairy cows. Aust J Exp Agric 48, 124-127.

Waghorn, G., Garnet, E.J., Pinares, C.S., Zimmerman, S., 2013. Implementation of GreenFeed for estimating methane in a dairy herd grazing pasture. Abstract, Greenhouse Gasses in Agriculture Conference. Advances in Animal Biosciences, Dublin, Ireland, p. 439.

Wallace, R.J., 2004. Antimicrobial properties of plant secondary metabolites. Proceedings of the Nutrition Society 63, 621-629.

Wang, Y., McAllister, T.A., 2002. Rumen microbes, enzymes and feed digestion - A review. Asian Austral J Anim 15, 1659-1676.

Wardrop, I.D., 1960. The post-natal growth of the visceral organs of the lamb Part II. The effect of diet on growth rate, with particular reference to the parts of the alimentary tract. J. Agric. Sci. 55, 127-132.

Wardrop, I.D., Coombe, J.B., 1960. The post-natal growth of the visceral organs of the lamb I. The growth of the visceral organs of the grazing lamb from birth to sixteen weeks of age. J. Agric. Sci. 54, 140-143.

Weisbjerg, M.R., Terkelsen, M., Hvelplund, T., Madsen, J., 2012. Increased productivity in Tanzanian cattle production is the main approach to reduce methane emission per unit of product. Book of Abstracts, 35th Annual scientific conference. Olasit Garden, Arusha, Tanzania, p. 7.

Welch, J.G., 1986. Physical parameters of fiber affecting passage from the rumen. J. Dairy Sci. 69, 2750-2754.

Williams, A.G., 1986. Rumen holotrich ciliate protozoa. Microbiol. Rev. 50, 25-49.

Williams, Y.J., Popovski, S., Rea, S.M., Skillman, L.C., Toovey, A.F., Northwood, K.S., Wright, A.D.G., 2009. A vaccine against rumen methanogens can alter the composition of archaeal populations. Appl. Environ. Microbiol. 75, 1860-1866.

Wolin, M.J., 1975. Interactions between the bacterial species of the rumen. . The University of new England, Armidale, Australia.

Wright, A.D.G., Kennedy, P., O'Neill, 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.

Zimmerman, S., Michal, J.J., White, R., Johnson, K.A., Guerouali, A., Zimmerman, P., 2013. Evaluation of a novel system to measure enteric methane emissions from beef cattle on pasture. Unpublished paper, Presented in the Joint Annual Meeting, Indianapolis, Indiana, USA. Available in http://c-lockinc.com/references.php.

Ørskov, E.R., 1986. Starch Digestion and Utilization in Ruminants. J. Anim. Sci. 63, 1624-1633.

Included Papers

Paper I

Development of methane emission from lambs fed milk replacer and cream for a prolonged period

Accepted in Animal Feed Science and Technology; Article in Press.
(DOI: http://dx.doi.org/10.1016/j.anifeedsci.2014.09.002).

Animal Feed Science and Technology xxx (2014) xxx–xxx

Contents lists available at ScienceDirect

Animal Feed Science and Technology

journal homepage: www.elsevier.com/locate/anifeedsci

Development of methane emission from lambs fed milk replacer and cream for a prolonged period

M.N. Haque[a,*,1], M. Roggenbuck[b], P. Khanal[c], M.O. Nielsen[c], J. Madsen[a,*]

[a] Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Groennegaardsvej 2, DK-1870 Frederiksberg C, Denmark
[b] Department of Biology, Microbiology, Faculty of Science, University of Copenhagen, Universitetsparken 15, 2100 København Ø, Denmark
[c] Department of Veterinary Clinical and Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Groennegaardsvej 3, DK-1870 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 17 March 2014
Received in revised form 5 September 2014
Accepted 8 September 2014
Available online xxx

Keywords:
Abundance
Artificial rearing
Breath sample
Carbon dioxide
Fibrous diet
Rumen development

ABSTRACT

Methane (CH_4) emission was investigated in artificially reared growing lambs fed milk replacer and cream. This study was part of a larger study with 70 lambs, of which 18 lambs with an average body weight of 21 ± 3.6 kg (mean ± SD) were used. The lambs were housed in individual pens (1.5 m × 1.5 m). From 3 until 180 days of age, they were fed either a restricted grass hay diet or a "Cream" diet (50% milk replacer and 50% cream) *ad libitum* until a daily maximum allocation of 2.5 L/d. In addition, rolled maize was fed *ad libitum* (maximum allowance 1 kg/d). After 180 days, two groups were placed together and supplied a hay diet. The CH_4 and carbon dioxide (CO_2) were measured in periods 1–4 (approx. 90, 150, 185 and 235 days of age, respectively). During periods 1 and 2, the measurements were performed on each of the 18 lambs individually for 8 h, equally distributed in three periods over a 24-h day. During periods 3 and 4, the measurements were performed on each of the 18 lambs consecutively for 30 min. Twenty-six lambs (out of 70), of which four lambs from the CH_4 measurement group, were slaughtered at the age of 180 days to collect rumen samples for microbiological study. The dry matter intake (DMI, g/d) was significantly lower ($P<0.001$) in the cream-fed group. The CH_4 production (g/d) was 84 and 87% lower in the cream group compared to the hay group during periods 1 and 2, respectively. The same group had a lower CH_4 emission per unit of DMI and DEI ($P<0.001$). The CH_4:CO_2 ratios were 0.0022 and 0.0036 in the cream group during periods 1 and 2, respectively. Within 4 days after changing the diet (period 3), the CH_4:CO_2 ratio of the ex-cream-fed lambs was 0.035, much higher compared to the CH_4:CO_2 ratio during period 1 ($P<0.001$). A significantly lower CH_4:CO_2 ratio ($P<0.001$) was observed in the cream group compared to the hay group during periods 3 and 4, respectively. The abundance of rumen methanogens was lower in the fluid portion of the cream group. In conclusion, the artificial rearing of lambs with milk

Abbreviations: ADFom, acid detergent fibre expressed exclusive of residual ash; aNDFom, neutral detergent fibre assessed with heat stable amylase and expressed exclusive of residual ash; BW, body weight; CO_2, carbon dioxide; CH_4, methane; CH_4:CO_2, ratio between methane and carbon dioxide; DE, digestible energy; DEI, digestible energy intake; DM, dry matter; DMI, dry matter intake; DNA, deoxyribonucleic acid; FA, fatty acid; HP, heat production; HPU, heat production unit; Lignin(sa), lignin determined by solubilisation of cellulose with sulphuric acid; LSM, least square mean; rRNA, ribosomal RNA; PCR, polymerase chain reaction; WG, weight gain.

* Corresponding authors. Tel.: +45 35 33 30 92; fax: +45 35 33 30 55.
E-mail addresses: naha@sund.ku.dk, najmulhaque_bau@yahoo.com (M.N. Haque), jom@sund.ku.dk (J. Madsen).
[1] Tel.: +45 35 33 28 68.

http://dx.doi.org/10.1016/j.anifeedsci.2014.09.002

replacer and cream nearly prevented CH_4 release. Switching from milk replacer and cream to a fibrous diet dramatically changed the CH_4:CO_2 ratio in the cream group within 4 days. The CH_4:CO_2 ratio remained lower for 50 days after the diet alteration.

1. Introduction

Enteric fermentation is the second largest source of greenhouse gas emissions, contributing approximately 40% to total emissions, of which the contribution of small ruminants is 10% (Gerber et al., 2013). Methane (CH_4) production from ruminants is a result of the microbial fermentation of feeds in the rumen. Moreover, enteric CH_4 production leads to a loss of productive energy ranging from 2 to 12% of the gross energy intake in ruminants, depending on the level of feed intake and diet composition (Johnson and Johnson, 1995). Therefore, due to nutritional and environmental considerations, efforts to mitigate CH_4 emissions from ruminants, especially through dietary manipulation, are receiving great attention. The addition of lipids or fats was recently proven to mitigate CH_4 emission while increasing the dietary energy content (Machmuller and Kreuzer, 1999; Machmuller et al., 2000). The CH_4-suppressing mechanism of fats is believed to be induced by the reduction of organic matter fermentation, fibre digestibility and, consequently, the methanogenic pathway and more importantly by the direct inhibition of methanogens in the rumen through the hydrogenation of unsaturated fatty acids (Johnson and Johnson, 1995). A strong effect of lipid supplementation on CH_4 reduction (up to 73%) has been reported in sheep (Machmuller and Kreuzer, 1999). Most studies have investigated a short-term CH_4 mitigation strategy, and their persistency is still in question. Therefore, it is crucial to determine a long-term mitigation strategy through dietary manipulation. Such an approach could be the artificial rearing of young ruminants through liquid feeding to avoid microbial fermentation in the rumen. In newborn ruminants, CH_4 production and energy loss through CH_4 are absent due to a non-functional rumen and lack of microbial fermentation (Eadie, 1962). The establishment of rumen microorganisms and fermentation in neonatal lamb begins at the age of 3–4 weeks (Wardrop and Coombe, 1960), during which the type of diet is the primary factor that affects the relative growth of the digestive organs (Wardrop, 1960). Moreover, the metabolic and physical development of the rumen during the growing phase depends on solid feed consumption (Baldwin et al., 2004). Increased the supply of milk to dairy calves produced a higher daily weight gain and slower rumen development (Khan et al., 2007). Similarly, Smith (1959) reported that rumen development will not occur in milk-fed calves for an abnormally prolonged time (up to 32 weeks). The initiation of fibrous feed consumption and fermentation processes is required to inoculate and establish the anaerobic rumen microbial ecosystem and develop proper rumen function in young ruminants (Baldwin et al., 2004). We hypothesised that feeding milk replacer for a prolonged period would affect the rumen microbial populations, fermentation rate and gas production, especially CH_4 production in lambs. We further hypothesised that the prolonged feeding of a liquid fat (dairy cream) would have a sustained suppressive effect on the enteric CH_4 production. Therefore, the objective of this study was to investigate the rumen fermentation and CH_4 emissions of lambs that were reared on a diet of milk and cream compared to lambs that were reared on a conventional grass-hay diet.

2. Material and methods

2.1. Animals, housing and feeding

This experiment complied with the guidelines of the Danish Ministry of Justice with respect to animal experimentation and care of animals under study. This study was part of a larger study with 70 lambs born to twin-pregnant ewes. Only 18 lambs with an average body weight 21 ± 3.6 kg (mean ± SD) were selected in this study to describe the effects of feeding milk replacer and cream on CH_4 emissions. Within each twin pair, one lamb was assigned to each of the two treatments, which were termed the "Hay" and "Cream" groups. The lambs suckled their dams until 3 days of age, after which the ewes were separated from their lambs. Initially, the lambs were housed in a large barn that was maintained with proper ventilation and at a temperature of approx. 18–22 °C. The lambs were fed individually in smaller pens (1.5 m × 0.75 m) with sawdust as a bedding material. When the lambs reached 60 days of age, they were transferred to larger pens (1.5 m × 1.5 m). From 3 days until 56 days of age, the "Hay" group was fed milk replacer (180 of milk powder g/L; Elitemilk Lamb, Vilofarm; DLA Group, Galten, Denmark) from a suckling bucket and received grass hay from 14 days of age. From 57 days of age, the hay group was fed only hay without any milk replacer (Fig. 1). The daily allowances of milk replacer and hay for the hay group were adjusted on a weekly basis to achieve moderate daily live weight gains of approx. 225 g/d.

Lambs in the second group, which was termed the "Cream" group, received 50% milk replacer and 50% dairy cream (Osted Ost og Mejeri ApS, Lejre, Denmark) *ad libitum* (until the daily predefined maximum of 2.5 L/d up to 180 days of age). Rolled maize (Maize flakes; R2 Feed Partner A/S, Hedensted, Denmark) was fed *ad libitum* (until the predefined maximum allowance of 1 kg/d). The milk replacer-dairy cream mix was fed from a suckling bucket. From 3 to 7 days of age, the lambs in both of the groups were fed four times a day and twice daily thereafter at approx. 07:00 h and 16:00 h, respectively. A small amount of barley straw (approx. 10 g/d) was fed to the both groups, and for cream lambs, this feeding was to prevent disorders of rumen function. The amount of DM from the ingested barley straw was considered insignificant. Therefore, this

Please cite this article in press as: Haque, M.N., et al., Development of methane emission from lambs fed milk replacer and cream for a prolonged period. Anim. Feed Sci. Tech. (2014), http://dx.doi.org/10.1016/j.anifeedsci.2014.09.002

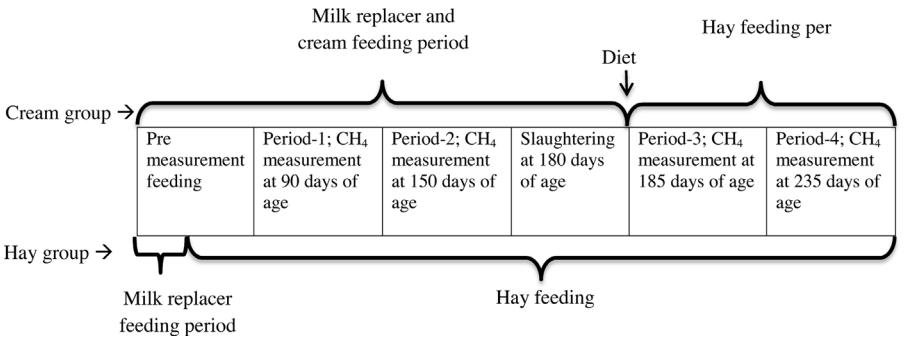


Fig. 1. Experimental feeding period of the lambs (n = 18) in the cream and hay group.

was not added to the total DMI for both of the groups. Water was available *ad libitum* at all times, and a vitamin-mineral mix was provided based on requirements (NRC, 2007). The daily amount that was fed to the lambs was recorded daily during periods 1 and 2. The feed residues and weight of the lambs were recorded once per week. The daily ingested amount was calculated considering the weekly supply and refusal. The weight gain was calculated considering the differences in the weekly body weight changes during periods 1 and 2. The feed intake and body weight were not recorded during periods 3 and 4. At 180 days of age, a total of 26 lambs (13 from each treatment) out of 70 were slaughtered for the collection and analysis of the rumen contents for rumen microbial diversity, of which four lambs (two from each treatment) were from the CH_4 measurement groups. The remaining lambs were thereafter managed together and were fed the hay diet. The CH_4 measurements were performed in four periods at approximately 90, 150, 185 and 235 days of age. Periods 3 and 4 were held at 4 and 50 days after the transfer of the cream lambs to a normal hay diet, respectively. In order to make a comparable CH_4 estimation during periods 3 and 4, two lambs were added to each group to make similar the number of animals as in periods 1 and 2. The added lambs with closer average BW in the respective groups were selected from the flock that was reared with a same feeding regime as in the cream and hay groups, respectively.

2.2. Sampling and analysis of feed samples

The chemical composition, nutritive value and energy content of the feed that was used during periods 1 and 2 are shown in Table 1. Feed samples were taken from both of the groups during each of the measurement periods. Immediately after collection, the samples were stored in a freezer. Before laboratory analysis, the samples were mixed together to create a composite sample. The samples were dried at 103 °C to determine the dry matter percentage. The crude ash content was determined according to EU (2009). The aNDFom, ADFom and lignin(sa) were determined according to Van Soest et al. (1991). Both aNDFom and ADFom were expressed exclusive of residual ash. Lignin(sa) was determined by solubilisation of cellulose with sulphuric acid. The crude protein content was determined according to Licitra et al. (1996). The crude fat content was determined following ISO-11085 (2008). The digestibility of the feed ingredients of the hay diet was calculated according to NRC (2001). The digestibility of the feed ingredients in the diet of the cream group was determined according to Møller et al. (2000). The digestible energy content was based on the chemical composition of the individual ingredients, and was calculated according to NRC (2001).

2.3. Sampling and microbial analysis

Both solid and liquid samples were collected from the rumen. Immediately after the collection of the total contents, the solid part was separated from the liquid by filtering with double-folded cheese cloth. The samples were immediately

Table 1
Chemical composition, digestible nutrients and energy content of the feeds.

Items	Grass hay	Cream	Milk powder	Rolled maize
[a]DM (g/kg)	931.0	429.2	956.1	895.0
Ash (g/kg DM)	68.2	8.3	71.0	6.2
aNDFom (g/kg DM)	504.0			41.1
ADFom (g/kg DM)	323.4			40.0
Lignin(sa) (g/kg DM)	35.0			9.0
CP (g/kg DM)	208.0	43.3	225.1	85.0
cFat (g/kg DM)	37.1	380.0	236.1	19.3
DE (MJ/kg DM)	12.7	35.8	20.4	15.9

DM, dry matter; aNDFom, neutral detergent fibre assessed with heat stable amylase and expressed exclusive of residual ash; ADFom, acid detergent fibre expressed exclusive of residual ash; Lignin(sa), lignin determined by solubilisation of cellulose with sulphuric acid; CP, crude protein; cFat, crude fat; DE; digestible energy.

[a] Calculated as g/kg of fresh material.

Please cite this article in press as: Haque, M.N., et al., Development of methane emission from lambs fed milk replacer and cream for a prolonged period. Anim. Feed Sci. Tech. (2014), http://dx.doi.org/10.1016/j.anifeedsci.2014.09.002

stored at −40 °C after collection. During the analysis phase, the DNA was extracted from 0.5 g of ruminal fluid and solid content using the Genomic Mini AX Soil Spin Kit (A&A Biotechnology). To study the archaeal composition in the ruminal samples, the variable regions V3 and V4 of the 16S rRNA gene phylogenetic marker were amplified using the primer 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′) by the Phusion Hot Start Polymerase 540L (Neefs et al., 1991; Yu et al., 2005). The polymerase reaction (PCR) mixture contained 5 μl of 5× Phusion HF buffer (7.5 mM $MgCl_2$, Finnzymes, Finland), 0.5 μl of 10 mM dNTP mixture, 0.25 μl of Phusion Hot Start DNA Polymerase (1 unit/μl, Finnzymes), 1.25 μl of each primer (10 μM), and 2 μl of template. The PCR reaction started at 98 °C for 30 s, followed by 30 cycles of 98 °C for 5 s, 56 °C for 20 s and 72 °C for 20 s. The reaction was finalised with 72 °C for 5 min. The amplicon fragment of 466 bp was elongated to 526 bp by adding sequencing adaptors and barcodes (Roche FLX) to the PCR product following the same conditions as for the first PCR with a shortened cycle number of 15. The sequences were generated using 454 GS FLX Titanium. For methanogen quantification, the sequences were cleaned of low quality reads and split into individual animal samples using the default settings of Qiime (Caporaso et al., 2010). Chimaera were removed by USEARCH UCHIME (Edgar et al., 2011). Operational taxonomic units (OTUs) were *de novo* picked by UCLUST, and the taxonomy was assigned to the OTUs with the RDP classifier method and Greengenes reference database (Liu et al., 2008).

2.4. Installation and gas measurement

The gas measurements were performed every 15 s in the four experimental periods. During periods 1 and 2, each of the 18 lambs was measured individually for 8 h equally distributed in three periods over a 24-h day. During periods 3 and 4, the measurements were taken on each of the 18 lambs consecutively for 30 min during the day without considering the potential diurnal variation. Prior to measuring the breath sample, the pens of the lambs were covered by Plexiglas to restrict the air movement as much as possible. However, the pens were not completely airtight. The glass was transparent to avoid blocking the views of the lambs from each other. The breath concentrations of CH_4 and CO_2 were measured using a continuous gas analyser Gasmet DX-4030 (Gasmet™, 2010) based on Fourier Transformed Infrared Radiation. The inlet filter of the Gasmet was fitted inside the pen to collect concentrated breath samples. After being received in the inlet, the breath sample passes through the filter and thereafter through the Gasmet analyser to determine the concentrations of CH_4 and CO_2. Before each measurement, the equipment was calibrated with known standard gases to verify the accuracy of the measurement. On each experimental day, the background concentrations of CH_4 and CO_2 were measured. The measurements were remotely monitored *via* the internet using TeamViewer (TeamViewer©, 2013).

2.5. Calculation

The CH_4 and CO_2 emissions from the lambs were calculated according to the CO_2 method (Madsen et al., 2010). The barn concentrations of CO_2 (590, ppm) and CH_4 (6.9, ppm) were subtracted from the exhaled concentrations of the lambs to obtain the actual breath concentration. A ratio between CH_4 and CO_2 (CH_4:CO_2) was determined. The heat production (watt) of the lambs was calculated following Eq. (1) as described by CIGR (2002). The excretion of CO_2 (L/d) was calculated according to Pedersen et al. (2008) and mentioned in Eq. (2). The amount of CH_4 (g/d) was calculated as described by Madsen et al. (2010) in Eq. (3).

$$\mathrm{HP(watt)} = 6.4 * \mathrm{BW}^{0.75} + 145Y \tag{1}$$

$$\mathrm{CO_2} = \mathrm{HPU} * 180 * 24 \tag{2}$$

$$\mathrm{CH_4} = \mathrm{CO_2} * \frac{\mathrm{CH_4}}{\mathrm{CO_2}} * 0.714 \tag{3}$$

where

HP = heat production of the animals;
$BW^{0.75}$ = metabolic body weight of the animals;
Y = daily weight gain of the animals;
HPU = heat-producing unit HP/1000;
180 = L of CO_2/HPU/h.

2.6. Statistical analyses

The raw data were processed to obtain the average emission per lamb per day. The day average data were then fitted with a linear model using the statistical software R version 3.0.0 (R Development Core Team, 2013). The primary model for periods 1 and 2 was fitted with all of the possible influential variables of interest {body weight, weight gain (WG), groups, periods and dry matter intake}. The final model in Eq. (4) for periods 1 and 2 was selected by the stepwise elimination of the non-significant variables. The model (Eq. 5) for periods 3 and 4 was fitted with groups and periods. The model was validated

Please cite this article in press as: Haque, M.N., et al., Development of methane emission from lambs fed milk replacer and cream for a prolonged period. Anim. Feed Sci. Tech. (2014), http://dx.doi.org/10.1016/j.anifeedsci.2014.09.002

Table 2
Least square means (LSM) of the body weight, weight gain and nutrient intake of the lambs (n = 18) of hay and cream group during periods 1 and 2.

Parameters	Hay		Cream		RSE	R^2	Significance
	Period 1	Period 2	Period 1	Period 2			
BW (kg)	20.9[a]	33.7[b]	21.8[a]	34.7[b]	5.33	0.59	<0.001
WG (g/d)	151.2[a]	204.1[b]	197.3[b]	250.1[c]	58.53	0.24	0.004
[1]DMI (g/d)	650.9[b]	1096.5[d]	237.1[a]	682.7[c]	176.60	0.75	<0.001
NDFI (g/d)	147.7[c]	341.6[d]	0.9[a]	2.8[b]	56.81	0.84	<0.001
FI (g/d)	12.7[a]	36.2[a]	197.7[b]	221.2[b]	60.47	0.70	<0.001
DEI (MJ/d)	8.0[a]	14.2[c]	8.8[b]	15.1[c]	2.87	0.56	<0.001

[1] DMI, dry matter intake excluding the amount from barley straw; NDFI, neutral detergent fibre intake; FI, fat intake; RSE, residual standard error; R^2, goodness of fit of the linear model; Significance indicates the model P value; [abcd] superscripts indicate differences (P<0.05) followed by multiple comparison within group and between the same period across the groups.

using an analysis of variance (ANOVA) on the Akaike Information Criterion. The model residuals were checked for normality and homoscedasticity by visual inspection, producing qqplots.

$$y_{ij} = \mu + \alpha_i + \beta_j + X\gamma_{ij} + \varepsilon_{ij} \quad (4)$$

$$y_{ij} = \mu + \alpha_i + \beta_j + \varepsilon_{ij} \quad (5)$$

where y_{ij} is the response variable, y = {CH_4 g/d, CH_4 g/kg WG, CH_4 g/kg DMI, CH_4 g/MJ DEI and CH_4:CO_2 ratio for periods 1 and 2; and only the CH4:CO2 ratio for periods 3 and 4} of group i, and period j, μ = overall mean, α_i = group (cream and hay), β_j = measurement period {1 and 2 (Eq. 4), and 3 and 4 (Eq. 5)}, $X\gamma_{ij}$ = total dry matter intake of group i, and period j, and ε_{ij} is the model residuals. Although the period as a fixed variable was not significant, this was included in the model to determine the trend of CH_4 production over the time. The least square means (LSM) were extracted from the model using the package lsmeans as described by Russell (2013). A multiple comparison was performed using Tukey's pairwise comparisons using the function glht from the multcomp package (Hothorn et al., 2008). For microbiological analysis, the rarified relative sequence counts of the methanogens were checked for normal distribution using the Shapiro Wilk test. Due to the non-normal distribution of the dataset (Shapiro Wilk; W = 0.703, P<0.001), the non-parametric two-sample Wilcoxon Rank Sum test (cut-off p-value, P<0.05) was used to evaluate the diet-induced differences in methanogen abundance between the variables.

3. Results

3.1. Body weight and dry matter intake

The body weight, daily weight gain and feed intake of the experimental lambs are shown in Table 2. The body weight (BW, kg) was not different in the two groups within the periods. During period 2, a significant increase in the BW was observed in both of the groups (P<0.001) compared to that in period 1. The average daily weight gain was 30% and 22% higher (P=0.02) in the cream compared to the hay group during periods 1 and 2, respectively. In the cream group during periods 1 and 2, the mixture of milk replacer and cream intake was 280 and 317 (DM, g/d), whereas the rolled maize intake was 80 and 241 (DM, g/d), respectively. The total dry matter intake (DMI, g/d) was significantly lower in the cream compared to the hay group (P<0.001). However, there was a period effect on the DMI for both of the groups, resulting in a greater intake (P<0.001) during period 2. The neutral detergent fibre (NDF, g/d) intake was extremely low, and the total dietary fat intake was much higher (P<0.001) in the cream compared to the hay group. The digestible energy intake (DEI, MJ/d) during period 1 was different between the groups (P=0.016). However, no difference (P>0.05) was found in DEI (MJ/d) between the groups during period 2, although there was a tremendous increase compared to the amount that was observed during period 1 (Table 2).

3.2. Methane production

The CH_4 output (g/d) was significantly (P<0.001) lower in the cream compared to the hay group (Table 3). Feeding milk replacer and cream resulted in 84% less CH_4 output compared to the hay group (P<0.001) during period 1. The reduction in the same group was greater (87%) during the 2nd period (P<0.001). However, the CH_4 (g/d) yield in each group was not different between periods 1 and 2.

The CH_4 release (g/kg WG) was significantly lower in the cream group (P<0.001) compared to that of the hay group between the periods. The same results were observed (P<0.001) for CH_4 expressed in terms of dry matter intake (DMI, g/d) and digestible energy intake (DEI, MJ/d) (Table 3). A regression analysis of CH_4 output (g/d) according to the body weight (kg) explained this difference more clearly (Fig. 2). This figure illustrates an increasing trend of CH_4 (g/d) in the hay group with increased BW over time. However, in the cream group, the CH_4 (g/d) yield was almost steady across time, with increased body weight.

Please cite this article in press as: Haque, M.N., et al., Development of methane emission from lambs fed milk replacer and cream for a prolonged period. Anim. Feed Sci. Tech. (2014), http://dx.doi.org/10.1016/j.anifeedsci.2014.09.002

Table 3
Least square means (LSM) of the CH_4 production from the lambs (n = 18) fed a hay- or cream-based diet at two time points during periods 1 and 2.

Parameters	Hay		Cream		RSE	R^2	Significance
	Period 1	Period 2	Period 1	Period 2			
CH_4 (g/d)	19.9^{dc}	19.1^{c}	3.2^{ab}	2.4^{a}	2.39	0.96	<0.001
CH_4 (g/kg WG)	116.3^{c}	113.9^{c}	11.5^{ab}	9.1^{a}	14.05	0.95	<0.001
CH_4 (g/kg DMI)	31.1^{c}	34.3^{cd}	4.3^{ab}	1.1^{a}	4.02	0.93	<0.001
CH_4 (g/MJ DEI)	2.5^{c}	2.6^{cd}	0.4^{ab}	0.2^{a}	0.29	0.94	<0.001

WG, weight gain; DMI, dry matter intake; MJ, megajoule; DEI, digestible energy intake; RSE, residual standard error; R^2, goodness of fit of the linear model; Significance indicates the model P value; ^{abcd} superscripts indicate differences (P<0.05) followed by multiple comparison within group and between the same period across the groups.

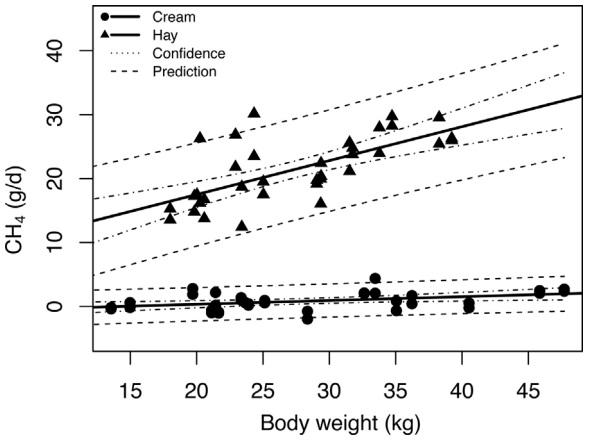


Fig. 2. Daily CH_4 production of individual lambs (n = 18) as affected by the body weight (kg) in the cream and hay group during periods 1 and 2.

The CH_4 output (g/d) was positively correlated (r = 0.74 and 0.71) with the digestible energy intake (DEI, MJ/d) in both of the groups during periods 1 and 2 (Fig. 3a). In case of cream group the correlation between CH_4 release (g/d) and DEI (MJ/d) was negative (r = -0.12 and -0.32, respectively) during periods 1 and 2. The CH_4 emission (g/MJ DEI) was very different in the two groups (P<0.001), even though a similar CH_4 (g/MJ DEI) output was observed in the cream group during periods 1 and 2. The DMI (g/d) was strongly correlated (r = 0.73 and 0.72) with the CH_4 (g/d) in the hay group during periods 1 and 2 (Fig. 3b). However, a negative correlation (r = -0.25 and -0.58) was observed between CH_4 (g/d) and DMI (g/d) in the cream group during period 2. This result indicates that the CH_4 (g/d) emission was steady in the cream group, although the DMI increased over time.

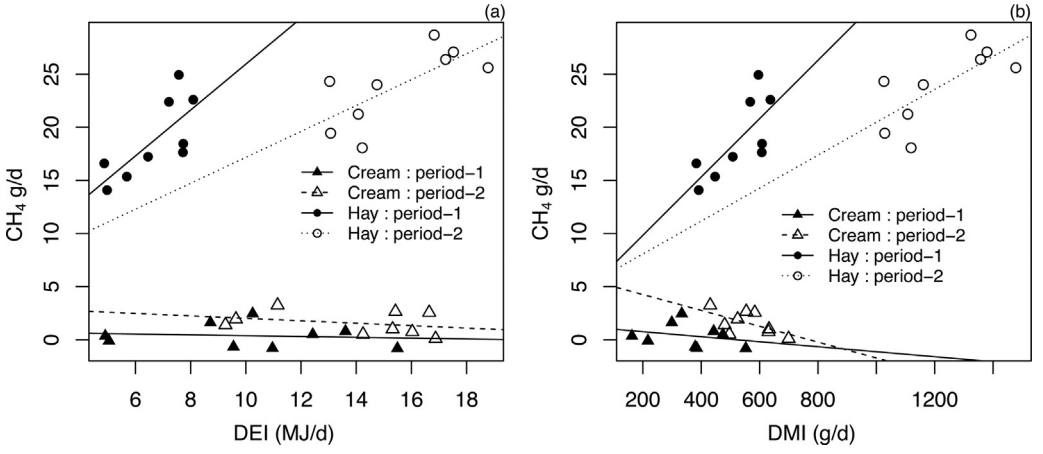


Fig. 3. Methane production (g/d) of individual lambs (n = 18) in relation to the digestible energy intake (MJ/d) and DMI (g/d) in two groups during periods 1 and 2.

Please cite this article in press as: Haque, M.N., et al., Development of methane emission from lambs fed milk replacer and cream for a prolonged period. Anim. Feed Sci. Tech. (2014), http://dx.doi.org/10.1016/j.anifeedsci.2014.09.002

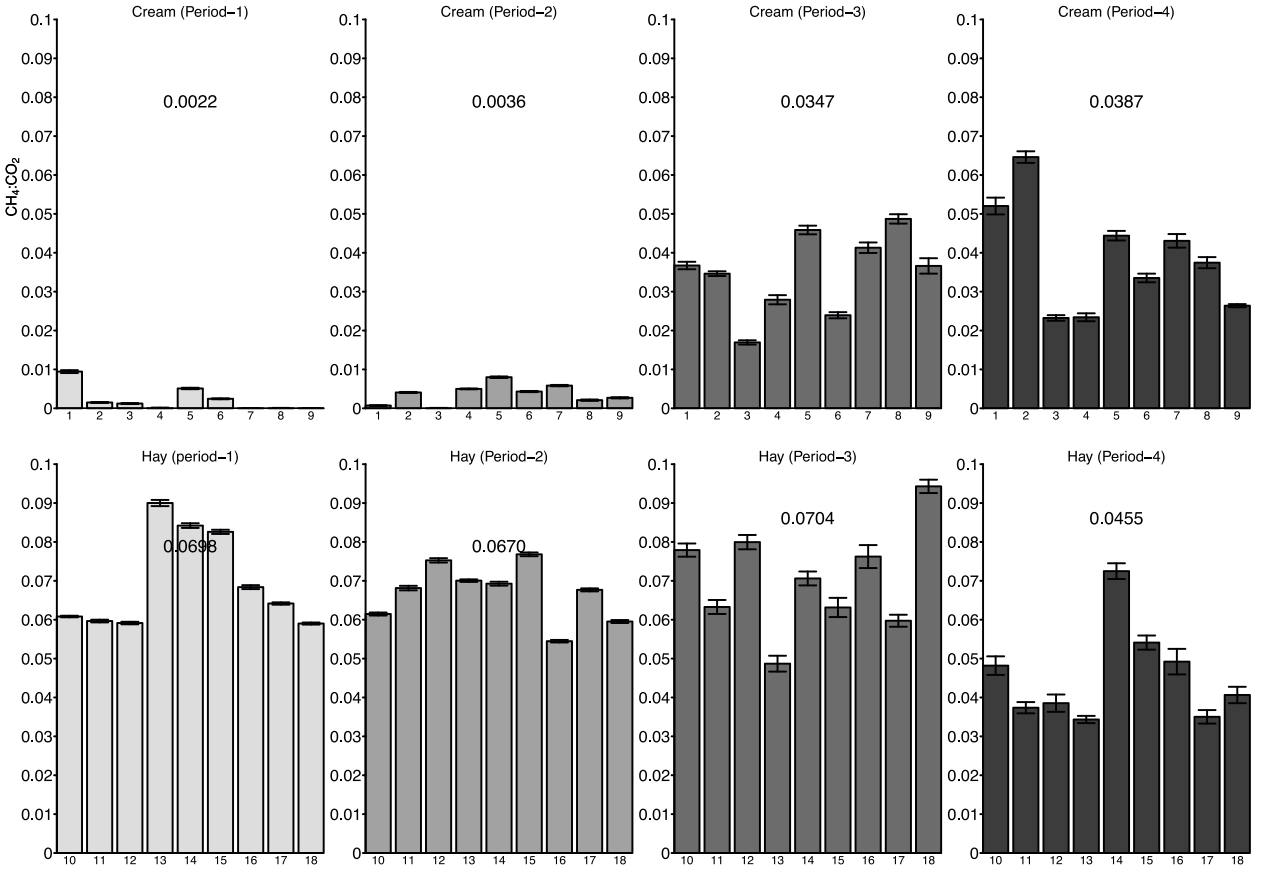


Fig. 4. The CH_4:CO_2 ratio of the lambs (n = 18) in two groups during periods 1, 2, 3 and 4 at the age of 90, 150, 185 and 235 days, respectively. The error bar indicates the standard error of the mean (SE). The values above the bars indicate the average per period.

3.3. CH_4 and CO_2 ratio (CH_4:CO_2) after changing the diet

An extremely quick response was observed in the CH_4 emission change in the 180-day-old cream-fed lambs after the lambs were transferred to a normal hay diet. Within four days of changing the diet (period 3), the CH_4:CO_2 ratio of the ex-cream-fed lambs was 0.035 ± 0.0011 (mean ± SE), which was much higher than 0.0022 ± 0.00036 (mean ± SE) in the same lambs during period 1 at 90 days of age ($P<0.001$). Fifty days after the diet alteration (period 4), the ratio increased ($P<0.001$) further to 0.039 ± 0.0015 (mean ± SE) compared to the ratio that was recorded during period 1 (Fig. 4). However, no difference in the CH_4:CO_2 ratio was found in the cream group between periods 1 and 2 ($P > 0.05$). Similar results of the CH_4:CO_2 ratios were also observed in the same group after the diet alteration during periods 3 and 4.

The CH_4:CO_2 ratios in the hay group were 0.069 ± 0.0013, 0.067 ± 0.0008, 0.070 ± 0.0014 and 0.046 ± 0.0013 (mean ± SE) in periods 1, 2, 3 and 4, respectively (Fig. 4). A multiple comparison of the CH_4:CO_2 ratio from the 4 measurement periods within the hay group showed no significant difference ($P>0.05$) between the ratios during periods 1, 2 and 3. However, the CH_4:CO_2 ratio in the same group was significantly lower ($P<0.001$) during period 4 compared to that during period 1. When comparing the CH_4:CO_2 ratio between the groups during periods 3 and 4, the cream group had a significantly lower CH_4:CO_2 ratio ($P<0.001$) compared to that of the hay group (Fig. 4).

3.4. Methanogens and morphology of rumen wall

The 16S rRNA gene was amplified with universal primers to study the archaeal community (further details in Section 2.3). The amplicon libraries were further processed using high-throughput sequencing and were quality trimmed according to the recommendations of the default settings of Qiime (Caporaso et al., 2010). After the basic data treatment, *de novo* OTU picking and taxonomy assignment, 4680 archaeal sequences (75 OTUs) were received for downstream analysis. The full data describing the bacteria will be presented in a separate publication. The majority of the generated sequences (3704) belong to the *Methanobacteriaceae* family, which includes most of the known methanogenic prokaryotes. In hay-fed lambs, the methanogens were composed of *Methanobrevibacter* (93.85%), *Methanosphaera* (1.5%), *Methanocorpusculum* (4.5%) and unassigned *Methanobacteriaceae* (0.15%) sequences. The cream-fed group was composed of only *Methanobrevibacter*. To

Please cite this article in press as: Haque, M.N., et al., Development of methane emission from lambs fed milk replacer and cream for a prolonged period. Anim. Feed Sci. Tech. (2014), http://dx.doi.org/10.1016/j.anifeedsci.2014.09.002

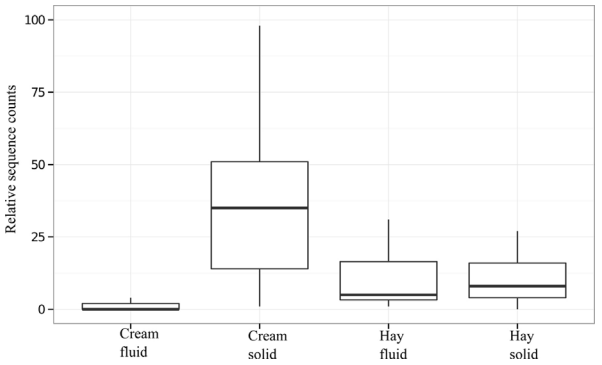


Fig. 5. Relative sequence counts of methanogens of the lambs (n = 26) in cream and hay groups. The ruminal solid and fluid phases of the diet groups are distinguished.

compare the relative abundances between both of the diet groups, the sequences were randomly and evenly subsampled, and the sequencing-induced differences were eliminated.

A small but significant increase in the methanogens count was observed in the solid phase of the rumen digesta in the cream compared to that in the hay-fed lambs (Wilcoxon-Rank-Sum test, P=0.036) (Fig. 5). In contrast, higher counts were found in the hay-fed lambs when looking at the ruminal fluid phase. The cream-fed lambs barely carried any methanogenic sequences in the rumen fluid (Wilcoxon Rank-Sum test P=0.013).

A visual inspection of rumen wall clearly demonstrate a poor development of rumen mucosa, with little papillary growth and light yellow colouration that were considered to be associated with a lack of microbial fermentation in the cream group (Fig. 6a). In contrast, in the hay group, excellent mucosal development and distinctive papillary growth were observed (Fig. 6b).

4. Discussion

4.1. Feed intake and daily weight gain

The diet of the cream group contained 50% dairy cream along with milk replacer and produced a very high total fat content. In addition, rolled maize is rich in starch and is a source of readily fermentable carbohydrate. These two components produced a diet with a high energy concentration and reduced the DMI (g/d) in cream group. Haddad and Younis (2004) reported a reduced DMI in lambs with 5% fat added to a high concentrate fattening diet. Similar results have been declared in lambs from 15 to 180 days by supplementing 25–75 g (per kg of concentrate) of coconut oil (Bhatt et al., 2011). Moreover, Machmuller and Kreuzer (1999) described that the DMI was not affected by feeding a ration with 30–35 g/kg coconut oil, whereas feeding 70 g/kg coconut oil reduced the DMI in adult sheep. The diet in this study was completely different from those that were mentioned earlier, but the markedly lower DMI in the cream group was due to a higher energy content in the diet. Although the DMI was lower in the cream group, the total digestible energy intake (MJ/d) was very similar in the cream and hay group, indicating that the reduction in the DMI was due to the higher digestible energy content in the milk replacer and cream diet. This result confirms that ruminants can be reared on a diet of milk and other liquids for a longer period without an adverse effect on the daily DE intake and the occurrence of digestive disorders. It should be noted that a

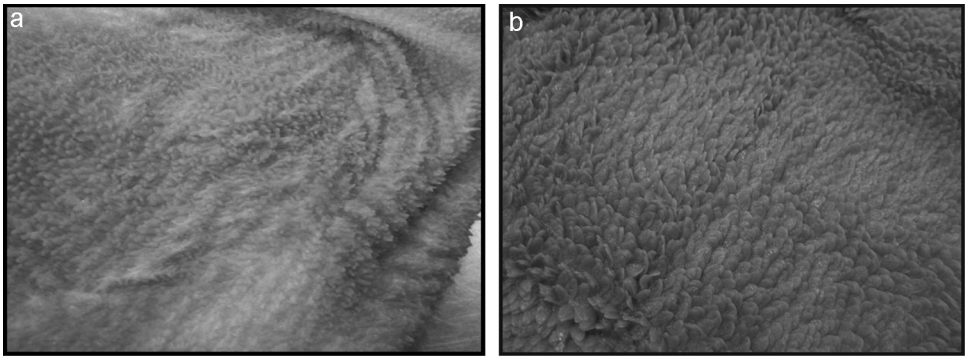


Fig. 6. (a) Photograph of the rumen wall from a lamb from the cream group at 180 days of age showing a poor development of rumen mucosa, with little papillary growth and light yellow colouration associated with a lack of microbial fermentation in the rumen. (b). Photograph of the rumen wall from a lamb from the hay group at 180 days of age showing excellent mucosal development with dark colouration due to microbial fermentation in the rumen.

Please cite this article in press as: Haque, M.N., et al., Development of methane emission from lambs fed milk replacer and cream for a prolonged period. Anim. Feed Sci. Tech. (2014), http://dx.doi.org/10.1016/j.anifeedsci.2014.09.002

negligible amount of barley straw was provided to the lambs that were fed the liquid diet to avoid digestive disturbances. The lambs in the cream group were fed the milk replacer and cream diet from birth. Therefore, these lambs were considered well adapted to the diet. The rate of daily weight gain (g/d) was higher in the cream group presumably reflecting better energy utilisation compared to that of the hay group. This difference in the daily weight gain between two groups might have caused by the moderate growth rate in the hay at a restricted feed intake. However, the gross body weight was not different between the groups presumably because of the low protein content, which restricted their lean growth to some extent.

4.2. Breath sampling and methane estimation

The estimation of CH_4 by the CO_2-method makes it possible to conduct measurements while keeping the animals in their natural environment (Madsen et al., 2010). However, in this study, we slightly modified the measurement technique. The individual pens were covered with transparent Plexiglas to restrict air movement, but the normal behaviour and movements of the lambs were confirmed by periodic inspection throughout the entire duration of the experiment. Therefore, the breath samples are assumed to be from the normal metabolism of the lambs. Another important aspect should be considered is the length of the measurement because the concentrations of the breaths are not always constant. During the development phase of the CO_2 method, Madsen et al. (2010) found that approx. 2–3% of the breath sample is enough to estimate CH_4 with the CO_2 method. Another study of dairy cows demonstrated that 2–3 days of measurement in the automatic milking system (AMS) is enough for CH_4 estimation (Lassen et al., 2012). In this study, we used 8 h of measurement during the first two periods and 30 min during periods 3 and 4. The length of the measurements is longer than in the previous study of Haque et al. (2014), who measured dairy cows for 5 days during milking in AMS with an average of 6 min, and the number of visits to the AMS was 2.6 per day, producing a measurement time of approx. 15.6 min per day per animal, which is less than the 30 min in this study during periods 3 and 4. Moreover, the variation in the concentration of CH_4 and CO_2 as measured in the pens is much less compared to those in the AMS (Madsen and Bertelsen, 2012). Therefore, the shorter measurements during the last two periods are considered reasonable.

4.3. Long-term dietary impact on methane emission

Highly significant differences in CH_4 production (g/d) were observed between the two experimental groups. It was expected that the CH_4 emission from the cream lambs would be markedly lower than that of the hay fed lambs because of the extremely high level of total fat and almost no fibre in the cream diet. Moreover, it can be assumed that the continuous feeding of milk and cream in liquid form may have influenced the significantly lower CH_4 release in cream group. The very different feeding in the cream group up to 180 days of age probably retarded the normal development of the rumen, and very negligible microbial fermentation occurred in lambs as evidenced by the extremely low CH_4 (g/d) emission. Yurtseven and Ozturk (2009) found that feeding a corn-based diet to adult sheep greatly increased the proportion of propionate, whereas the proportion of acetate decreased. Feeding milk replacer and cream along with rolled maize in this study may have enhanced the proportion of propionic acid production by reducing the proportion of acetate, thereby reducing the levels of CH_4 output. Several studies of adult sheep and lambs have reported remarkable reductions of up to 73% in the CH_4 emissions by supplementing coconut oil (Machmuller and Kreuzer, 1999; Machmuller et al., 2000). In this study, the CH_4 release was 84–87% lower in the cream group, which is fairly consistent with the results of (Machmuller and Kreuzer, 1999), who reported a 73% reduction by the addition of 3.5–7% coconut oil to the diet. The same author in another study reported a 43–57% CH_4 reduction by supplementing 3 and 6% coconut oil (Machmuller, 2006). Although a different fat source was used in this study, cream is also characterised by high levels of medium-chain fatty acids, similar to coconut oil products. In fact, coconut oil is one of the only other fat sources except for milk fat from ruminants that contains very large amounts of medium-chain (C_{12}:C_{16}) fatty acids. Furthermore, Czerkawski et al. (1966) found that a momentarily high concentration of fatty acid in the rumen fluid might be more important to suppress CH_4 release. In this study, the remarkable CH_4 suppression in the cream group was probably linked to an accumulation of medium-chain saturated fatty acids in the rumen fluid, which reduced the supply of organic matter and had inhibitory effects against the methanogens. This result is also supported by the result of the microbial analysis in this study, in which almost no methanogens were found in the fluid portion in the cream-fed group (Fig. 5). Moreover, very little solid contents were found in the cream group compared to the hay group, indicating that the total abundance of methanogens in the cream group was lower.

4.4. Rumen morphology and ruminal microbial biodiversity

The rumen wall was collected at slaughter. No histomorphological measurement was performed. Photographs of the rumen wall (Fig. 6) clearly demonstrate the visible differences in the morphological structure. It is possible that the limited roughage diet and supply of liquid milk in the cream group for a prolonged period suppressed the morphological development of the rumen wall, the growth of rumen papillae and, more importantly, the growth of the rumen microbial population. The same argument was mentioned by Jasper and Weary (2002) in dairy calves. Similarly, Baldwin et al. (2004) found that fibrous feed consumption and fermentation are crucial for rumen development and for the establishment of rumen microbes. In this study, although the cream group received rolled maize and a small amount of barley straw, the ingested amount was

Please cite this article in press as: Haque, M.N., et al., Development of methane emission from lambs fed milk replacer and cream for a prolonged period. Anim. Feed Sci. Tech. (2014), http://dx.doi.org/10.1016/j.anifeedsci.2014.09.002

too low to initiate rumen development. In the hay group, the proper development of the rumen was obvious because of the presence of a sufficient amount fibre in the diet. Similarly, Khan et al. (2011) reported that the provision of hay to calves promotes rumen development.

Methanogenic archaea do not directly assimilate cellulose and are dependent of the fibrolytic bacteria, which provide carbon dioxide and hydrogen during fermentation (Liu and Whitman, 2008). Previous studies have demonstrated that fatty acids supplied to the diet are toxic for fibrolytic bacteria and protozoa and consequently resulted in CH_4 depletion (Machmuller et al., 2003; Hook et al., 2010). Thus, it was expected that the relative numbers of methanogens would significantly be reduced in the cream-fed group. The results suggest that the extreme cream diet feeding did not eliminate the archaea but rather reduced the diversity of the methanogens to the genus *Methanobrevibacter*. The methanogens appeared to be adapted to the solid substrate in the cream group. A significant shift of the methanogens from the ruminal fluid to the solid material was observed in the cream group compared to the hay group, indicating an adaptation of the microbial community to the dietary conditions. The rolled maize that was added to sustain crucial microbial metabolic activities in the rumen system of the cream group certainly enhanced the possibility of survival of the methanogenic archaea in the solid phase of the rumen contents. However, the level of CH_4 release decreased probably due to the increased production of propionate as described by Yurtseven and Ozturk (2009), suggesting possible interactions of the methanogens with other microorganisms in the solid rumen material and their adaptation to the diet, which needs to be confirmed by future studies.

4.5. Response of lambs to the fibrous feed

The intense response of the CH_4:CO_2 ratio in the cream group within 4 days of changing the diet indicates that feeding a fibrous diet would quickly initiate the rumen fermentation, which appears to be independent of the feeding regime earlier in life. The significantly lower CH_4:CO_2 ratio in the cream group 50 days after the diet alteration attributes an important assumption, *i.e.*, although the cream group responded very quickly to the fibrous diet, we assume that artificially reared ruminants will take longer to emit an equal amount gas (CH_4 and CO_2) as from the ruminants reared under normal feeding regime. The effect of artificial feeding would last for an extended period, presumably because of the possibility of residual effects. Therefore, the result in the ex-cream-fed group does not necessarily imply that the CH_4 suppressing effect of an extreme diet is transitory. Further studies are required to confirm this hypothesis. It should be acknowledged that the CH_4:CO_2 ratio in the hay group during period 4 was slightly lower, which might not be a representative gas emission from normal metabolism, presumably because the lambs were free in the barn, and some handling was involved to get the lambs into the measurement pens. In addition, a 30-min measurement time was followed during this period. The situation again demonstrates that to obtain a normal breath sample for accurate CH_4 estimation, it is important to maintain the animals in their normal movement.

5. Conclusions

The artificial rearing of lambs with milk replacer and cream nearly prevented CH_4 release. The abundance of rumen methanogens was lower in the fluid portion of the cream group, and the rumen archaea were mostly adapted in the solid phase of the rumen content. Switching from milk replacer and cream to a fibrous diet dramatically changed the fermentation pattern and, consequently, the CH_4:CO_2 ratio in the cream group within 4 days. The CH_4:CO_2 ratio remained lower for 50 days after the diet alteration. Feeding milk replacer and cream to the lambs up to 180 days reduced the CH_4 emissions for an extended period. However, it cannot be excluded that the effect is long-lasting.

Conflict of interest

None.

Acknowledgements

The authors sincerely acknowledge all of the members of the project “Impact of pre- and postnatal dietary interactions on postnatal metabolic and endocrine function” for their support of this study.

References

Baldwin, R.L., McLeod, K.R., Klotz, J.L., Heitmann, R.N., 2004. Rumen development. Intestinal growth and hepatic metabolism in the pre- and postweaning ruminant. J. Dairy Sci. 87, E55–E65.

Bhatt, R.S., Soren, N.M., Tripathi, M.K., Karim, S.A., 2011. Effects of different levels of coconut oil supplementation on performance, digestibility, rumen fermentation and carcass traits of Malpura lambs. Anim. Feed Sci. Technol. 164, 29–37.

Caporaso, J.G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F.D., Costello, E.K., Fierer, N., Pena, A.G., Goodrich, J.K., Gordon, J.I., Huttley, G.A., Kelley, S.T., Knights, D., Koenig, J.E., Ley, R.E., Lozupone, C.A., McDonald, D., Muegge, B.D., Pirrung, M., Reeder, J., Sevinsky, J.R., Turnbaugh, P.J., Walters, W.A., Widmann, J., Yatsunenko, T., Zaneveld, J., Knight, R., 2010. QIIME allows analysis of high-throughput community sequencing data. Nat. Methods 7, 335–336.

CIGR, 2002. Climatization of animal houses – heat and moisture production at animal and house level. In: Pedersen, S., Sällvik, K. (Eds.), 4th Report of CIGR Working Group. Horsens, Denmark, Research Centre Bygholm, Danish Institute of Agricultural Sciences, P.O. Box 536, DK-8700 Horsens, Denmark.

Please cite this article in press as: Haque, M.N., et al., Development of methane emission from lambs fed milk replacer and cream for a prolonged period. Anim. Feed Sci. Tech. (2014), http://dx.doi.org/10.1016/j.anifeedsci.2014.09.002

Czerkawski, J.W., Blaxter, K.L., Wainman, F.W., 1966. The effect of linseed oil and of linseed oil fatty acids incorporated in the diet on the metabolism of sheep. Br. J. Nutr. 20, 485–494.
Eadie, J.M., 1962. The development of rumen microbial populations in lambs and calves under various conditions of management. J. Gen. Microbiol. 29, 563–578.
Edgar, R.C., Haas, B.J., Clemente, J.C., Quince, C., Knight, R., 2011. UCHIME improves sensitivity and speed of chimera detection. Bioinformatics 27, 2194–2200.
EU, 2009. Determination of crude ash. Off. J. Eur. Union 152, 50–52 (Commission Regulation).
Gasmet™, 2010. Gasmet Technologies Oy Pulttitie 8 A, FI-00880 Helsinki, Finland, http://www.gasmet.com/products/ftir_gas_analyzers/on-site_%28portable%29/gasmet_dx4030 (accessed 17.04.13).
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), Rome, Italy.
Haddad, S.G., Younis, H.M., 2004. The effect of adding ruminally protected fat in fattening diets on nutrient intake, digestibility and growth performance of Awassi lambs. Anim. Feed Sci. Technol. 113, 61–69.
Haque, M.N., Cornou, C., Madsen, J., 2014. Estimation of methane emission using the CO_2 method from dairy cows fed concentrate with different carbohydrate compositions in automatic milking system. Livest. Sci. 164, 57–66.
Hook, S.E., Wright, A.D., McBride, B.W., 2010. Methanogens: methane producers of the rumen and mitigation strategies. Archaea 2010, 1–11.
Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biom. J. 50, 346–363.
ISO-11085, 2008. Animal Feeding Stuffs – Determination of Crude Fat and Total Fat Content by the Randall Extraction Method. ISO-11085. International Organization for Standardization, Geneva, Switzerland.
Jasper, J., Weary, D.M., 2002. Effects of ad libitum milk intake on dairy calves. J. Dairy Sci. 85, 3054–3058.
Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. J. Anim. Sci. 73, 2483–2492.
Khan, M.A., Lee, H.J., Lee, W.S., Kim, H.S., Ki, K.S., Hur, T.Y., Suh, G.H., Kang, S.J., Choi, Y.J., 2007. Structural growth, rumen development, and metabolic and immune responses of Holstein male calves fed milk through step-down and conventional methods. J. Dairy Sci. 90, 3376–3387.
Khan, M.A., Weary, D.M., von Keyserlingk, M.A., 2011. Hay intake improves performance and rumen development of calves fed higher quantities of milk. J. Dairy Sci. 94, 3547–3553.
Lassen, J., Lovendahl, P., Madsen, J., 2012. Accuracy of noninvasive breath methane measurements using Fourier transform infrared methods on individual cows. J. Dairy Sci. 95, 890–898.
Licitra, G., Hernandez, T.M., VanSoest, P.J., 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Technol. 57, 347–358.
Liu, Y., Whitman, W.B., 2008. Metabolic, phylogenetic, and ecological diversity of the methanogenic archaea. Ann. N. Y. Acad. Sci. 1125, 171–189.
Liu, Z., DeSantis, T.Z., Andersen, G.L., Knight, R., 2008. Accurate taxonomy assignments from 16S rRNA sequences produced by highly parallel pyrosequencers. Nucleic Acids Res. 36, e120.
Machmuller, A., 2006. Medium-chain fatty acids and their potential to reduce methanogenesis in domestic ruminants. Agric. Ecosyst. Environ. 112, 107–114.
Machmuller, A., Kreuzer, M., 1999. Methane suppression by coconut oil and associated effects on nutrient and energy balance in sheep. Can. J. Anim. Sci. 79, 65–72.
Machmuller, A., Ossowski, D.A., Kreuzer, M., 2000. Comparative evaluation of the effects of coconut oil, oilseeds and crystalline fat on methane release, digestion and energy balance in lambs. Anim. Feed Sci. Technol. 85, 41–60.
Machmuller, A., Soliva, C.R., Kreuzer, M., 2003. Effect of coconut oil and defaunation treatment on methanogenesis in sheep. Reprod. Nutr. Dev. 43, 41–55.
Madsen, J., Bertelsen, M.F., 2012. Methane production by red-necked wallabies (*Macropus rufogriseus*). J. Anim. Sci. 90, 1364–1370.
Madsen, J., Bjerg, B.S., Hvelplund, T., Weisbjerg, M.R., Lund, P., 2010. Methane and carbon dioxide ratio in excreted air for quantification of the methane production from ruminants. Livest. Sci. 129, 223–227.
Møller, J., Thøgersen, R., Kjeldsen, A.M., Weisbjerg, M.R., Søegaard, K., Hvelplund, T., Børsting, C.F., 2000. Feedstuff Table, Composition and Feeding Value of Feedstuffs for Cattle. Report No. 91, The Danish Agricultural Advisory Centre., pp. 5–10.
Neefs, J.M., Van de Peer, Y., De Rijk, P., Goris, A., De Wachter, R., 1991. Compilation of small ribosomal subunit RNA sequences. Nucleic Acids Res. 19 (Suppl.), 1987–2015.
NRC, 2001. Nutrient Requirements of Dairy Cattle, Seventh Revised Ed. The National Academies Press, Washington, DC.
NRC, 2007. Nutrient Requirements for Small Ruminants. The National Academies Press, Washington, DC.
Pedersen, S., Blanes-Vidal, V., Joergensen, H., Chwalibog, A., Haeussermann, A., Heetkamp, M.J.W., Aarnink, A.J.A., 2008. Carbon dioxide production in animal houses: a literature review. Agric. Eng. Int.: CIGR J. 8, 8.
R Development Core Team, 2013. R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Vienna, Austria, R version 3.0.0. http://www.R-project.org
Russell, V.L., 2013. Least-Squares Means. R-Package, vol. 14, http://cran.r-project.org/web/packages/lsmeans/ (accessed 05.13).
Smith, R.H., 1959. The development and function of the rumen in milk-fed calves. J. Agric. Sci. 52, 72–78.
TeamViewer©, 2013. Kuhnbergstr, 16 73037 Göppingen, Germany, http://www.teamviewer.com/en/news/343-New-version-7-for-Windows-7.0.12979-and-Linux-7.0.9348-released.aspx (accessed 17.04.13).
Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and nonstarch polysaccharides in relation to animal nutrition. J. Dairy Sci. 74, 3583–3597.
Wardrop, I.D., 1960. The post-natal growth of the visceral organs of the lamb: Part II. The effect of diet on growth rate, with particular reference to the parts of the alimentary tract. J. Agric. Sci. 55, 127–132.
Wardrop, I.D., Coombe, J.B., 1960. The post-natal growth of the visceral organs of the lamb: I. The growth of the visceral organs of the grazing lamb from birth to sixteen weeks of age. J. Agric. Sci. 54, 140–143.
Yu, Y., Lee, C., Kim, J., Hwang, S., 2005. Group-specific primer and probe sets to detect methanogenic communities using quantitative real-time polymerase chain reaction. Biotechnol. Bioeng. 89, 670–679.
Yurtseven, S., Ozturk, I., 2009. Influence of two sources of cereals (corn or barley), in free choice feeding on diet selection, milk production indices and gaseous products (CH_4 and CO_2) in lactating sheep. Asian J. Anim. Vet. Adv. 4, 76–85.

Please cite this article in press as: Haque, M.N., et al., Development of methane emission from lambs fed milk replacer and cream for a prolonged period. Anim. Feed Sci. Tech. (2014), http://dx.doi.org/10.1016/j.anifeedsci.2014.09.002

Paper II

Estimation of methane emission using the CO_2 method from dairy cows fed concentrate with different carbohydrate compositions in automatic milking system

Article published in Livestock Science.

Livestock Science 164 (2014) 57–66

Contents lists available at ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

Estimation of methane emission using the CO_2 method from dairy cows fed concentrate with different carbohydrate compositions in automatic milking system

M.N. Haque *, C. Cornou, J. Madsen **

Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Groennegaardsvej 2, DK-1870 Frederiksberg C, Denmark

ARTICLE INFO

Article history:
Received 24 July 2013
Received in revised form
4 March 2014
Accepted 6 March 2014

Keywords:
Breath
CO_2-method
Methane
Production
Total mixed ration

ABSTRACT

Two concentrates (MELK and VEM) with two different carbohydrate compositions were supplemented during milking in an Automatic Milking System (AMS). The objectives of this study were to estimate the effect of the concentrates on CH_4 emission from dairy cows and to investigate the precision of the CO_2-method when measuring in an AMS for different length of time. Holstein cows ($n=36$) were used with mean body weight of 660 kg (SD=75.13) and average milk production of 31.7 kg (SD=8.98), mixed parity and mixed lactation. Cows were allocated in two groups ($n=18$). After an adaptation period (period 1), each group received either 100% MELK (More Energy Lactating Cows; a newly introduced feeding system) or 100% VEM (Feed Value System for milk production) during periods 2 and 3. Besides, both groups were fed the same Total Mixed Ration (TMR) *ad libitum* in the stable. Air samples in the AMS from a point near the cows head were analysed every 20 s using the Gasmet equipment based on Fourier Transform Infrared (FTIR) Spectroscopy Technique. The equipment ran continuously for 15 days over the three measurement periods (5 days × 3 periods) with a 14 days waiting time in between the periods. Individual records of the CH_4 and CO_2 concentrations in the cows breath was calculated after subtracting the CH_4 and CO_2 concentrations in the stable air from the measured concentrations. The CH_4:CO_2 ratio was then multiplied with the calculated total CO_2 production by the individual cows to get the quantitative CH_4 production. Milk production and total dry matter intake (DMI, kg/day) were very similar in the two groups. The supplemented concentrate was allocated according to the individual milk yield and the intake ranged from 1.60 to 7.30 kg/day in MELK cows and from 2.06 to 7.20 kg/day in VEM cows. No significant difference was found for CH_4 production in MELK and VEM groups over the three periods. A linear positive relation between the CH_4 (g/day) and energy corrected milk (ECM, kg/day) production and the feed intake (DMI, kg/day) was observed for the entire period. The calculated CO_2 and CH_4 production were very similar in the two groups throughout the entire measurement period. The analysis of the precision of the CO_2-method, using a 95% significance level, indicated that showing a difference of 9 or 5% in methane production requires a measuring period of 5 or 15 days, respectively, when using 18 cows per group. The study shows no effect of a limited change in supplementation of starch and sugar on CH_4 production through feeding concentrates MELK or VEM in the AMS. To obtain an effect of changing the

* Corresponding author. Tel.: +45 35 33 28 68.
** Corresponding author. Tel.: +45 35 33 30 92.
E-mail addresses: naha@sund.ku.dk (M.N. Haque), jom@sund.ku.dk (J. Madsen).

http://dx.doi.org/10.1016/j.livsci.2014.03.004

carbohydrate composition of the diet on the CH_4 production, it is likely that a larger change in the diet is necessary. This can only efficiently be done by changing the TMR part of the diet.

1. Introduction

Methane emissions by the ruminant animals are not only an environmental hazard but represent also a loss of energy from the animal. Globally, 287 Mt of CH_4 are annually released from anthropogenic sources, about 50% of which is from agriculture, and the largest biogenic source of CH_4 is enteric fermentation from ruminant livestock (EPA, 2008). Methane is a natural end product of rumen fermentation. It arises as a result of anaerobic digestion of feed and the removal of hydrogen from the rumen by methanogens. Methane emission from ruminant depends on the diet composition and quantity of feed consumed (Johnson and Johnson, 1995). In dairy cows, the CH_4 energy loss (% of gross energy intake) is about 5.3–6.1% (Benchaar et al., 2013; Hassanat et al., 2013). Efficient dairy production is characterized by high milk production per cow and aims at efficient conversion of feed energy and nutrients to human-edible food, such as milk and meat. This includes efforts to reduce the loss of energy by CH_4 release. Recently, several authors have reviewed a number of strategies to mitigate enteric CH_4 production such as the use of nutritional strategies and genetic modifications in order to change the rumen microbial biodiversity (Martin et al., 2008; Beauchemin et al., 2009; Gerber et al., 2013). At the time being, it seems that the most promising approach for reducing methane emissions from ruminants is by improving productivity and efficiency through better nutritional management (Steinfeld et al., 2006; Gerber et al., 2013). Among nutritional strategies, high concentrate and lipid supplementation is considered most effective in lowering CH_4 production per unit of energy intake (Johannes et al., 2011). The biological mechanism is to shift rumen fermentation towards propionogenesis, whereas fibrous diets result in a preferential production of acetate, butyrate, and CH_4. Feeding of high yielding dairy cows aiming at maximizing milk production often results in high-starch diet. Rapidly degradable starch supplementation leads to a drop in acetate-to-propionate ratio with an ultimate result of reduced methane (Plaizier et al., 2008). Several studies have investigated the effect of different sources of carbohydrate on methane emission (Hristov et al., 2013), but few have examined the possible effect of changing the composition of concentrate allocated to cows in the Automatic Milking System (AMS) where only limited amount of concentrate can be fed. It is hypothesized that feeding starch rich concentrate in the AMS would be effective to reduce methane. A new feed planning system for dairy cows (MELK) has been developed in The Netherlands to substitute the old system (VEM). The new system should favour propionate production, thereby reducing the methane production in the rumen. In addition, there are now a number of methods in focus to estimate the CH_4 production from ruminants e.g. traditional respiration chamber method (Blaxter and Clappert, 1965) and SF6 tracer technique (Johnson et al., 1994). These methods have different pros and cons. Some methods, for instance respiration chamber, can measure with high accuracy but only on few animals and not in their natural environment. Other methods as the CO_2-method newly developed by Madsen et al. (2010) can measure on many animals and give the opportunity to evaluate the variation between animals and differences between diets in practice with a reasonable precision. The CO_2-method is a pertinent technique for measuring on many animals and evaluating differences between feeds. Therefore, the objectives of this study are (i) to investigate the effect of changing the starch, sugar and fibre content on methane emission and milk production using two concentrates MELK and VEM fed in an AMS and (ii) to investigate the precision of the CO_2-method when measuring in an AMS for different lengths of time.

2. Materials and methods

2.1. Experimental design, animals and housing

Holstein-Friesian dairy cows ($n=36$) with an average body weight of 660 (SD=75.13) kg and an average milk yield of 31.7 (SD=8.98) kg/day were selected from a private dairy farm (Dalfsen, The Netherlands) for this study. The cows were initially selected as 18 pairs based on age, parity, daily milk production, body condition and average methane excretion per day. Cows from each pair were randomly assigned into two groups. During a first period, each group received 50% of each concentrate. From day 18th, one group was fed MELK concentrate; the other was fed VEM concentrate. Animals were housed in a closed housing system fitted with one AMS.

2.2. Diets, experimental period and feeding

Both groups were fed the same Total Mixed Ration (TMR) *ad libitum* in the stable and two different concentrate mixtures in AMS named MELK and VEM (Tables 1 and 2). MELK stands for “More Energy Lactating Cows” and is a new feeding system for high yielding dairy cow in the Netherlands. VEM is the “Feed Value System”, considered to be a traditional feeding system which has been used in the Netherlands in the last few decades. TMR was supplied to all cows in the stable two times a day. The concentrates MELK and VEM were automatically supplied during the milking in AMS. The amount of fed concentrates was based on the daily milk yield of individual cows controlled by “The dynamic feeding system” developed by Agrovision©. The experiment was divided into three periods, each of 5 days duration, with 14 days waiting time in

Table 1
Ingredients of Total Mixed Ration (TMR) and the concentrates MELK and VEM.

TMR (% of dry matter)		Concentrates (% of DM)		
Ingredients		Ingredients	MELK	VEM
Grass silage	43.02	Sugar beet pulp	–	31.4
Maize silage	39.85	Palm kernel expeller	–	17.4
Grass seed hay	2.80	Citrus molasses	10.0	10.0
Brewery grain	7.30	Rapeseed expeller	–	9.1
Unimix 922[a]	6.64	Soya hulls	14.8	8.1
Univit Mobiel[b]	0.26	Wheat	6.3	5.7
Calcium carbonate	0.13	Rapeseed meal	16.1	5.1
		Cane molasses	10.0	5.0
		Rapeseed meal	9.0	3.5
		Soya meal	–	2.6
		Premix	2.3	1.6
		Urea	0.3	0.3
		Vegetable oil	0.2	0.2
		Maize	31.1	–

[a] Rapeseed meal and soya meal (1/2% each).
[b] Minerals and vitamins mix.

between each period. During period 1, all cows were supplied both of the concentrates MELK and VEM (50% of each), whereas in periods 2 and 3, 100% MELK or VEM were allocated separately according to the groups.

2.3. Gas measurement

Methane and CO_2 from the cows were analysed using a continuous gas analyser Gasmet DX-4030 (Gasmet™, 2010) based on Fourier Transformed Infrared Radiation. Three days prior to each measurement period, Gasmet was installed to the Delaval AMS to ensure the correctness of measurements. The inlet filter of the Gasmet was fitted on the feeding pen of AMS in order to get concentrated breath sample from cows. The breath sample passes through the filter and thereafter through Gasmet analyser to determine the concentrations of CH_4 and CO_2. The measurements were performed every 20 s over 24 h for the entire 5 days experimental periods. The methane production for the individual cows was based on the methane–carbon dioxide ratio (CH_4:CO_2) when the specific cow was in the AMS. Each cow was visiting the AMS at least two times a day (2.6 times in average), with an average milking time of 6 min. The individual methane production was calculated based on 294 ± 106, 236 ± 74 and 266 ± 88 (mean $\pm$ SD) observations per cow during period 1, 2 and 3, respectively. Before the first measurement in each periods, Gasmet was calibrated with standard gas to check the accuracy of the measurement. During period 1, Gasmet was stopped for 10 min each day to get the stable concentration of CH_4 and CO_2. This concentration of CH_4 and CO_2 was subtracted from the measured concentration to get the real breath concentration of CH_4 and CO_2. Remote monitoring of the measurements was performed via internet using TeamViewer (TeamViewer©, 2013).

2.4. Sampling and analysis of feed samples

One sample of the TMR and of the concentrates was taken during each of the measurement periods. Immediately after collection, the samples were stored in a freezer. Before laboratory analysis, the three fractions of the same sample were mixed together to make a composite sample. All of the TMR samples were dried at 65 °C and the concentrates at 103 °C to determine the dry matter percentage. Crude fibre was determined according to EU (2009b) and crude ash at 550 °C according to EU (2009a). Neutral detergent fibre (NDF) was determined following ISO-16472 (2006) where heat stable amylase and ash correction were considered and EFOS through FO-19 (2005). Acid detergent fibre and acid detergent lignin was determined according to ISO-13906 (2008). Crude protein and rate of degradation of protein

Table 2
Chemical composition and nutritive values of TMR the concentrates MELK and VEM.

Chemical composition	TMR (% of dry matter)	Concentrates (% of dry matter)	
		MELK	VEM
Dry matter (% of fresh feed)	47.3	86.3	85.8
Crude protein	12.1	17	17.1
Crude fat	2.8	3.7	3.9
Crude fibre	25.3	11.2	14.4
Ash	6.2	6.5	6.9
Sugar	5.8	7.6	11.9
Starch	11.48	29.66	6.27
ADF	26	16	22
NDF	45.1	20.7	30.8
Lignine	2.4	3.2	3.9
Calcium	0.48	0.84	0.81
Phosphorus	0.31	0.39	0.37
Nutritive values			
EFOS (% of organic matter)	71.4	94	91.7
Buffer solubility (% of crude protein)	51.2	21.0	25.5
Digestible energy[a], MJ/kg DM	13.42	16.5	16.2
Metabolizable energy[b], MJ/kg DM	10.74	13.2	13.0
Scandinavian Feed Units[c], SFU/kg DM	0.87	1.22	1.19

ADF = acid detergent fibre.
NDF = Neutral detergent fibre.
DM = dry matter.
EFOS = enzyme solubility of organic matter.

[a] Digestible energy = 24.237 × digestible crude protein (kg/kg DM) + 34.116 × digestible crude fat (kg/kg DM) + 17.300 × digestible carbohydrate (kg/kg DM) − 0.766 × sugar (kg/kg DM).
where

Digestible organic matter for TMR (%) = 0.204 + 0.727 EFOS.
Digestible organic matter for concentrate (%) = 5.38 + 0.867 EFOS. (Weisbjerg et al., 2007).
Digestible crude protein (kg/kg DM) = (0.93 × % crude protein in DM − 3)/100.
Digestible crude fat (kg/kg DM) = (0.96 × % crude fat in DM − 1)/100.
Digestible carbohydrate (kg/kg DM)) = (% digestibility of organic matter/100) × (100 − % crude ash in DM)/100 − digestible crude protein − digestible crude fat.

[b] Metabolizable energy = Digestible energy × 0.80.
[c] Scandinavian Feed Units = −0.369 + 0.0989 × Digestible energy − 0.347crude fibre (kg/kg DM).

through buffer solubility test was determined according to Licitra et al. (1996). Crude fat was determined following ISO-11085 (2008) using petroleum ether. Enzymatic method (ISO-15914, 2004) was followed to determine the amount of starch whereas the titration method (EU, 2009c) was followed to determine the amount of sugar.

2.5. Calculations

The data for air composition was matched with the cow identification numbers and data for entrance and exit times of the individual cows into the AMS by using the time recorded in a computer connected to the AMS. All calculations regarding CH_4 and CO_2 emissions from cows were done according to the CO_2-method (Madsen et al., 2010). The stable concentrations of CO_2 (605 ± 88.3 ppm) and CH_4 (26 ± 10.3 ppm) (mean $\pm$ SD) obtained from period 1 were subtracted from the exhaled concentration of the cows to get the corrected breath concentration of each sample. After correction, all values of corrected CO_2 below 400 ppm were removed in order to avoid the influence of samples containing a very low concentration of breath. The ratio between CH_4 and CO_2 (CH_4:CO_2) was thereafter determined. This ratio represents an index of feed gross energy loss in CH_4 as well as a factor for quantifying CH_4 from the animals (Madsen and Bertelsen, 2012).

The body weight (BW) (kg) of the animals was determined according to Remmelink et al. (2011), as shown in Eq. (1). The dry matter intake (DMI) kg/day of concentrate was set to the amount allocated on individual and daily basis. The average TMR intake for the cows in the two groups was set as the herd average. The individual TMR intake of the cows was calculated following Eq. (2) described by Kristensen and Ingvartsen (2003), where the intake is corrected according to the amount of concentrate allocated and the parity of the individual cows. The individual total dry matter intake (TDMI) was calculated by adding the individually allocated concentrate dry matter intake (CDMI) to individually calculated TMR dry matter intake (TMRDMI). The heat production (HP) watt of the cows was calculated following Eq. (3), described by CIGR (2002). The excretion of CO_2 (L/day) was calculated according to Pedersen et al. (2008), as shown in Eq. (4). The amount of methane (g/day) was calculated as described by Madsen et al. (2010) using Eq. (5). Energy corrected milk (ECM) (kg) was calculated following the Eq. (6) described by Sjaunja et al. (1991).

$$BW(kg) = 0.000275 \times Breast\ size\ in\ \mathrm{cm}^{2.76} \tag{1}$$

$$TMRDMI\left(\frac{\mathrm{kg}}{\mathrm{day}}\right) = a + 0.5(b-c) + d \tag{2}$$

$$HP(\mathrm{watt}) = 5.6 \times BW^{0.75} + \{(Y \times 22) + (1.6 \times 10^{-5} \times P^3)\} \tag{3}$$

$$CO_2(L/\mathrm{day}) = HPU \times 180 \times 24 \tag{4}$$

$$CH_4(g/\mathrm{day}) = CO_2 \times \frac{CH_4}{CO_2} \times 0.714 \tag{5}$$

$$ECM\ (\mathrm{kg}) = Y \times (0.383 \times milk\ fat + 0.242 \times milk\ protein + 0.7832)/3.14 \tag{6}$$

where

a is the measured average TMR intake;
b is the measured average concentrate intake;
c is the allocated concentrate intake of the individual cows during the experimental periods;
d is the correction factor for the lactation number: $d = -1.61$ was considered for first lactation and $d = 0.39$ for the second and subsequent lactations;
HP is heat production from the animals;
$BW^{0.75}$ is metabolic body weight of the animals;
Y is Milk yield of cow kg/day
P is days of pregnancy
HPU = Heat producing unit ($HP/1000$);
180 = L of CO_2/HPU/h;
ECM = Energy corrected milk.

2.6. Statistical analyses

Statistical analyses were performed using the software R (R Development Core Team, 2013). The data were fitted using mixed models using the lme function from the package nlme (Bates and Sarkar, 2009).

The analyses focused on making inference about the effect of the concentrates (MELK and VEM) and about the length of the treatment for changes in levels of CH_4 (g/day), CH_4:CO_2, CH_4 (g/kg DMI) and CH_4 (g/kg ECM). Therefore all periods 1, 2 and 3 were included in the analysis. For all of the response variables an average data per cow and per day were used. Group, period of measurement, the interaction group $\times$ period, BW, DMI, ECM and lactation numbers were included as fixed effects in the primary model. Both cow number and day of measurement were included as random effects. Different serial correlation structures were tested for the effect of day. The final model was confirmed by stepwise removing of the non-significant variables. Model validation was performed using analysis of variance (ANOVA) on the Akaike Information Criterion. Model residuals were checked for normality and homoscedasticity by visual inspection, qqplots and Bartlett test. The final model was:

$$y_{ijk} = \mu + \alpha_i + \beta_j + X\boldsymbol{\gamma}_{ijk} + Y\theta_{ijk} + C_k + \varepsilon_{ijk}, \tag{7}$$

where y_{ijk} is the response variable $y = \{CH_4$ g/day, CH_4:CO_2, CH_4 g/kg DMI, CH_4 g/kg ECM$\}$ of i group, for period j and cow k and μ is the overall mean. The fixed effects are the group α_i with $i = \{$MELK, VEM$\}$, the period β_j with $j = \{$period 1, period 2, period 3$\}$, the ECM (kg) for cow k, $X\boldsymbol{\gamma}_{ijk}$, and the BW for cow k, $Y\theta_{ijk}$; C_k is the random effect of cow k and ε_{jkl} is the residual errors. Even though only ECM was significant, BW was included since it has a direct influence on CO_2 production. Group and period were also included since both of them are of interest for this study. The least square means (LSM) were extracted from the model by using the package lsmeans as described by Russell (2013). Multiple comparison was done using Tukey's pairwise

Table 3
Average body weight, milk production (corrected and uncorrected), TMR and concentrate intake of cows per day; n is the number of observations.

Parameters	MELK [mean (SE)]	VEM [mean (SE)]	*n*
BW (kg)	647 (19.2)	674 (15.9)	18
Milk production (kg/day)	31.8 (0.56)	31.6 (0.54)	270
ECM (kg/day)	33.1 (0.48)	33.7 (0.51)	270
TMRDMI (kg/day)	18.7 (0.06)	18.7 (0.06)	270
CDMI (kg/day)	4.5 (0.12)	4.8 (0.12)	270
TDMI (kg/day)	23.2 (0.14)	23.5 (0.14)	270

SE=standard error.
BW=body weight.
ECM=energy-corrected milk.
TMRDMI=total mixed ration dry matter intake.
CDMI=concentrate dry matter intake.
TDMI=total dry matter intake.

comparisons using the function glht from the multcomp package (Hothorn et al., 2008). One way ANOVA were carried out to get the model *P* values. Finally, using the information from this study, precision based power calculation was performed in order to estimate the minimum mean difference that indicates a significant effect between groups, according to the number of observations (Pandis et al., 2011).

3. Results

3.1. Dry matter intake and milk production

Data for BW, milk production, DMI for TMR and concentrates are shown in Table 3. Total DMI were 23.2 (SE=0.08) and 23.5 (SE=0.08) kg/day in MELK and VEM respectively, with individual cows values ranging from 21.5 to 25.6 kg/day. There was a large variation among the cows in milk production and consequently concentrate dry matter intake, as the amount of concentrate was supplied according to individual milk production.

3.2. CH_4:CO_2 ratio and methane emission

Measurements of air composition were performed in the AMS every 20 s throughout the entire experimental period. The frequent air analysis was done in order to get ample data for the best possible estimation of CH_4 production as the individual observations of breath sample analysis show large variation from each other. Fig. 1 illustrates the variation in concentration of breath in the analysed air sample by showing the concentration of CO_2 in 52 measurements of air for a single cow during three visits. Likewise the variation in the corrected CH_4:CO_2 ratio for the same 52 observations is shown in Fig. 2. There is still a variation between samples and this can be ascribed to the different concentrations of CH_4 in the breath. All exhaled air from the cows contains CH_4 and the high values of more than 0.2 indicate that the CH_4:CO_2 ratio can get close to the ratio in the rumen.

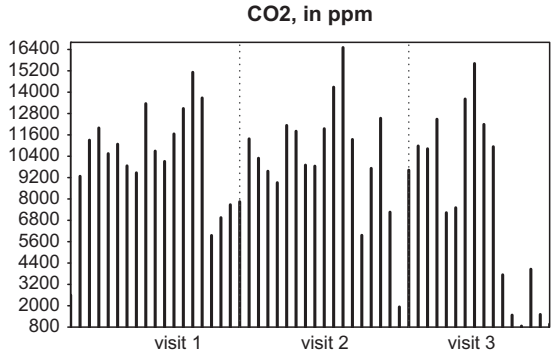


Fig. 1. Individual observations of CO_2 (ppm) concentration in analysed air from three visits of a cow from group VEM during period 1.

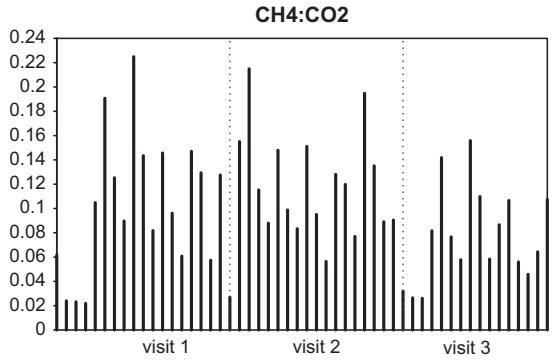


Fig. 2. Individual values of the calculated CH_4:CO_2 ratio corresponding to the measurements from Fig. 1.

Results of the mixed models (Table 4) indicate that there was no significant difference in the effect of supplementation of the two concentrates on the CH_4 production ($P=0.97$). The difference between the three periods was also non-significant ($P=0.49$). In addition, about 43% of the random variation in CH_4 (g/day) production is due to the individual cow effect, the rest being due to the variation between measurements. Results for the ratio CH_4:CO_2 also indicate that both the effects of the group ($P=0.75$) and period ($P=0.62$) are non-significant. In order to visualize the day to day variation, the arithmetic mean of CH_4:CO_2 and of CH_4 production (g/day) in the two groups and over the three periods are shown in Fig. 3.

The methane production per cow increased with increased ECM as a result of the higher DM intake with increased milk production. The coefficient for ECM ($P<0.001$) from the mixed model indicates an incremental emission of methane of 6.1 g/kg increase in ECM. As the concentrate is fed according to milk yield, the difference of ingested starch content is smaller for low yielding cows and larger for high yielding cows. Therefore, a different effect on low and high yielding cows could be expected in the current study.

Scatterplots of CH_4 production (g/day) according to ECM is shown in Fig. 4. A simple linear regression using average CH_4 release (g/day) showed very similar slopes in two groups during period 1, where both groups got the

Table 4
Least square means (LSM) of CH_4:CO_2 and CH_4 production g/day, g/kg DMI and g/kg ECM.

	CH_4:CO_2 ratio [LSM (SE)]	CH_4 g/day [LSM (SE)]	CH_4 g/kg feed DMI [LSM (SE)]	CH_4 g/kg ECM [LSM (SE)]
MELK				
Period 1	0.0993 (0.00435)	444 (19.5)	19.0 (0.98)	13.8 (0.78)
Period 2	0.1001 (0.00436)	449 (19.5)	19.9 (0.98)	14.0 (0.78)
Period 3	0.0997 (0.00436)	450 (19.5)	19.6 (0.98)	14.1 (0.78)
Mean[a]	0.1000 (0.00405)	447 (18.2)	19.6 (0.98)	14.2 (0.70)
VEM				
Period 1	0.0987 (0.00436)	436 (19.6)	18.1 (0.84)	13.5 (0.78)
Period 2	0.0995 (0.00436)	442 (19.5)	19.0 (0.98)	13.7 (0.78)
Period 3	0.0991 (0.00436)	442 (19.5)	18.7 (0.98)	13.8 (0.78)
Mean[a]	0.0993 (0.00405)	438 (18.2)	18.7 (0.84)	13.9 (0.70)

SE=standard error.
ECM=energy corrected milk.
DMI=dry matter intake.

[a] Mean values considering both periods 2 and 3.

same diet consisting of MELK and VEM (50% of each). Based on the linear regression, in periods 2 and 3 when two groups were either MELK or VEM (100%), a tendency is shown between the groups ($P=0.07$ for both subsets of period 2 and 3). The VEM group tended to show a sharper slope than the MELK group. This supports the hypothesized highest effect of a high starch concentration in the concentrate on lowering CH_4 when feeding the highest amount of concentrate.

3.3. Precision of the CH_4 estimates

Table 5 reports the results of precision based power calculations for CH_4 production (g/day) using the results SD=74 based on the mean values per cow per day from the present experimental conditions. The results indicate that in order to get a significant difference at 5% level, the minimum mean difference of CH_4 (g/day) between the groups should be at least $\pm$ 40, 28 and 23 equivalent to a 9, 6 and 5% for 5, 10 and 15 days of measurement, respectively, with 18 cows per group.

4. Discussion

4.1. Breath sample measurement

The air samples analysed are influenced by the concentration of breath in the air samples as the position of the nose of the cows in relation to the inlet filter varies. Most samples have a CO_2 concentration of between 5000 and 10,000 ppm which shows that samples contain between 10 and 30% of breath considering that the average concentration of CO_2 in breath typically ranges between 30,000 and 50,000 ppm (Elliott-Martin et al., 1997; Smith et al., 2009). When calculating the corrected CH_4:CO_2 ratio after subtracting the concentration of CH_4 and CO_2 in the surrounding air, the variation due to the position of the nose in relation to the inlet filter is removed. Part of the variation in the CH_4:CO_2 ratio is also caused by the proportion of the ruminal fermentation gases and gases from normal breathing. This variation requires several measurements to obtain a reliable mean value. A recent large scale study for breeding purposes describes that more than 3 days measurement is expensive and impractical for getting better estimation of the CH_4:CO_2 ratio (Lassen et al., 2012). Furthermore, Madsen et al. (2010) mentioned that about 2–3% of breath in the analysed air sample is sufficient to get a precise estimation of CH_4 production. The latter also indicate that it is sufficient to get a relative diluted breath to get a reliable determination of the CH_4:CO_2 ratio.

4.2. Methane production

As described by Hindrichsen et al. (2004), the mode of fermentation of starch and sugar and their end products indicate that there should be a lower CH_4 production in the MELK group. In the current study, there was a tendency observed that the cows receiving the highest amount of MELK concentrate (high starch) produced less CH_4 (g/day). Nevertheless, no significant differences were found in CH_4 output between the groups. The reason for the absence of reduced CH_4 production in the MELK group is probably due to the very limited change in the total carbohydrate composition of the diet. As seen in Table 6 the starch content that was expected to be lowered the CH_4 output was four times as high in the MELK concentrate as in the VEM concentrate. When calculated on the total diet basis (concentrate+TMR) the total starch content increases only with 25%. The starch proportion of the total potential digestible carbohydrates (sugar, starch and NDF) is 21.7% in VEM to 27.5% in MELK. It can therefore be assumed that only 5.8% increase in the starch content of the total diet has not been enough to change the CH_4 emission. Similar effects have been presented by Aguerre et al. (2011) where dietary starch content was reduced by increasing fibre concentration in the diet of dairy cows. Mc Geough et al. (2010) reported a decreased CH_4 (g/kg DMI) emission in beef cows with increased amount of starch feeding through whole-crop wheat silages. In the same line, Hassanat et al. (2013) reported a reduced methane emission in dairy cows by supplementing 30% starch

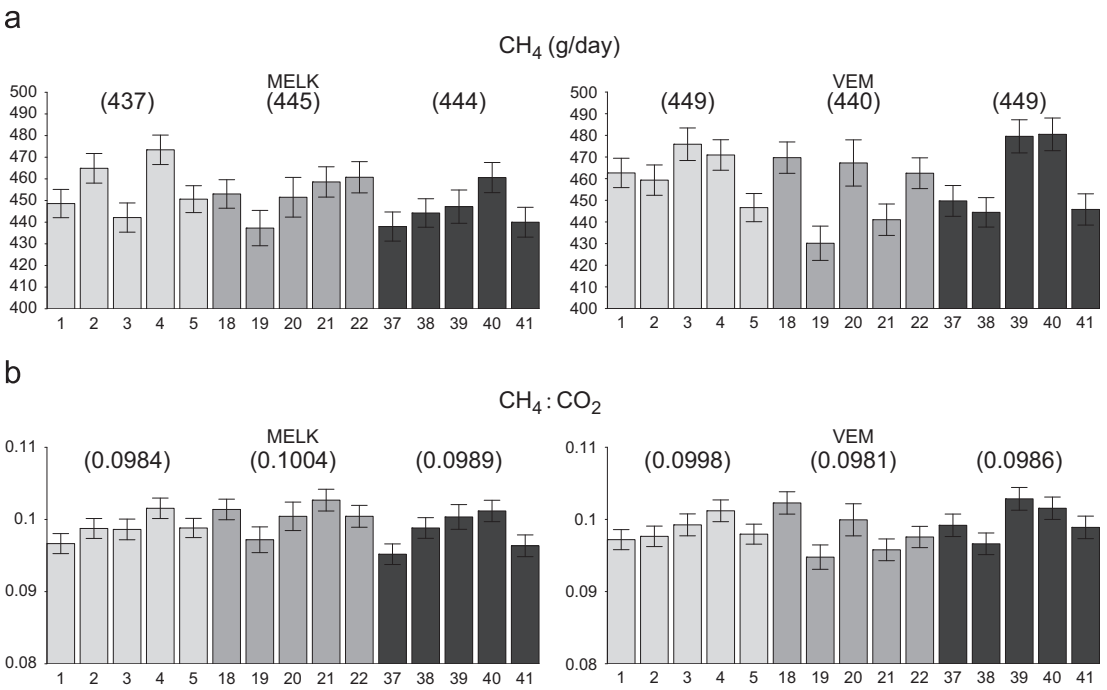


Fig. 3. Daily averages (± SE) of CH_4:CO_2 ratio and CH_4 production (g/day) for the MELK and VEM groups for the 15 days measurement period. Each period has a different shade of grey and average per period is indicated in brackets. The horizontal axis indicates the experimental day.

through 100% corn silage, whereas no effect on CH_4 has found by increasing starch from 17–27% starch through supplementation of 0–50% corn silage. As mentioned earlier, in the current study only 25% increase in starch (on total ration basis) resulted in no effect on CH_4 (g/day) reduction, which is in accordance with Hassanat et al. (2013). The latter suggested that the methane reduction effect of starch is linked with acidic ruminal environment due to low pH (<6.0) and with the shift of the volatile fatty acid pattern toward proportionally more propionate and less acetate and butyrate. Furthermore, Fahey and Berger (1988) pointed out starch as the most propionate producer in rumen fermentation than any other carbohydrates. In the present study, the supplemented amount of starch through concentrate in AMS was certainly too low to show a response on changing rumen environment with increase propionate proportion and consequently CH_4 reduction. Besides, supplying diet containing high quantities of starch via grain or cereal forages has been proposed as a mean of methane reduction (Beauchemin et al., 2008). The current study made a similar effort to reduce methane by increasing the amount of starch. However, it appears that an inapt way was chosen to supplement starch through concentrate in AMS. Feeding starch through TMR could have been more effective to visualize the methane reduction effect.

4.3. *Precision of the CH_4 estimates*

The CO_2-method used in the experiment is relatively newly developed (Madsen et al., 2010). It offers opportunities to get measurements from many animals within
a short time, which is an advantage in many situations. A large number of animals is typically required for breeding experiments (Lassen et al., 2012). Furthermore, increasing the number of individuals can improve the precision of CH_4 measurement for feeding experiments.

When comparing the effect of different diets on groups of cows, it is of uttermost importance that the precision of the estimates are high whereas the level (or accuracy) is of less importance to validate the effect of diets or treatments.

Danielsson et al. (2012) showed a large individual variation between cows using the SF_6 method. The variation of CH_4 ranges from 12.3 to 21.8 for one diet, and from 11.8 to 25.7 (g/kg DMI) for another diet, for averages based on a five days measurement period. To reduce this variation would require increasing the number of animals or days of measurement. In this study, the individual cow variation of CH_4 (g/kg DMI), based on the 15 days experimental period ranged from 17.0 to 23.0 in the MELK group and from 14.0 to 21.6 for VEM group. The individual variation of CH_4 production was highlighted as most important by Grainger et al. (2007) and has also been seen in own experiment (Haque et. al., 2014, unpublished data).

The shown variation in the CH_4:CO_2 ratio and the estimated daily CH_4 output are assumed to be related to the time of the day the cows visit the AMS. Moreover, the time of the day when they have eaten TMR may influence the actual CH_4 production.

In this study, the resulted least square means difference of CH_4 (g/day) between groups (Table 4) was about 8,

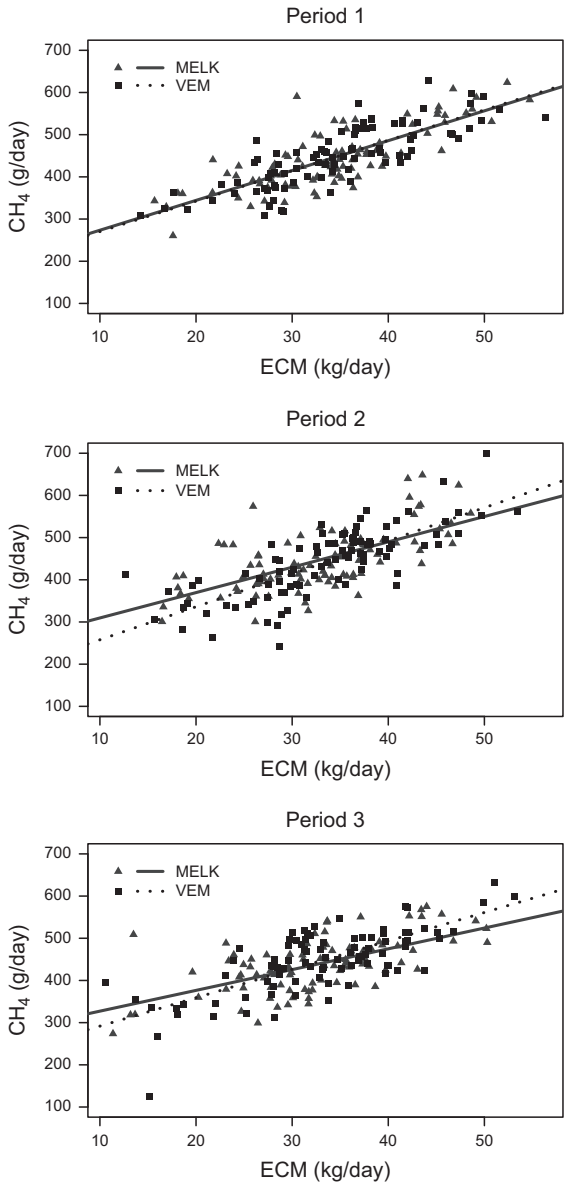


Fig. 4. Linear regression of CH_4 production (g/day) for cows of MELK and VEM groups in the three periods of measurement.

7 and 8 for period 1, 2 and 3 respectively, and 9 when considering both period 2 and 3 (10 days in total). At the light of the findings of precision-based power calculation, the results show no significant difference was obtained between groups. In order to improve the precision of CH_4 quantification, more individuals or measurements for a longer period of time is needed.

4.4. Accuracy of the methane production

For an experiment as the present, another aspect is whether the estimates are accurate, i.e. whether they give values of the right magnitude. The accuracy is also influenced by the accuracy of the calculated CO_2 production.

Table 5
Expected mean difference in methane production (g/day) according to the number of observations (cows × days) per group in order to get a significant difference between the groups at a 0.05 level of significance and a power of 95%.

Number of cows	Days of measurement	Expected mean difference[a]	Expected mean difference[b] (%)
18	1	89	20
18	5	40	9
18	10	28	6
18	15	23	5

[a] Calculated from $d = \sqrt{f(\alpha,\beta)\{2 \times SD^2\}/n}$. Where α=*significance level*, β=*Power*, $f(\alpha,\beta)$=13.0 at 5% level of significance for a power of 95%. *SD*=standard deviation of the response (74) and n=number of observations per group.

[b] Calculated considering a mean values 442, for periods 2 and 3 (see Table 4).

The formula used is based on the work of an international commission of agriculture (CIGR, 2002) and of Pedersen et al. (2008), and is considered reasonably accurate and the best available. Comparing the results of this experiment with recently published studies using the SF_6 (O'Neill et al., 2011; Danielsson et al., 2012) and chambers methods (Aguerre et al., 2011; van Zijderveld et al., 2011) indicate comparable magnitude and associated precision of CH_4 emission (g/kg DMI). In this study, the emissions range from 18.7–19.6 (MELK–VEM), whereas the other studies report values ranging from 16.9 (Danielsson et al., 2012) to 25.9 (Aguerre et al., 2011) g/kg DMI. The corresponding SEM for CH_4 (g/kg DMI) reported for the chamber experiments are 0.65 (van Zijderveld et al., 2011) and 1.21 (Aguerre et al., 2011); for the SF6, the values are 0.57 (O'Neill et al., 2011) and 2.9 (Danielsson et al., 2012; only SED was reported). The value reported in the present study (0.84 for 10 days measurement) indicates that the CO_2-method is as precise as the other methods and produces results of the same magnitude. In O'Neill et al. (2011), for which the SEM is the lowest, it should be noted that the number of individuals per group (n=24; 10 days measurements) was larger than in the present study (n=18; 10 days measurements).

As high yielding cows have a lower emission of CH_4 per kg milk, lowering the marginal emission may also be an objective in itself. Tamminga et al. (2007) made a prediction of the expected CH_4 production per kg milk at different levels of milk production. At the milk production level corresponding to the cows of this study (33.5 kg/day) the predicted CH_4 production was 12.8 g/kg ECM. In this respect, the CH_4 production reported in this study, ranging from 13.9 to 14.2 g/kg ECM (Table 4) can be considered estimated with an acceptable accuracy.

5. Conclusions

This study showed no effect of changing the composition of concentrate fed in AMS to higher starch content and less fibre and sugar on the methane output. The absence of hypothesized reduction in the CH_4 (g/day) release is most likely due to the small proportion of dry matter consumed from the allocated concentrate in the AMS,

Table 6
Average carbohydrate intake of the diets, kg DM/cow/day.

	MELK			VEM			Change (%) in nutrient, VEM to MELK	
	Total	TMR	Concentrate	Total	TMR	Concentrate	Total	Concentrate
Sugar	2.8	2.4	0.4	3.1	2.4	0.7	−10	−43
Starch	6.4	4.7	1.7	5.1	4.7	0.4	+25	+325
NDF	19.8	18.6	1.2	20.5	18.6	1.9	−3	−37
Total DM	23.2			23.5				
% Starch in DM	27.5			21.7			+5.8	

TMR=total mixed ration.
NDF=neutral detergent fibre.

which is scanty in relation to the dry matter intake from the TMR. To obtain an effect on CH_4 yield by dietary manipulation with carbohydrate composition, it is recommended to change the composition of the TMR part of the diet, as this can result in a greater change in the composition of the total carbohydrate intake. The results from the used CO_2-method illustrate that higher precision can be obtained by either having more cows in the experiment or by measuring for a longer period.

Conflict of interest statement

The authors report that there is no conflict of interest relevant to this publication.

Acknowledgements

The study was supported by a stipend from University of Copenhagen, Denmark and a research grant from the Dutch Ministry of Finance and Innovation through the company Feed Innovation Services, the Netherlands.

References

Aguerre, M.J., Wattiaux, M.A., Powell, J.M., Broderick, G.A., Arndt, C., 2011. Effect of forage-to-concentrate ratio in dairy cow diets on emission of methane, carbon dioxide, and ammonia, lactation performance, and manure excretion. J. Dairy Sci. 94, 3081–3093.
Bates, D.M., Sarkar, D., 2009. lme4: Linear Mixed-effects Models Using S4 Classes, R package version 0.99875-6. . ⟨http://cran.r-project.org/web/packages/lme4/index.html⟩ (accessed 17.04.13).
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., McAllister, T.A., McGinn, S.M., 2009. Dietary mitigation of enteric methane from cattle. CAB Rev.: Perspect. Agric., Vet. Sci., Nutr. Nat. Resour. 4, 1–18.
Benchaar, C., Hassanat, F., Gervais, R., Chouinard, P.Y., 2013. Effects of increasing amounts of corn dried distillers grains with solubles in dairy cow diets on methane production, ruminal fermentation, digestion, N balance, and milk production. J. Dairy Sci. 96, 2413–2427.
Blaxter, K.L., Clappert, J.L., 1965. Prediction of amount of methane produced by ruminants. Br. J. Nutr. 19, 511–522.
CIGR, 2002. Climatization of animal houses—heat and moisture production at animal and house level. In: Pedersen, S., Sällvik, K. (Eds.), 4Th Report of CIGR Working Group. Horsens, Denmark. Research Centre Bygholm, Danish Institute of Agricultural Sciences, P.O Box 536, DK-8700 Horsens, Denmark.
Danielsson, R., Schnurer, A., Arthurson, V., Bertilsson, J., 2012. Methanogenic population and CH_4 production in swedish dairy cows fed different levels of forage. Appl. Environ. Microbiol. 78, 6172–6179.
Elliott-Martin, R.J., Mottram, T.T., Gardner, J.W., Hobbs, P.J., Bartlett, P.N., 1997. Preliminary investigation of breath sampling as a monitor of health in dairy cattle. J. Agric. Eng. Res. 67, 267–275.
EPA, 2008. Inventory of U.S. Greenhouse Gas Emissions and Sinks. U.S. Environmental Protection Agency, Washington, DC 20460, U.S.A. (1200 Pennsylvania Avenue, N.W).
EU, 2009a. Determination of crude ash. Off. J. Eur. Union Comm. Regul. 152, 50–52.
EU, 2009b. Determination of crude fibre. Off. J. Eur. Union Comm. Regul. 152, 40–42.
EU, 2009c. Determination of sugar. Off. J. Eur. Union Comm. Regul. 152, 45–52.
Fahey, G.C., Berger, L.L., 1988. Carbohydrate nutrition of ruminants. In: Church, D.C. (Ed.), The Ruminant Animal: Digestive Physiology and Nutrition, Prentice Hall Inc., Englewood Cliffs, NJ.
FO-19, 2005. Changing the Method of Analysis to the Provisions of EFOS Cattle. Ministry for food agriculture and fisheries, Denmark.
Gasmet™, 2010. Gasmet Technologies Oy Pulttitie 8A, FI-00880 Helsinki, Finland (accessed 17.04.13).
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), Rome, Italy.
Grainger, C., Clarke, T., McGinn, S.M., Auldist, M.J., Beauchemin, K.A., Hannah, M.C., Waghorn, G.C., Clark, H., Eckard, R.J., 2007. Methane emissions from dairy cows measured using the sulfur hexafluoride (SF6) tracer and chamber techniques. J. Dairy Sci. 90, 2755–2766.
Hassanat, F., Gervais, R., Julien, C., Massé, D.I., 2013. Replacing alfalfa silage with corn silage in dairy cow diets: Effects on enteric methane production, ruminal fermentation, digestion, N balance, and milk production. J. Dairy Sci. 96, 4553–4567.
Hindrichsen, I.K., Wettstein, H.R., Machmuller, A., Soliva, C.R., Knudsen, K.E.B., Madsen, J., Kreuzer, M., 2004. Effects of feed carbohydrates with contrasting properties on rumen fermentation and methane release in vitro. Can. J. Anim. Sci. 84, 265–276.
Hothorn, T., Bretz, F., Westfall, P., 2008. Simultaneous inference in general parametric models. Biometrical J. 50, 346–363.
Hristov, A.N., Oh, J., Lee, C., Meinen, R., Montes, F., Ott, T., Firkins, J., Rotz, A., Dell, C., Adesogan, A., Yang, W., Tricarico, J., Kebreab, E., Waghorn, G., Dijkstra, J., Oosting, S., 2013. In: Gerber, J. Pierre, Henderson, Benjamin, Makkar, H.P.S (Eds.), Mitigation of Greenhouse gas Emissions in Livestock Production—A Review of Technical Options for Non-CO_2 Emissions, FAO Animal Production and Health, FAO, Rome, Italy.
ISO-11085, 2008. Animal Feeding Stuffs—Determination of Crude Fat and Total Fat Content by the Randall Extraction Method. International Organization for Standardization, Geneva, Switzerland (ISO-11085:2008).
ISO-13906, 2008. Animal Feeding Stuffs—Determination of Acid Detergent Fiber and Acid Detergent Lignin Contents. International Organization for Standardization, Geneva, Switzerland (ISO 13906:2008).
ISO-15914, 2004. Animal Feeding Stuffs—Enzymatic Determination of Total Starch Content. International Organization for Standardization, Geneva, Switzerland (ISO 15914:2004).

ISO-16472, 2006. Animal Feeding Stuffs—Determination of Amylase-treated Neutral Detergent Fibre Content. International Organization for Standardization, Geneva, Switzerland (ISO 16472:2006).
Johannes, M., Louise, P.L.A., Hellwing, F., Weisbjerg, M.R., 2011. Hvad betyder fodring for metanemission?. In: Kristensen, T., Lund, P. (Eds.), Kvæg og Klima. Videnskabelig Rapport no. 1. Aarhus Universitet, DCA—Nationalt Center for Fødevarer og Jordbrug, Denmark.
Johnson, K., Huyler, M., Westberg, H., Lamb, B., Zimmerman, P., 1994. Measurement of methane emissions from ruminant livestock using a sulfur hexafluoride tracer technique. Environ. Sci. Technol. 28, 359–362.
Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. J. Anim. Sci. 73, 2483–2492.
Kristensen, V.F., Ingvartsen, K.L., 2003. Forudsigelse af foderoptagelsen hos malkekøer og ungdyr. In: Hvelplund, T., Nørgaard, P. (Eds.), Kvægets Ernæring og Fysiologi. Bind 1. Ministeriet for Fødevarer Landbrug og Fiskeri, Næringsstofomsætning og fodervurdering: DJF rapport, Husdyrbrug, Denmark, pp. 511–564.
Lassen, J., Lovendahl, P., Madsen, J., 2012. Accuracy of noninvasive breath methane measurements using Fourier transform infrared methods on individual cows. J. Dairy Sci. 95, 890–898.
Licitra, G., Hernandez, T.M., VanSoest, P.J., 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Technol. 57, 347–358.
Madsen, J., Bertelsen, M.F., 2012. Methane production by red-necked wallabies (*Macropus rufogriseus*). J. Anim. Sci. 90, 1364–1370.
Madsen, J., Bjerg, B.S., Hvelplund, T., Weisbjerg, M.R., Lund, P., 2010. Methane and carbon dioxide ratio in excreted air for quantification of the methane production from ruminants. Livest. Sci. 129, 223–227.
Martin, C., Rouel, J., Jouany, J.P., Doreau, M., Chilliard, Y., 2008. Methane output and diet digestibility in response to feeding dairy cows crude linseed, extruded linseed, or linseed oil. J. Anim. Sci. 86, 2642–2650.
Mc Geough, E.J., O'Kiely, P., Hart, K.J., Moloney, A.P., Boland, T.M., Kenny, D.A., 2010. Methane emissions, feed intake, performance, digestibility, and rumen fermentation of finishing beef cattle offered whole-crop wheat silages differing in grain content. J. Anim. Sci. 88, 2703–2716.
O'Neill, B.F., Deighton, M.H., O'Loughlin, B.M., Mulligan, F.J., Boland, T.M., O'Donovan, M., Lewis, E., 2011. Effects of a perennial ryegrass diet or total mixed ration diet offered to spring-calving Holstein-Friesian dairy cows on methane emissions, dry matter intake, and milk production. J. Dairy Sci. 94, 1941–1951.
Pandis, N., Polychronopoulou, A., Eliades, T., 2011. Sample size estimation: an overview with applications to orthodontic clinical trial designs. Am. J. Orthod. Dentofac. 140, 141–146.
Pedersen, S., Blanes-Vidal, V., Joergensen, H., Chwalibog, A., Haeussermann, A., Heetkamp, M.J.W., Aarnink, A.J.A., 2008. Carbon dioxide production in animal houses: a literature review. Agric. Eng. Int.: CIGR Ej. X, 1–19. (Manuscript BC 08 008).
Plaizier, J.C., Krause, D.O., Gozho, G.N., McBride, B.W., 2008. Subacute ruminal acidosis in dairy cows: the physiological causes, incidence and consequences. Vet. J. 176, 21–31.
R Development Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. R version 3.0.0. ⟨http://www.R-project.org⟩.
Remmelink, G., Blanken, K., Van Middelkoop, J., Ouweltjes, W., Wemmenhove, H., 2011. Handboek Melkveehouderij. Handboek. Wageningen UR Livestock Research. (p. 6-6).
Russell, V.L., 2013. Least-squares means, R-package. ⟨http://cran.r-project.org/web/packages/lsmeans/⟩ (accessed 14.05.13).
Sjaunja, L.O., Baevre, L., Junkkarinen, L., Pedersen, J., Setala, J., 1991. A Nordic proposal for an energy corrected milk (ECM) formula. In: Gaillon, P., Chabert, Y. (Eds.), Performance Recording of Animals State of the Art 1990. EAAP Publication 50, Centre for Agricultural Publishing and Documentation, Wageningen, pp. 156–157.
Smith, D., Pysanenko, A., Spanel, P., 2009. The quantification of carbon dioxide in humid air and exhaled breath by selected ion flow tube mass spectrometry. Rapid Commun. Mass Spectrom. 23, 1419–1425.
Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., Rosales, M., de Haan, C., 2006. Livestock's Long Shadow: Environmental Issues and Options. Food and Agriculture Organization of the United Nations. FAO, Rome, Italy.
Tamminga, S., Bannink, A., Dijkstra, J., Zom, R., 2007. Feeding Strategies to Reduce Methane Loss in Cattle. Report: Animal Nutrition and Animal Sciences Group, Wageningen UR.
TeamViewer©, 2013. Kuhnbergstr, 16 73037 Göppingen Germany. ⟨http://www.teamviewer.com/en/news/⟩ (accessed 17.04.13).
van Zijderveld, S.M., Fonken, B., Dijkstra, J., Gerrits, W.J.J., Perdok, H.B., Fokkink, W., Newbold, J.R., 2011. Effects of a combination of feed additives on methane production, diet digestibility, and animal performance in lactating dairy cows. J. Dairy Sci. 94, 1445–1454.
Weisbjerg, M.R., Søegaard, K., Thøgersen, R., Mikkelsen, M., Brunsgaard, G., 2007. Bestemmelse af Fordøjelighed af Organisk stof i Grovfoder ved brug af In vitro-Metoder Baseret på Vomvæske eller Enzymer. DJF Husdyr No. 76. Aarhus Universitet, Det Jordbrugsvidenskabelige Fakultet.

Paper III

Method based comparative methane estimation from cattle fed three different diets

Manuscript ready to submit.

Method based comparative methane estimation from cattle fed three different diets

M. N. Haque,* I. M. L. D. Storm, H. H. Hansen, and J. Madsen,*

Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Denmark;

*Corresponding authors
Email addresses: jom@sund.ku.dk (J. Madsen), naha@sund.ku.dk (M. N. Haque)

ABSTRACT

The objectives of this study were to estimate the effect of different source of carbohydrate supplements on the CH_4 emission and compare the precision of the CO_2 method with respiration chamber and in vitro gas production technique. The present study was conducted as a 3x3 Latin Square design where three Dexter heifers were allocated to the balance cages and subsequently in the respiration chamber (RC_1) over 3 periods consist of two weeks of adaptation period followed by one week of measurement. The average body weight (BW) of the heifers was 226±11 kg (mean±SD) and the dry matter intake (DMI) was 5.1±0.3 kg/d (mean±SD). The heifers were fed *ad libitum* a total mixed ration (TMR) consisted (on DM basis) of 49% grass-clover silage, 14% soybean meal along with 35% of one of three supplements: wheat (W), molasses (M), and molasses + sodium bicarbonate (Mbic). Breath samples of the heifers were continuously analyzed in the metabolic cage every 20 seconds to determine the concentrations of CH_4 and CO_2. The calculations regarding the CH_4 and CO_2 emissions from the heifers were done according to the CO_2 method (CO_2T). The dry matter intake (DMI, kg/d) was significantly higher ($P<0.001$) in the metabolic cage compared to the intake in the respiration chamber. The absolute CH_4 (L/d) production estimated by CO_2T was significantly different ($P<0.05$) between three diets. The wheat based diet "W" produced significantly lower CH_4 compared to the molasses based "M" and "Mbic" diets. The ranking of the diets based on the absolute CH_4 (L/d) production were W<M<Mbic. The emissions of CH_4 (L/kg DMI) followed the same ranking ($P<0.05$). The absolute CH_4 (L/d) emissions obtained by the CO_2T and RC_1 were strongly correlated ($r = 0.83$). Positive correlation was also found between the estimated CH_4 by the CO_2T and IPCC recommended prediction model. The daily CH_4 (L/kg DMI) emission was lower in the CO_2T compared to the respiration chamber (RC_2) technique with Holstein cows. A substantial animal variation of the daily average CH_4 production was observed within the same diet. The coefficient of variation of CH_4 production between animals (CV_b) was 7.4-8.0% and lower than the within animal variation (CV_w) 17.3-17.4% estimated by the CO_2T. The measured CO_2 in the RC_1 and the calculated CO_2 according to the CO_2T was strongly ($r = 0.85$) correlated. In conclusions, the DMI was lower in the respiration chamber. The wheat based diet showed significantly lower CH_4 emission (L/d and L/kg DMI) compared to the Molasses based diets. All three diets showed a numerically lower CH_4 (L/kg DMI) estimated by the CO_2T than the

study with RC_2. The CO_2T can predict CH_4 emission with a reasonable accuracy and precision compared to the chamber technique.

Keywords: CO_2-method, Dexter cattle, Methane, Respiration chamber, Precision

1. Introduction

Methane (CH_4) is an undesirable by-product of rumen fermentation. This is produced in the rumen by a particular group of microbes, the methanogenic archaea (Baldwin and Allison, 1983). Methanogens use hydrogen (H_2) to reduce carbon dioxide to CH_4, thus keeps H_2 partial pressure low and favours rumen fermentation. Among the ruminants cattle are the main contributor to the CH_4 emissions with about 4.6 gigatonnes CO_2 equivalent, represents 65% emissions from the livestock sector (Gerber et al., 2013). This particular greenhouse gas has received a great deal of attention in the recent years because it is not only responsible for environmental pollution but also associated with feed energy loss from the animals (Hongmin Dong et al., 2006). Typically, methane emission associated about 2 to 12% gross energy loss (Johnson and Johnson, 1995), a portion of energy that can potentially be used by the animals. The amount of CH_4 release depends on the level of feed intake, diet composition, use of feed supplements and additives (Johnson and Johnson, 1995). Enteric CH_4 production in ruminants is the process very closely related to the amount of volatile fatty acids production as the end product of rumen fermentation (Hungate, 1982; Johnson and Johnson, 1995). The primary substrate for methanogenesis is H_2, which is produced during fermentation of structural carbohydrates to acetate and butyrate (Moss et al., 2000). In contrast, the fermentation of starch and other non-structural carbohydrates favour propionate production, which serves as a competitive pathway for H_2 use in the rumen (Benchaar et al., 2001). It is well established that feeding more starch to ruminants reduces enteric CH_4 production and energy losses from the animals (Benchaar et al., 2001; Beauchemin et al., 2009). The underlying mechanism is that fermentation of starch lower the ruminal pH and favors the production of propionate instead of acetate in the rumen, ultimately results in decreased CH_4 production (Hassanat et al., 2013). Furthermore, feeding high-starch diets may result in decrease rumen protozoa, which reduce H_2 transfer from protozoa to methanogens, thereby contribute to reduce CH_4 production (Wolin and Miller, 1988; Hegarty, 1999). A recent study indicated a significant reduction of CH_4 emissions in dairy cows fed higher amount of starch through a 100% corn silage (Hassanat et al., 2013). Unlike starch, feeding sugar has been reported to increase CH_4 production (Hindrichsen et al., 2004). Fermentation of sugar leads to a preferential production of butyrate instead of propionate (Friggens et al., 1998). Butyrate formation provides free H_2 and facilitates methanogenesis. Moreover, van Kessel and Russell (1996) reported that the methanogenic effect of sugars will increase only at a high ruminal pH, because both the producers (fibrolytic bacteria and protozoa) and the consumers (methanogens) of H_2 are highly susceptible at a lower pH. A number of methods have been developed to estimate the actual emissions from the livestock. They are based on different principles and have a wide range of applicability (Storm et al., 2012b). A new techniques called the CO_2 method (CO_2T) has recently been described for the estimation of CH_4 emission from ruminants (Madsen et al., 2010). The hypothesis of this study was that the CO_2T is suitable for the

investigation of the CH_4 mitigation potential of different diets. We further hypothesized that the precision of the CH_4 estimates are comparable with other methods. Therefore, the present study was designed to estimate the effects of different source of carbohydrate supplements on CH_4 emissions measured by the CO_2T and compare the precision of the CO_2T with the respiration chamber and in vitro gas production technique.

2. Materials and methods

2.1. Experimental design, animals and feeding

This study is one of the three separate experiments that conducted with the same three feeds. The CO_2 method (CO_2T), in vitro gas production technique (Storm et al., 2012a) and respiration chamber technique (Hellwing et al., 2012) using Holstein dairy cows. The present study was conducted as a 3x3 Latin Square design, where three Dexter heifers were allocated to the balance cages and subsequently in the respiration chamber over 3 periods consist of two weeks of adaptation period followed by one day each in cage 1, cage 2, respiration chamber 1 and 2. A spare heifer was kept and data was recorded. From this heifer only the feed intake data was used for each diet. The average body weight (BW) of the heifers was 226±11 kg (mean±SD) and dry matter intake (DMI) was 5.1±0.3 kg/d (mean±SD). The heifers were fed *ad libitum* a total mixed ration (TMR) consist of (on DM basis) 49% grass-clover silage, 14% soybean meal along with 35% of one of three supplements: wheat (W), molasses (M), and molasses + sodium bicarbonate (Mbic). All diets for the entire experiment were prepared once from the same batch of ingredients. The TMRs were immediately vacuum-packed in portions for 1 day and frozen. Each portion was thawed at room temperature over night before being fed *ad libitum* with one daily feeding. The chemical compositions of the three supplements is shown in Table 1, were analyzed in the study conducted by Hellwing et al. (2012). The daily feed intake was measured by the difference between the amounts of supply and the refusal. The weight gain of the heifers was not determined. However, daily weight gain of 0.5 kg/d was considered for calculation of heat production.

2.2. Methane and carbon dioxide measurement

Breath concentrations of CH_4 and CO_2 from the heifers were continuously analyzed in the metabolic cage in every 20 seconds. A portable continuous gas analyser GASMET DX-4030 (Gasmet Technologies Oy, Helsinki, Finland) was used for the analysis based on Fourier Transformed Infrared detection. The sampling inlet was fitted in the balance cage, at the nose level of the animals (Figure 1) in order to get concentrated breaths. The record of the breath concentrations were stored in a data logger connected with computer. All gas volumes are reported at 0^oC and 100 KPa. The measurements were performed for approximately 22 hours after which the heifers were moved to the respiration chamber (RC_1) to measure the CH_4 and CO_2 emissions. However, the CH_4 measurements in the chamber were not recorded due to human error. Therefore, these data were excluded from the analysis. In order to get the background concentration of the air

in the barn, the sampling inlet was disconnected from the Gasmet for 10 minutes during each experimental day.

2.3. Calculations

The calculations for CH_4 and CO_2 emissions from heifers were done according to the CO_2-method named as CO_2T (Madsen et al., 2010). The average barn concentrations of CO_2 (705±88.3 ppm) and CH_4 26±10.3 ppm (mean±SD) were subtracted from the exhaled air concentrations to get the actual breath concentration of each sample. After the corrections, all values of corrected CO_2 below 400 ppm were removed in order to avoid the influence of the samples that contained very low concentration of CH_4 and CO_2. The ratio between CH_4 and CO_2 (CH_4:CO_2) was thereafter determined. The heat production (HP, watt) of the heifers were calculated following Equation 1, described by CIGR (2002). The excretion of CO_2 (L/d) was calculated according to Pedersen et al. (2008), as shown in Equation 2. The amount of methane (L/d) was calculated as described by Madsen et al. (2010) using Equation 3. The CH_4 emissions of the heifers were further calculated using the measured CO_2 in the respiration chamber (RC_1) and the CH_4:CO_2 ration from the breath sample analyses using Gasmet. Data for the CH_4 emission (L/kg DMI) from the respiration chamber (RC_2) study with Holstein cows (Hellwing et al., 2012) and in vitro gas production technique (IVGT) (Storm et al., 2012a) were used to compare the precision of the CO_2T.

$$HP\ (watt) = 7.64 * BW^{0.69} + Y\left[\frac{23}{M} - 1\right]\left[\frac{57.27+0.302*BW}{1-0.171Y}\right] + 1.6 * 10^{-5} * P^3 \quad (1)$$

$$CO_2 = HPU * 180 * 24 \quad (2)$$

$$CH_4 = CO_2 * \frac{CH_4}{CO_2} \quad (3)$$

Where,
HP = heat production of the animals;
BW = body weight of the animals;
M = energy contents of the diet;
Y = daily weight gain set as 0.5 kg/d;
P = days of pregnancy of the heifers;
HPU = heat producing unit considered as $\frac{HP}{1000}$;
180 = L of CO_2/HPU/h;
$\frac{CH_4}{CO_2}$ = CH_4 and CO_2 ration of the breath sample analysis.

2.4. Statistical analysis

The raw data were reduced to make an average values per hour for the individual heifer. This data were fitted with a linear mixed model using the lmer function from the package "lme4" (Bates and

Sarkar, 2009) with the software R (R Development Core Team, 2013). An extension package "lmerTest" was used to get the P values directly from the model (Kuznetsova et al., 2012). The primary model was fitted by maximum likelihood including the body weight, diets [3 levels] and DMI as the fixed variables and the heifers as the random variable. The final model in Equation (4) was selected by the stepwise elimination of the non-significant variables. The estimates of the responses were obtained by fitting the final model with Restricted Maximum Likelihood (REML). The model was validated using an analysis of variance (ANOVA) based on the Akaike Information Criterion. The model residuals were checked for normality and homoscedasticity by visual inspection of qqplots.

$$y_{ij} = \mu + \alpha_i + X\gamma_{ij} + H_j + \varepsilon_{ij} \quad (4)$$

Where y_{ij} is the response variable, y = [CH_4 L/d, CH_4 L/kg DMI, CH_4 L/MJ DEI and CH_4:CO_2 ratio] of diet i, and heifer j, μ = overall mean, α_i=diets (wheat, molasses and molasses+bicarnonate), $X\gamma_{ij}$ = dry matter intake of heifer j, for diet i and ε_{ij} is the model residuals.

3. Results

3.1. Feed intake

The dry matter intake (DMI, kg/d) was significantly higher (P<0.001) in the metabolic cage compared to the intake in the respiration (RC_1) chamber (Figure 2). There were no difference (P>0.1) in DMI of different diets. However, there was a difference (P<0.05) in the DMI between the RC_1 and the CO_2 method (CO_2T).

The CH_4 estimates following the four different methods (CO_2T, RC_1, RC_2 and IVGT) are presented in Table 2. The absolute CH_4 (L/d) production estimated by CO_2T was significantly different (P<0.05) between three diets. The wheat based diet "W" produced significantly lower emission compared to the molasses based "M" and "Mbic" diets. The ranking of the diets based on the absolute CH_4 (L/d) production were W< M< Mbic. Likewise, the emissions of CH_4 (L/kg DMI) followed the similar ranking (P<0.05). The W diet emitted significantly lower CH_4 (L/MJ DEI/d) compared to the M and Mbic diet. However, no difference was found in CH_4 (L/MJ DEI/d) between the molasses based M and Mbic diet. When comparing the absolute values of CH_4 (L/d) obtained by the CO_2T and RC_1, a strong correlation (r=0.83) was found between the two measurement techniques (Figure 3). Moreover, regression analysis showed a strong positive correlation of CH_4 (L/d) production estimated by the CO_2T and RC_1 compared with the recommended prediction model by IPCC (2006) (Figure 4).

When comparing the daily CH_4 (L/kg DMI) emissions between the respiration chamber technique (RC_2) with Holstein cows (Hellwing et al., 2012), the estimates were numerically lower for the CO_2T compared to the RC_2 (Table 2). However, the CH_4 (L/kg DMI) using the RC_1 was extremely higher compared to the values obtained by the CO_2T. Furthermore, comparative values of CH_4

(L/kg DMI) between the CO_2T and the IVGT indicated a higher emissions obtained by the CO_2T. A substantial animal variation of CH_4 production (L/d and L/kg DMI) was observed within the diet (Figure 5) on the basis of hourly mean emissions. The coefficient of variation of CH_4 emission (L/d and L/kg DMI) between animals (CV_b) was 7.4-8.0% whereas the within animal variation (CV_w) was 17.4 % using the CO_2T (Table 2).

The measured CO_2 (L/d) production in the RC_1 was 1784±193.5 (mean±SD), numerically higher than the calculated CO_2 production 1709±52.1 (mean±SD) according to the CO_2T. The measured and calculated CO_2 production was strongly (r=0.85) correlated (Figure 6). Moreover, the CO_2 emissions according to the body weight of the animals showed a linear positive correlation between the CO_2T and the RC_1 (Figure 7).

4. Discussion

4.1. Diet effect on methane emissions

Among the diets the lowest methane emission was observed in wheat-based diet "W" compared to the molasses based "M" and "Mbic" diets. The CH_4 reduction effect was probably due to the higher propionate production instead of acetate. Beauchemin et al. (2008) proposed that supplying diets containing high quantities of starch via grain or cereal forages would be an effective mean for CH_4 reduction. All carbohydrate fractions contribute to the CH_4 production of which starch results in the least emission (Fahey and Berger, 1988). The core mechanism is that the starch is the most efficient propionate producing carbohydrate, which probably lower the rumen pH and reduce the proportion of acetate and butyrate (Hassanat et al., 2013), thus, reduce CH_4 release from the rumen. Moreover, Lechartier and Peyraud (2011) described that feeding starch reduce fibrolytic activity and change in the VFA proportion, which might lead to a lower CH_4 emission. The CH_4 reduction effect of wheat based diet in the current study is in the line with Hassanat et al. (2013), who found a significant methane reduction in dairy cows by feeding 30% of starch through 100% of corn silage. In the same line Benchaar et al. (2013) reported that methane production linearly decreased with an increased levels of corn dried distillers grain at the rate of 0, 10, 20 and 30%. In contrast, decreased proportion of starch in the diet with increased level of forage increased the CH_4 emissions in dairy cows (Aguerre et al., 2011).

Unlike starch, sugar was reported to have a higher methanogenic potential (Hindrichsen et al., 2005). In vitro study showed a higher CH_4 emission per unit of OM from sucrose instead of starch (Hindrichsen et al., 2004). The same author describe that the higher methanogenic potential of sugar is probably related to the higher production of butyrate. Likewise, in the present study the molasses based diet (M) showed a higher CH_4 emission compared to the wheat based diet. On the other hand molasses based diet with sodium bicarbonate showed the highest CH_4 yield among the diets. This is probably due to the fact that sugar contents in Mbic diet together with bicarbonate helped to maintain a neutral rumen pH approximately 7 and enhance the activity of the fibrolytic bacteria.

4.2. Method comparison

Respiration chamber is the oldest method in the metabolic study and CH_4 estimation. It accounts for the total emissions from the animals. In contrast, the CO_2T is a newly developed technique where the total CO_2 emission is used as a marker to quantify the CH_4 emission by taking into account the quantitative CH_4:CO_2 ratio from breath of the animals. Most of the CH_4 produced in the rumen are emitted through the eructation (Place and Mitloehner, 2010) and negligible fermentation (up to 13%) occur in the hind gut (Ellis et al., 2008). Therefore, the CO_2T considers majority of the emissions through the breath sample analysis. As shown in Figure 3, the estimated amount of CH_4 (L/d) is strongly correlated between the two techniques (CO_2T and RC_1). However, the high values for CH_4 (L/kg DMI) for the RC_1 (Table 2) is misleading as the CH_4:CO_2 was observed in the cages where the DMI was higher than in the respiration chamber. The calculated CH_4 was divided by the low DMI in the chamber caused a very high CH_4 (L/kg DMI). In the same line, an earlier study reported a lower DMI during the CH_4 measurement in the respiration chamber (Pinares-Patino and Clark, 2008). Furthermore, Thorbek (1980) found that animals fed *ad libitum* in the barn showed a significantly lower DMI when moved into the chamber. The absolute daily emissions in the chamber are varies due to the difference in DMI compare to the normal condition. Therefore, the absolute daily CH_4 production is not suitable for comparison of the methods. However, the amount of CH_4 per unit of DMI is comparable between the methods. In this study the CH_4 (L/kg DMI) was 18% lower in the CO_2T compared to the measured CH_4 (L/kg DMI) in RC_2. The in vitro method was also showing 12% lower CH_4 (L/kg DMI) compared to the RC_2. The estimated CH_4 (L/kg DMI) using CO_2T is within the same range of the results described by Aguerre et al. (2011). Therefore, it can be ascribed that the CO_2T is able to estimate CH_4 with a reasonable accuracy.

4.3. Calculation of Carbon dioxide production and methane estimation

The total CO_2 production of the animals can be calculated using either animal's body mass, growth rate and milk production or using the nutrients intake and utilization of the animals. The calculation of CO_2 from the animals is based on knowledge from 100 years of metabolism experiments. The CO_2 production of the animals is determined by the type of diet and nutrient concentration, levels of intake and body activity. This is very closely related to the nutrient metabolism or heat production of the animals (CIGR, 2002). The accuracy of CH_4 estimation using the CO_2T depends on the accuracy of CO_2 production (Madsen et al., 2014). The calculated (using CO_2T) and measured CO_2 (using RC_1) in this study showed a strongly correlated (r=0.85) with a deviation (±53 L/d) between the techniques. Therefore, it can be stated that the CO_2T precise enough to predict the total CO_2 production with a reasonable accuracy that is comparable with the gold standard respiration chamber. In the CO_2T, the total CO_2 emission is multiplied with the CH_4:CO_2 ratio from the breath samples in order to get the daily CH_4 emission. The CH_4 estimation can be influenced by the diurnal variation of the CH_4:CO_2 ration (Bjerg et al., 2012), which might have an influence on the CH_4 estimation. However, the diurnal variation was considered in the present study by analysing breath samples over several hours a day. From the comparative values of CH_4 (L/d) and CH_4 (L/kg DMI) estimated the CO_2T, RC_1 and RC_2, it appears that the CO_2T is able to estimate CH_4 emission with a

reasonable accuracy and precision. A previous study using CO_2T justified the accuracy and precision of the CH_4 estimates (Haque et al., 2014), in agreement with the present results.

4.4. Animal variation and precision of the methods

Apart from the dietary factors, the genetics of the animals has an influence on the CH_4 emission. Even after correction for the daily feed intake the variation in CH_4 emission remains which referred to the genetic variation or heritability of that trait (Pinares-Patino et al., 2013; Bell et al., 2014). Using respiration chamber, data from sheep were analysed based on 2500 determinations of the 24-h CH_4 production (Blaxter and Clapperton, 1965). This study produced a day to day variation (CV) of CH_4 emissions 7% within animals and 7-8% between animals. Sixteen calorimetric studies with dairy cows showed a wider range of CV (3 to 34 %) for the CH_4 output (Ellis et al., 2010). In a comparative study using SF_6 and chamber technique, Grainger et al. (2007) reported a within animal variation of CH_4 production of 19.6% for SF_6 techniques and 17.8 % for the chamber techniques. The observed between animal variations in this study is analogous to the earlier study by Blaxter and Clapperton (1965) using respiration chamber. The within animal variation of CH_4 emission was in the same line of those reported earlier Grainger et al. (2007) and Ellis et al. (2010). The animal variation of CH_4 emission of this study was in the similar range of the results in a previous study with lactating dairy cows using the same technique (unpublished data). Therefore, it can be stated that the CO_2T is able to estimate CH_4 production from the animals with a reasonable accuracy that is comparable to the chamber technique.

5. Conclusions

The results showed a lower DMI in the respiration chamber (RC_1). The wheat based diet showed significantly lower CH_4 emission (L/d and L/kg DMI). All three diets displayed a lower CH_4 (L/kg DMI) estimated by the CO_2T than the study with respiration chamber (RC_2). Comparable animal variations of CH_4 production were obtained using the CO_2T. Compared to the chamber technique the CO_2T can predict CH_4 emission with a reasonable accuracy and precision. The precision can be improved either by using more animals or longer measurement period.

Conflict of interest

None

References

Aguerre, M.J., Wattiaux, M.A., Powell, J.M., Broderick, G.A., Arndt, C., 2011. Effect of forage-to-concentrate ratio in dairy cow diets on emission of methane, carbon dioxide, and ammonia, lactation performance, and manure excretion. J. Dairy Sci. 94, 3081-3093.

Bates, D.M., Sarkar, D., 2009. lme4: Linear mixed-effects models using S4 classes, R package version 0.99875-6. Retrieved on 17 April 2013, from http://cran.r-project.org/web/packages/lme4/index.html

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.

Bell, M.J., Potterton, S.L., Craigon, J., Saunders, N., Wilcox, R.H., Hunter, M., Goodman, J.R., Garnsworthy, P.C., 2014. Variation in enteric methane emissions among cows on commercial dairy farms. animal 8, 1540-1546.

Benchaar, C., Hassanat, F., Gervais, R., Chouinard, P.Y., 2013. Effects of increasing amounts of corn dried distillers grains with solubles in dairy cow diets on methane production, ruminal fermentation, digestion, N balance, and milk production. J. Dairy Sci. 96, 2413-2427.

Bjerg, B., Zhang, G.Q., Madsen, J., Rom, H.B., 2012. Methane emission from naturally ventilated livestock buildings can be determined from gas concentration measurements. Environ. Monit. Assess. 184, 5989-6000.

Blaxter, K.L., Clapperton, J.L., 1965. Prediction of the amount of methane produced by ruminants. The British journal of nutrition 19, 511-522.

CIGR, 2002. Climatization of animal houses – heat and moisture production at animal and house level. In: Pedersen, S., Sällvik, K. (Eds.), 4Th Report of CIGR Working Group (Research Centre Bygholm, Danish Institute of Agricultural Sciences, P.O Box 536, DK-8700 Horsens, Denmark.

Ellis, J.L., Bannink, A., France, J., Kebreab, E., Dijkstra, J., 2010. Evaluation of enteric methane prediction equations for dairy cows used in whole farm models. Global Change Biol. 16, 3246-3256.

Ellis, J.L., Dijkstra, J., Kebreab, E., Bannink, A., Odongo, N.E., McBride, B.W., France, J., 2008. Aspects of rumen microbiology central to mechanistic modelling of methane production in cattle. J. Agric. Sci. 146, 213-233.

Fahey, G.C., Berger, L.L., 1988. Carbohydrate nutrition of ruminants. In The Ruminant animal : digestive physiology and nutrition. D. C. Church, ed. Prentice Hall Inc., Englewood Cliffs, NJ.

Friggens, N.C., Oldham, J.D., Dewhurst, R.J., Horgan, G., 1998. Proportions of volatile fatty acids in relation to the chemical composition of feeds based on grass silage. J. Dairy Sci. 81, 1331-1344.

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), Rome, Italy.

Grainger, C., Clarke, T., McGinn, S.M., Auldist, M.J., Beauchemin, K.A., Hannah, M.C., Waghorn, G.C., Clark, H., Eckard, R.J., 2007. Methane emissions from dairy cows measured using the sulfur hexafluoride (SF6) tracer and chamber techniques. J. Dairy Sci. 90, 2755-2766.

Haque, M.N., Cornou, C., Madsen, J., 2014. Estimation of methane emission using the CO2 method from dairy cows fed concentrate with different carbohydrate compositions in automatic milking system. Livestock Science 164, 57-66.

Hassanat, F., Gervais, R., Julien, C., Massé, D.I., 2013. Replacing alfalfa silage with corn silage in dairy cow diets: Effects on enteric methane production, ruminal fermentation, digestion, N balance, and milk production. J. Dairy Sci. 96, 4553-4567.

Hellwing, A.L.F., Brask, M., Lund, P., Weisbjerg, M.R., 2012. Effect of carbohydrate source and rumen pH on enteric methane from dairy cows. In: Hassouna, M., Guingand, N. (Eds.), International symposium on Emission of gas and dust from livestock (EMILI 2012), Saint-Malo, France, pp. 206-208.

Hindrichsen, I.K., Wettstein, H.R., Machmuller, A., Jorg, B., Kreuzer, M., 2005. Effect of the carbohydrate composition of feed concentratates on methane emission from dairy cows and their slurry. Environ. Monit. Assess. 107, 329-350.

Hindrichsen, I.K., Wettstein, H.R., Machmuller, A., Soliva, C.R., Knudsen, K.E.B., Madsen, J., Kreuzer, M., 2004. Effects of feed carbohydrates with contrasting properties on rumen fermentation and methane release in vitro. Can. J. Anim. Sci. 84, 265-276.

Hongmin Dong, Joe Mangino, Tim A. McAllister, Jerry L. Hatfield, Donald E. Johnson, Keith R. Lassey, Lima, M.A.d., Romanovskaya, A., 2006. Emissions from livestock and manure management.

IPCC, 2006. 2006 IPCC Guidelines for National Greenhouse Gas Inventories. Intergovermental Panel on Climate Change. Accessed January, 2012, http://www.ipcc-nggip.iges.or.jp/public/2006gl/index.html.

Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. J. Anim. Sci. 73, 2483-2492.

Kuznetsova, A., Brockhoff, P.B., Christensen, R.H.B., 2012. Tests for random and fixed effects for linear mixed effect models (lmer objects of lme4 package). Retrieved on 27 April 2013, from http://cran.r-project.org/web/packages/lmerTest/index.html.

Lechartier, C., Peyraud, J.L., 2011. The effects of starch and rapidly degradable dry matter from concentrate on ruminal digestion in dairy cows fed corn silage-based diets with fixed forage proportion. J. Dairy Sci. 94, 2440-2454.

Madsen, J., Bjerg, B.S., Hvelplund, T., Weisbjerg, M.R., Lund, P., 2010. Methane and carbon dioxide ratio in excreted air for quantification of the methane production from ruminants. Livest. Sci. 129, 223-227.

Madsen, J., Chwalibogb, A., Hvelplundc, T., Weisbjergc, M.R., 2014. Use of carbon dioxide as a breath marker. Book of abstracts of 65th Annual Meeting of the European Federation of Animal Science, Copenhagen, Denmark, p. 408.

Pedersen, S., Blanes-Vidal, V., Joergensen, H., Chwalibog, A., Haeussermann, A., Heetkamp, M.J.W., Aarnink, A.J.A., 2008. Carbon dioxide production in animal houses: A literature review. Agricultural Engineering International: CIGR Ejournal. Retrieved on 07 July 2014, from http://www.cigrjournal.org/index.php/Ejounral/article/viewFile/1205/1132

Pinares-Patino, C.S., Clark, H., 2008. Reliability of the sulfur hexafluoride tracer technique for methane emission measurement from individual animals: an overview. Aust J Exp Agric 48, 223-229.

Pinares-Patino, C.S., Hickey, S.M., Young, E.A., Dodds, K.G., MacLean, S., Molano, G., Sandoval, E., Kjestrup, H., Harland, R., Hunt, C., Pickering, N.K., McEwan, J.C., 2013. Heritability estimates of methane emissions from sheep. Animal 7 Suppl 2, 316-321.

Place, S.E., Mitloehner, F.M., 2010. Invited review: Contemporary environmental issues: A review of the dairy industry's role in climate change and air quality and the potential of mitigation through improved production efficiency. J. Dairy Sci. 93, 3407-3416.

R Development Core Team, 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. R version 3.0.0. Retrieved on 17 April 2013, from http://www.R-project.org.

Storm, I.M.L.D., Haque, M.N., Madsen, J., Hansen, H.H., 2012a. Comparison of methane emissions from cattle assessed by three different methods: open-circuit respiration chambers, in vitro gas production and the CO2-method. In: Hassouna, M., Guingand, N. (Eds.), International symposium on Emission of gas and dust from livestock (EMILI 2012), Saint-Malo, France, pp. 346-349.

Storm, I.M.L.D., Hellwing, A.L.F., Nielsen, N.I., Madsen, J., 2012b. Methods for measuring and estimating methane emission from ruminants. Animals 2, 160-183.

Thorbek, G., 1980. Studies on Protein and Energy Metabolism in Growing Calves. Landhusholdingsselskabets forlag: Copenhagen, Denmark, .

van Kessel, J.A.S., Russell, J.B., 1996. The effect of pH on ruminal methanogenesis. FEMS Microbiol. Ecol. 20, 205-210.

Table 1. Ingredients and chemical composition of three diets

Components	W	M	Mbic
Composition of the ration (g/kg DM)			
Grass-clover silage	494	494	490
wheat	353		
Sugar beet molasses		353	350
$NaHCO_3$			9.3
Soybean meal	141	141	140
Mineral and vitamins	12	12	12
Chemical composition (g/kg DM)			
Ash	60.6	97.5	103
Protein[1]	172	177	175
Fat	25.8	16.5	16.7
Starch	243	7.6	3.8
Sugar	34.2	241	238
NDF	318	280	277
Energy concentration in diet (MJ/kg DM)			
Gross energy[2]	18.8	18.0	17.9

W = diet with ground wheat; M = diet with sugar beet molasses; MBic: diet with sugar beet molasses and bicarbonate; [1] Feed Table value; [2] Calculated according to Volden and Nielsen (2011).

Table 2. Methane production and ratio between CH_4 and CO_2 for three diets

Parameters	Systematic effect			Random effect	
	W	M	Mbic	CV_{bc}	CV_{wc}
CH_4 (L/d)	126.7[a]	144.8[b]	154.0[c]	8.0	17.4
[1]CH_4 (L/d)	142.9[a]	148.6[a]	151.5[b]	5.7	18.0
CH_4 (L/kg DMI)	25.1[a]	28.2[b]	30.2[c]	7.4	17.4
[1*]CH_4 (L/kg DMI)	43.7[a]	44.6[a]	46.6[b]	5.3	18.9
[2]CH_4 (L/kg DMI)	32.1	33.0	35.9	-	-
[3]CH_4 (L/kg DMI)	29.6	29.0	29.6	-	-
CH_4 (L/MJ DEI/d)	1.7[a]	2.0[b]	2.1[b]	7.6	17.3
CH_4:CO_2	0.072[a]	0.085[b]	0.091[c]	8.5	17.6

[1]Calculated absolute CH_4 using CO_2 production measured in the respiration Chamber (RC_1); [1*]Methane per kg of DMI followed by the absolute CH_4 in the RC_1; [2]Measured in the respiration chamber (RC_2) study with Holstein cows (Hellwing et al., 2012); [3]Estimated by in vitro gas production technique (IVGT) (Storm et al., 2012a); W = wheat; M = Molasses; Mbic = Molasses + bicarbonate; CV_{bc} = coefficient of variation between cows; CV_{wc} = coefficient of variation within cows.

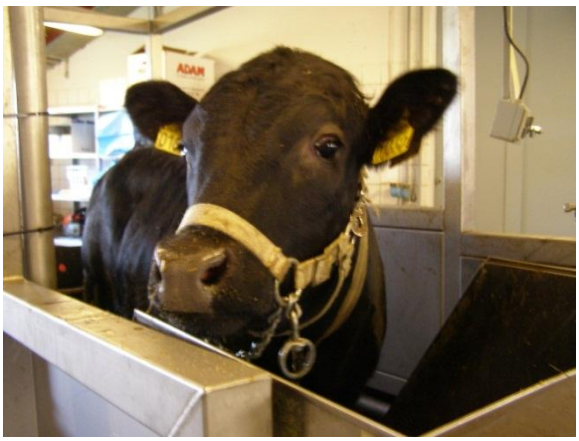


Figure 1. Methane measurement in the metabolic cage

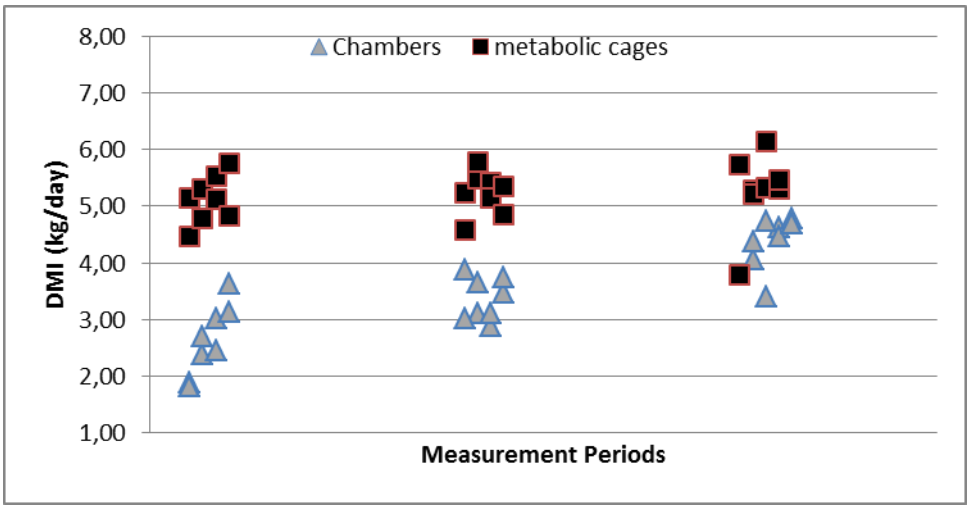


Figure 2. Dry matter intake in the metabolic cages and respiration chambers (RC_1). The DMI values were considered from two days in cage and two days in respiration chamber for each heifer. In addition to the three heifers, the DMI for one extra animal were included.

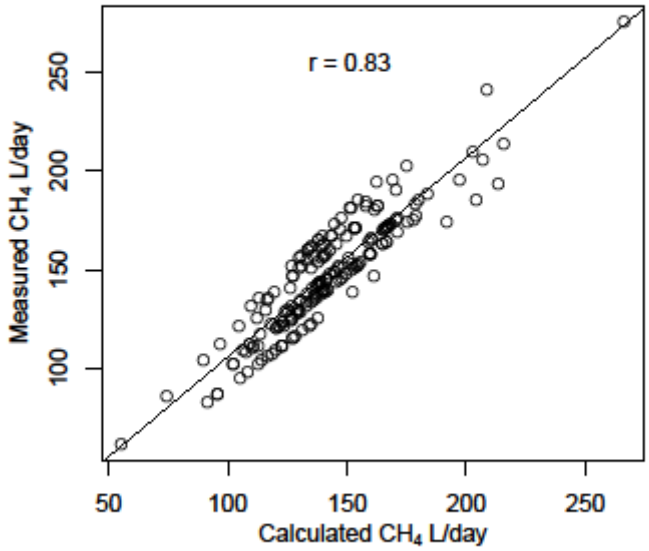


Figure 3. Regression analysis of CH_4 emissions (L/d) from the heifers obtained by the CO_2T (calculated) and RC_1 (measured).

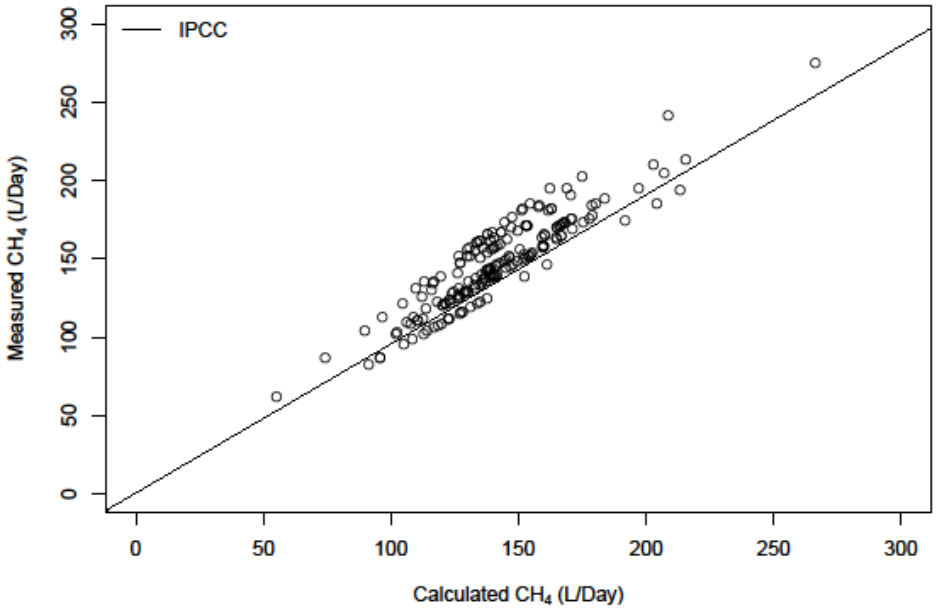


Figure 4. Regression analysis of CH_4 (L/d) emissions from the heifers followed by the CO_2T (calculated) and RC_1 (measured). The regression line is fitted for the calculated CH_4 according to IPCC recommendation.

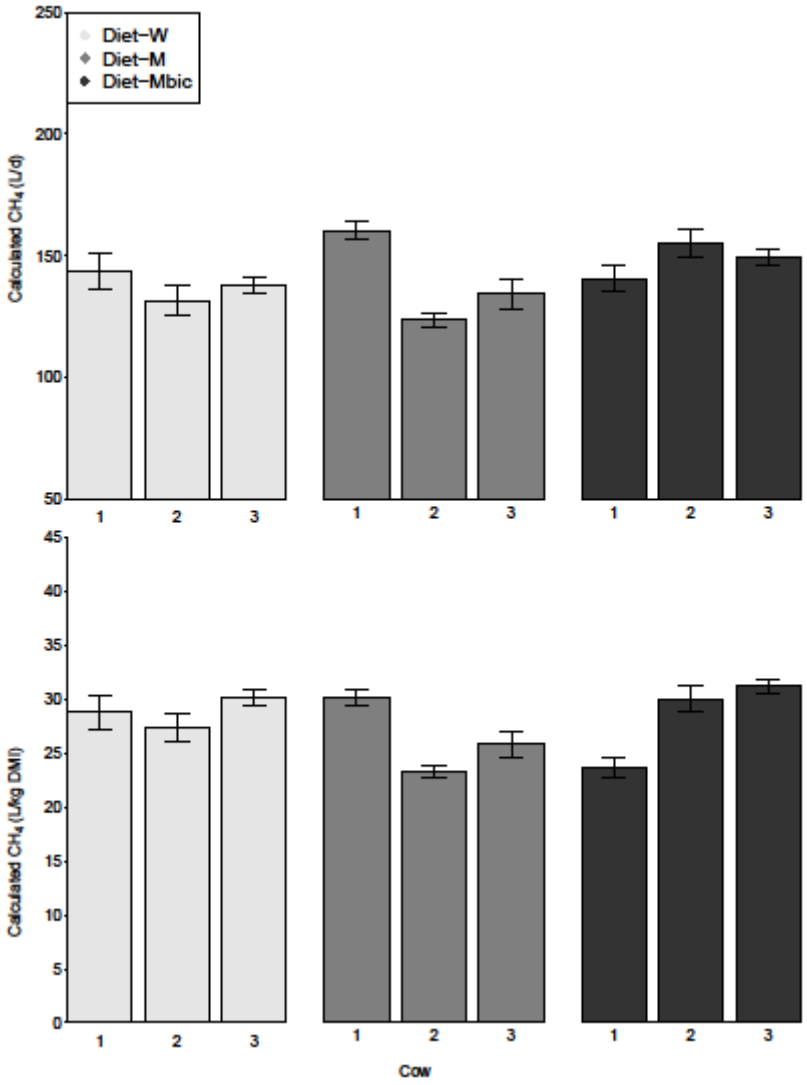


Figure 5. Diet effects on CH_4 output from the experimental heifers. The bars indicate mean±SE.

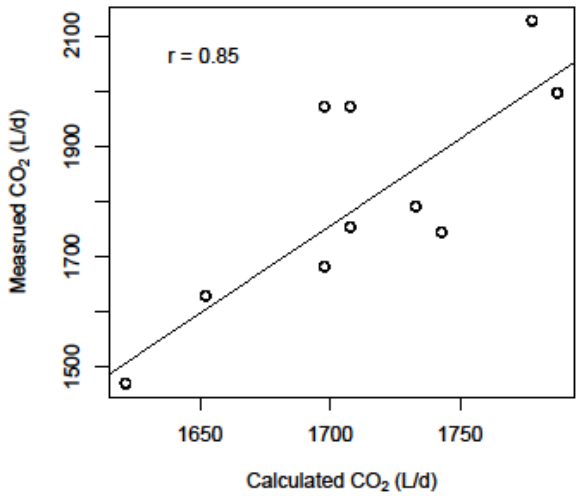


Figure 6. Regression of the daily average CO_2 (calculated by the CO_2T) and the measured CO_2 in the RC_1.

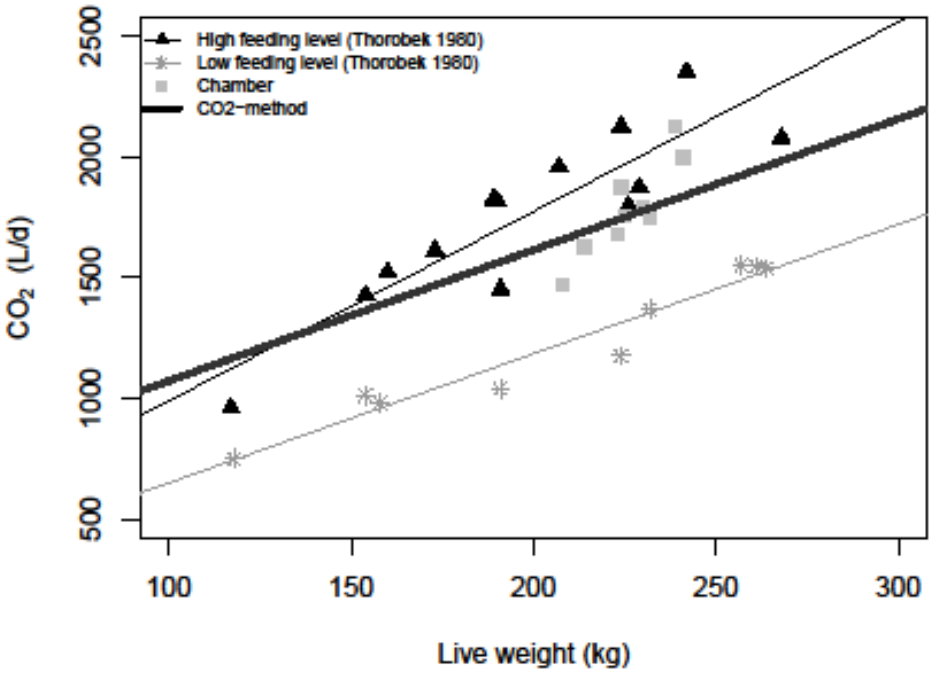


Figure 7. Calculated and measured CO_2 obtained by the CO_2T and RC_1 compared with the respiration chamber (Thorbek, 1980) results at low and high feeding levels.

Paper IV

Individual variation and repeatability of methane production from dairy cows estimated by the CO_2-method in automatic milking system

Manuscript submitted to Animal: An International Journal of Animal Bioscience (ANIMAL-14-50764).

Individual variation and repeatability of methane production from dairy cows estimated by the CO_2-method in automatic milking system

M. N. Haque*, C. Cornou, J. Madsen*

Department of Large Animal Sciences, Faculty of Health and Medical Sciences, University of Copenhagen, Groennegaardsvej 2, DK-1870 Frederiksberg C, Denmark

Running head: Individual variation of CH_4 emission in dairy cows

*Corresponding authors
Email addresses: naha@sund.ku.dk (M. N. Haque), jom@sund.ku.dk (J. Madsen)

ABSTRACT

The objective of this study was to investigate the individual variation, repeatability and phenotypic correlation of methane (CH_4) emission from dairy cows measured in two different years. A total of 21 cows were used with an average body weight of 621 ± 14.0 and 640 ± 8.0 kg (mean ± s.d.), and milk production of 29.1 ± 6.54 and 33.4 ± 6.00 kg/day (mean ± s.d.) during the 1st and 2nd year, respectively. The cows were housed in a loose housing system fitted with automatic milking system (AMS). The total mixed ration (TMR) was fed to the cows *ad libitum* in both of the years. In addition, they were offered concentrate in the AMS based on their milk yield. The CH_4 and CO_2 production from the cows were analysed using gas analyser "Gasmet DX-4030". The dry matter intake (DMI) was 19.8 ± 0.96 and 23.1 ± 0.78 kg/day (mean ± s.d.) and the energy corrected milk (ECM) production was 30.8 ± 8.03 and 33.7 ± 5.25 kg/day (mean ± s.d.) during the 1st and 2nd year, respectively. The DMI and ECM had a significant influence ($P < 0.001$) on the CH_4 (l/day) yield during both of the years. The daily CH_4 emission was significantly higher ($P < 0.05$) during the 2nd year compared to the 1st year. The DMI (described by the ECM production) appeared to be the key factor of the variation in CH_4 release. A fair correlation (r = 0.54) of the CH_4 productions was observed between the years. A strong positive phenotypic correlation (r = 0.70) was found in the CH_4 emission between the years when it was standardized using the ECM production (30 l/day). The diurnal variation of CH_4 (l/h) output showed a significantly lower ($P < 0.05$) emission during the night (0000 to 0800 h). The between cows variations of the CH_4 (l/day, l/kg DMI and l/kg ECM) were lower compared to the within cow variation for the 1st and 2nd year, respectively. The repeatability of CH_4 yield (l/day) was 0.36 and 0.41 in the 1st and 2nd year, respectively. In conclusion, the herd average CH_4 (l/day) was significantly lower in the second years due to higher DMI (kg/day). The between cow variation of CH_4 (l/day) was lower than the within cow variation. A strong positive phenotypic correlation of CH_4 (l/day) was found between the years at a standardized ECM production.

Keywords: breath, diurnal variation, methane, phenotypic correlation, dairy cows

Implications

Methane (CH_4) production between cows is always variable. The interference of the animal's behaviour can easily influence this variation. The variation of CH_4 production remained even after standardization of the feed intake and milk yield. When selecting cows with low CH_4 output for long term CH_4 mitigation, it is necessary to consider the animal variations. The yearly relation of CH_4 production could also be an indicator to select low emitter cows. The result of this study regarding animal variation and yearly correlation of CH_4 emission will provide valuable information for CH_4 mitigation through animal breeding.

Introduction

The livestock sector represents a significant source of greenhouse gas (GHG) emissions worldwide, generating carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (N_2O) throughout the production process. This sector often comes in focus because of its greater impact on the environment. A recent report by Gerber *et al.* (2013) described that the majority of the CH_4 emissions occurred from the livestock sector due to enteric fermentation and feed production. In the livestock sector, cattle are the highest contributor of greenhouse gas (GHG) emission, which account for 65% (4.6 gt CO2 eq). Out of the total emission, cattle emit most of the enteric CH_4, i.e. about 77%, followed by the other domesticated species (Gerber *et al.*, 2013). Another consideration beside environmental pollution is that between 2 and 12% of the ingested gross energy is loss through CH_4 emission (Johnson and Johnson, 1995); a loss of energy that could potentially be used by the animals. The CH_4 emissions from the animals vary according to the level of feed intake, type of carbohydrate, feed processing, addition of lipids, alteration of rumenal microflora (Johnson and Johnson, 1995) and measurement techniques (Vlaming *et al.*, 2008). Besides, it can also vary due to the genetic variation of the animals (Pinares-Patino *et al.*, 2013). One of the earlier study using standard respiration chamber reported a coefficient of variation (CV) of 7% for within-animal variation in the CH_4 production and of 7-8% for between-animal variation (Blaxter and Clapperton, 1965). More recently, several authors reported a CV of 4.3% for within-animal and 17.8% for between-animal using open-circuit calorimetry (Grainger *et al.*, 2007). Using the SF_6 technique, Vlaming *et al.* (2008) mentioned a wider range of variation in CH_4 emission for two different diets (6.91 - 10.09 within cow and 6.23 - 27.79% between cow, respectively). Moreover, in grazing condition, Lassey *et al.* (1997), Boadi *et al.* (2002) and McNaughton *et al.* (2005) reported a between-animal variation of, respectively, 11.5, 15.5 and 25% of CV, using the SF_6 technique. In a comparative study using two different techniques, Grainger *et al.* (2007) mentioned a higher within cow variation (CV = 19.6%) for SF_6 techniques compared to the chamber technique (CV = 17.8%). Till date, most of the studies estimated the animal variation in CH_4 production either by using the traditional chamber technique or the SF_6 techniques, where handling and confinement of the animals is required. A drawback of these methods is that they might have an influence on the normal metabolism of the animals. In this study, we assume that the animal should be free from any influential factors in order to get a real feature of the individual variability in CH_4 emission. We hypothesize that the animal variation in CH_4 emission would be lower if the measurements are taken from their natural environment. In dairy industry, the automatic milking systems (AMS) reduce

human's involvement and interaction with the cows, and thus allows the cows free movements. Therefore, under this condition, normal feeding and milking behaviour as well as rumen metabolism and gas production can be expected. The "CO_2-method", a newly developed technique for CH_4 estimation, was used in this study. This method is non-invasive and measures the CH_4 emissions from the cows by keeping them in their natural environment. The objectives of this study were i) to investigate the individual variation and the repeatability of CH_4 production measured in the AMS and ii) to investigate the phenotypic correlation of CH_4 emission of the individual cows in two different years.

Materials and method

Animals, experimental design and feeding

A total of 21 dairy cows with an average body weight of 619 ± 14.2 and 640 ± 8.0 kg (mean ± s.d.) and milk production of 29.1 ± 6.5 and 33.4 ± 6.0 kg/day (mean ± s.d.) were used in the 1st and 2nd year, respectively. The cows were housed in a loose housing system with adequate ventilation and automatic milking system (AMS). The study was conducted without interfering with the feeding and management planned by the farm. During both years, the measurements were taken from the same cows and in the same AMS. For the seven days experimental period, the temperature in the barn followed the outside temperature (15 and 22°C). The cows were offered a total mixed ration (TMR) *ad libitum* (Table 1) in both of the years. In addition to the TMR, cows were offered concentrate in the AMS based on their daily average milk production. A total of 57 cows were milked in the AMS, out of which 23 cows were common in both of the years. Among the common cows, two cows showed abnormal milking behaviour. One cow had just calved and only visited the AMS for 3 days out of the 7 days measurements. The other cow was visited the AMS once per day and were treated with lameness. These two cows were therefore excluded from the analysis, which resulted in 21 studied cows.

Gas measurement

The CH_4 and CO_2 emissions from the cows were analysed using a continuous gas analyser "Gasmet DX-4030" based on the Fourier Transformed Infrared Radiation. The inlet filter of the Gasmet was fitted on the feeding pen of the AMS in order to get the concentrated breath samples from the individual cows. The breath samples pass through the inlet filter and thereafter through the Gasmet to determine the concentrations of the CH_4 and CO_2. The measurements were performed every 15 seconds over 24 hours and 7 consecutive days during milking in the AMS. Each cow was visiting the AMS at least two times a day (ranging from 1 to 4, average 2.54). Before the first measurement, the Gasmet was calibrated with standard gases to check the accuracy of the measurements. During each measurement day, the Gasmet was disconnected from the sampling inlet for 10 minutes to get the barn concentration of CH_4 and CO_2. This concentration of CH_4 and CO_2 was used as a correction factor for the entire experimental period to get the actual breath concentration of CH_4 and CO_2. The measurements were remotely monitored via the internet using TeamViewer.

Calculations

Identification numbers, entrance and exit times of each individual cow were recorded in a computer connected to the AMS. These data were matched with the breath analysis data from the Gasmet. All calculations regarding the CH_4 and CO_2 emissions from the cows were done according to the CO_2-method (Madsen *et al.*, 2010). The barn concentrations of CO_2 (495.8 and 625.5 ppm) and CH_4 (23.2 and 25.8 ppm) were obtained during the measurements in 1st and 2nd year, respectively. These concentrations were subtracted from the exhaled air concentrations of the cows to get the actual breath concentration of each sample. All values of the corrected CO_2 below 400 ppm were removed in order to avoid the influence of the samples containing a very low concentration of CH_4 and CO_2. The ratio between CH_4 and CO_2 (CH_4:CO_2) was thereafter calculated.

The concentrate intake in the AMS was measured individually on a daily basis while the TMR intake was considered as herd average. The individual total DMI was calculated by adding the individually recorded concentrate DMI to the corrected TMR dry matter intake (TMRDMI) using Equation (1) according to Kristensen and Ingvartsen (2003). The heat production (HP) (watt) of the cows was calculated following Equation (2), described by CIGR (2002). The excretion of CO_2 (l/day) was calculated according to Pedersen *et al.* (2008), shown in Equation (3). The amount of CH_4 (l/day) was calculated as described by Madsen *et al.* (2010) using Equation (4). Energy corrected milk (ECM) (kg) was calculated following Equation (5), according to Sjaunja *et al.* (1991).

$$TMRDMI = a + 0.5(b - c) + d \quad (1)$$

$$HP = 5.6 * BW^{0.75} + [(Y * 22) + (1.6 * 10^{-5} * P^3)] \quad (2)$$

$$CO_2 = HPU * 180 * 24 \quad (3)$$

$$CH_4 = CO_2 * \frac{CH_4}{CO_2} \quad (4)$$

$$ECM = Y * (0.383 * milk\ fat + 0.242 * milk\ protein + 0.7832)/3.14 \quad (5)$$

where:
a = average TMR intake;
b = average concentrate intake;
c = concentrate intake of the individual cows during the experimental periods;
d = correction factor for the lactation number: d = -1.61 was considered for first lactation and d = 0.39 for the second and subsequent lactations
HP = heat production of the animals
$BW^{0.75}$ = metabolic body weight of the animals
Y = milk yield of the cows

P = days of pregnancy of the cows
HPU = heat producing unit $\frac{HP}{1000}$
180 = L of CO_2/HPU/h
ECM = energy corrected milk

Statistical analyses

Data were analysed with linear mixed models using the lmer function fitted by Restricted maximum likelihood (REML) from the package "lme4" (Bates and Sarkar, 2009) using the software R (R Development Core Team, 2013). An extension package "lmerTest" was used to get the P value directly from the function lmer (Kuznetsova *et al.*, 2012). Individual daily mean emissions were considered for the interpretation of the results. The analyses focused on making inference on the individual variation and repeatability of CH_4 production (l/day), CH_4 (l/kg DMI) and CH_4 (l/kg ECM). Models were fitted on yearly subsets of the data. The body weight (BW), dry matter intake (DMI), energy corrected milk (ECM), parity and days of pregnancy were included as fixed effects in the primary model fitted with maximum likelihood method (ML). Cow and number of visits to the AMS were included as random effects. The final model (Equation 6) was confirmed by stepwise removal of the non-significant variables. The significance of the fixed effects was assessed by F-ratio test and the random effects by likelihood-ratio tests. The model validations were performed with analysis of variance (ANOVA) based on the Akaike Information Criterion. The model residuals were checked for normality by visual inspection of qqplots. The final model is:

$$y_j = \mu + X\beta_j + X\gamma_j + \delta_j + C_j + \varepsilon_j \quad (6)$$

Where, y_j is the response variable y = [CH_4 l/day, CH_4 l/kg DMI, CH_4 l/kg ECM and CH_4: CO_2 ratio] of cows j and μ is the overall mean. The fixed effects are the $X\beta_j$ = DMI (kg/day) of cow j; $Y\boldsymbol{\gamma}_j$ = ECM (kg/day) of cow j; δ_j = parity of cow j; C_j = random effect of cow j and ε_j are the residual errors. Model estimates were extracted using the glht function from the "multcomp" package (Hothorn *et al.*, 2008). Coefficients of variation of CH_4 emissions between-cows (CV_{bc}) and within cow (CV_{wc}) were calculated from the variance components of the model (Equation 6) using Equations 7 and 8. The variance components were defined as the individual random effects (σ_α^2) and the variance of the random error (σ_ε^2).

$$CV_{bc} = \frac{\sigma_\alpha}{\bar{x}} * 100 \quad (7)$$

$$CV_{wc} = \frac{\sigma_\varepsilon}{\bar{x}} * 100 \quad (8)$$

The same model (Equation 6) was fitted to obtain the repeatability (R), calculated as the proportion of between animals variance with respect to the total variance as:

$$R = \frac{\sigma_{\alpha}^2}{\sigma_{\alpha}^2+\sigma_{\varepsilon}^2} \quad (9)$$

In order to investigate the phenotypic correlation of individual cows, the differences of CH_4 emissions in the two successive years were assessed by using the following model:

$$y_{ij} = \mu + \lambda_i + X\beta_{ij} + X\gamma_{ij} + \delta_j + C_j + \varepsilon_{ij} \quad (10)$$

Where, λ_i is the year of measurement with i = (1:2 years); $X\beta_{ij}$ = DMI (kg/day) of year i and cow j; $Y\boldsymbol{\gamma}_i$ = ECM (kg/day) of year i and cow j; δ_j = parity of for cow j; C_j = random effect of cow and ε_{ij} are the residual errors.

Yearly data subsets of the daily mean emissions during milking were considered for visualization of the diurnal variation of CH_4 production following:

$$y_{ij} = \mu + \partial_i + X\beta_j + Y\boldsymbol{\gamma}_j + \delta_j + C_j + \varepsilon_{ij}, \quad (11)$$

where, μ is the overall mean; ∂_i = hours of measurements in a day with i = (1:24 hours); $X\beta_j$ = DMI (kg/day) of cow j; $Y\boldsymbol{\gamma}_j$ = ECM (kg/day) of cow j; δ_j = parity of cow j; C_j = random effect of cow j and ε_{ij} are the residual errors.

Results

Feed intake, milk production and CH_4 emission in two years

Means and standard deviations from the 1st and 2nd year are shown in Table 2. The body weight (BW, kg), milk production (kg/day), energy corrected milk (ECM, kg/day) and dry matter intake (DMI, kg/day) was higher during the 2nd year compared to the 1st year. The dry matter intake was 19.8 ± 0.96 kg/day (mean ± s.d.) with a range of 17.5 to 20.6 kg/day during the 1st year, whereas DMI (kg/day) in the 2nd year was higher 23.1 ± 0.78 (mean ± s.d.) with a range of 22.5 to 25.3 kg/day. The ECM production during the 1st year was 30.8 ± 8.00 kg/day (mean ± s.d.), ranged from 18.1 to 48.6, and was lower than the production in the 2nd year 33.3±5.33 (mean ± s.d.) that ranged from 25.2 to 47.0 kg/day. A positive correlation was found when the CH_4 output (l/day) was plotted according to the ECM (kg/day) (Figure 1a). Moreover, the correlation between the CH_4 outputs (l/day) and the DMI (kg/day) was positive (Figure 1b). A clear difference of CH_4 emission, ECM production and DMI was observed between the two years. When the CH_4 emissions were expressed per unit of ECM production, a negative correlation was found between the CH_4 outputs (l/kg ECM) and the ECM kg/day (Figure 1c). When the amount of CH_4 (l/kg DMI) was plotted against the DMI kg (Figure 1d), a positive correlation was observed for the 1st year, whereas the observed correlation was negative for the 2nd year.

Phenotypic correlation of CH_4 emission between two years

When data from the two years are plotted against each other, a fair correlation (r = 0.54) was observed in the CH_4 emissions between the years (Figure 2a). This correlation was higher (r = 0.70) when calculated at a standardized ECM production (30 l/day) (Figure 2b). This correlation can be referred to the phenotypic trait of CH_4 (l/day). A similar correlation was observed for the $CH_4 : CO_2$ ratio between the years (Figure 2 c and d). The values of the response variables (CH_4 l/day, CH_4 l/kg ECM and $CH_4 : CO_2$) were significantly lower ($P < 0.05$) in the 1st year compared to the 2nd year. However, the CH_4 (l/kg DMI) was similar in both of the years.

Variation and repeatability of CH_4 production in two years

The CH_4 emissions along with its variability and repeatability were obtained from the fitted model (6) using the yearly data subsets (Table 3). The daily emission of CH_4 (l/day, l/kg DMI and l/kg ECM) were significantly lower ($P < 0.05$) during the 1st year compared to that of the 2nd year. The between cows variation of the CH_4 emission (l/day, l/kg DMI and l/kg ECM) was lower (CV_{bc} = 8.8 - 9.1%) than the within cow variation (CV_{wc} = 15.7 - 16.4) during the 1st year. The range of the variation during the 2nd year was narrower (CV_{bc} = 5.9 - 6.1 and CV_{wc} = 8.6 - 9.1) as compared to the 1st year. Similarly, the variation of the $CH_4 : CO_2$ ratio was lower during the 2nd year (CV_{bc} = 6.2 and CV_{wc} = 8.8) as compared to the variation during the 1st year (CV_{bc} = 8.4 and CV_{wc} = 15.9).
The repeatability (R) for CH_4 emission (l/day, l/kg DMI and l/kg ECM) was lower (0.35 - 0.37) during the 1st year than during the 2nd year (0.40 - 0.41). Likewise, the $CH_4 : CO_2$ ratio was more repeatable in the 2nd year (0.41), as compared to the observed repeatability during the 1st year (0.34).

Diurnal variation of CH_4 emission

The diurnal variation of CH_4 (l/h) during the two years is shown in Figure 3. During the 2nd year, the diurnal variation indicated a declining emission between 0000 h 0800 h with the lowest emission at 0800 h. The emission suddenly reached a peak level at 0900 h and continued with the same magnitude up to 1600 h. The CH_4 output at this time ranged from 24-27 l/h. After 1600 h the emission rate declined. During the 1st year, a similar pattern was found with a more variable emission over the time.

When the CH_4 emission rate (l/h) was aggregated into time intervals (0000 – 0600 h = night; 0601 - 1200 h = morning; 1201 - 1800 h = afternoon, and 1801 - 2359 h = evening), a significant difference (data were not shown) was found over the 6 hours intervals ($P = 0.01$) during 2nd year. However, during the 1st year the CH_4 (l/h) emission rate was not different except for the lower emission at night ($P = 0.02$).

Discussion

The results of this study have an implication in the selection of cows with low CH_4 emission for breeding purpose. For the first time, the CH_4 emission has been quantified from two different years for the same cows in a large scale dairy farm providing a similar diet in both years. Data from the same cows in two years were used to test different aspects of variability in CH_4 emission over the time.

Key source of variation for CH_4 emission

Concentration of breath samples

The estimation of CH_4 emission using breath samples of cows indicates a considerable variation in the total CH_4 emissions. The concentration of the breaths collected by the inlet filter of the GASMET^{TM} depends on the position of the nose of the animals. More importantly, the concentration of CH_4 depends on whether or not the breaths are coming from the rumen. This study showed a higher CV of the individual breath concentration (Figure 4a). The same evidence was described by Haque *et al.* (2014) in a previous study. The substantial variation in the individual breath concentrations are the reflection of the normal biological rhythm. In this connection, Garnsworthy *et al.* (2012a) stated a certain variation in eructation frequency and that the CH_4 concentration in eructation is correlated with the differences in daily CH_4 emissions. Unlike the respiration chamber technique, the non-invasive methods considered samples which have an ambient exposure. Hence, some changes in the concentration might occur. The average concentration of the CO_2 in breath typically ranges between 30,000 and 50,000 ppm. To get a typical breath concentration through sampling inlet filter is very rare and is mostly influenced by the physiology of the animals and the exposure of the breath samples to the ambient air. However, trapping 2 to 3% of breath samples through the sampling device was mentioned to be sufficient for a precise estimation of CH_4 emission in animals (Madsen *et al.*, 2010). In terms of variation, the individual breath concentration shows very large fluctuations, which make it improper for CH_4 estimation. As shown in Figure 4, the coefficient of variation gradually decreased when the visit average (Figure 4b) or day average (Figure 4c) was considered. Therefore, in order to be precise in the CH_4 estimation through the breath sample analysis, it is important to consider the mean of several individual samples such as emission rate per visit or per day.

Dry matter intake and ECM production

Most of the studies agreed that DMI is the key factor of daily CH_4 output (Blaxter and Clapperton, 1965, Johnson and Johnson, 1995, Grainger *et al.*, 2007) which is secondly being determined by the diet digestibility (Blaxter and Clapperton, 1965, Johnson and Johnson, 1995) and the amount of concentrate or lipid supplement (Beauchemin, 2009). In this study, the DMI and ECM had a significant influence on CH_4 yield during both years. The effect was very likely because the increased amount of DMI was mediated with the increased of body mass and ECM production.

Therefore, under a commercial farming situation, where recording individual DMI is rare, the ECM can be used to explain the variation of CH_4 production. Higher ECM production and DMI in the 2nd year resulted in significantly ($P < 0.05$) higher CH_4 (l/day). The CH_4 (l/kg DMI) was similar in both years, which supports the fact that more CH_4 is released at a higher DMI. In this connection, Boadi and Wittenberg (2002) also mentioned that 64% of the variation in CH_4 production is explained by the DMI. The results of this study are also in line with several recent findings where the diet effects were investigated on the CH_4 emissions (Beauchemin, 2009, Doreau *et al.*, 2011). In addition, Grainger *et al.* (2007) and Garnsworthy *et al.* (2012b) described similar results where DMI was mentioned as the primary determinant of CH_4 production. Moreover, the negative correlation between CH_4 (l/kg ECM) and amount of ECM (kg/day) in this study supports previously described results (Tamminga *et al.*, 2007).

Levels of variation

In a typical feed evaluation study using a respiration chamber, the animal variation of CH_4 production is minimized by a fixed amount of feed provided to the animals. Nevertheless, a significant variation between the animals remained. A large scale on farm CH_4 measurement study (Garnsworthy *et al.*, 2012b) indicated a within cow variation of 23% (CV), which is well above the between cow variation of 6%. Based on the same data and using a mixed effect, the latter showed a larger within cow variation (15%) compared to between cow variations (8%). Between animals variation of 26.6% and 25.3% have been reported for dairy and beef heifers with an *ad libitum* and restricted feeding, respectively (Boadi and Wittenberg, 2002).

Blaxter and Clapperton (1965) analysed the results of 23 investigations in which sheep were offered the same amount of the same diet and of other 30 investigations in which the intake was scaled according to the BW. In both analyses the reported CV in CH_4 emission were 7 to 8% between-animal and 5 to 7% within-animal. A wider range of between animals variability in CH_4 output was reported in some studies where feed was offered *ad libitum*. Results of 16 calorimetric studies in dairy cows showed a wider range of coefficients of variation (3 to 34 %) for the CH_4 output. The large range of DMI was put forward as the main reason for the large variation in CH_4 emissions (Ellis *et al.*, 2010). Using respiration chamber and SF_6 tracer technique to measure the CH_4 output from lactation dairy cows fed *ad libitum*, Grainger *et al.* (2007) reported variability of 19.6% for SF_6 techniques and of 17.8 % for the chamber techniques. A study conducted by Vlaming *et al.* (2008) with four non-lactating dairy cows fed two different diets, which used the SF_6 technique indicated a variance within and between cows of 6.91 and 6.23% in one diet and of 10.09 and 27.79% in another diet, respectively.

In the current study, the observed variation in the CH_4 (l/day) emissions between cows (5.9 – 8.8%) in two years is much lower than those reported by Bell *et al.* (2014), Grainger *et al.* (2007) and Ellis *et al.* (2010), and greater than those reported by Vlaming *et al.* (2008) and Garnsworthy *et al.* (2012b). In the present study, the within cow variation in CH_4 (l/day) ranged between 8.6 - 15.5%

in two years. This is considerably narrower than what has been reported earlier (Grainger *et al.*, 2007, Garnsworthy *et al.*, 2012b).

Compared to the standard respiration chamber (Blaxter and Clapperton, 1965), the current study resulted in similar levels of variations between cows and slightly higher variation within cow. Moreover, the variation in the CH_4 emissions observed in this study was less than the variation reported using the SF_6 technique (Grainger *et al.*, 2007). The slightly wider range of within cow variation reported in this study might be linked to the greater range of individual DMI and ECM production, which are assumed to be the key determinant of CH_4 production.

Repeatability and phenotypic relation of CH_4 output in two years

Repeatability expresses the total variation that is reproducible among the repeated measures of the same subject (Nakagawa and Schielzeth, 2010). In this study, the repeatability of CH_4 (l/day) emission was 0.36 and 0.41 during the 1st and 2nd years, respectively. During the 1st year, the repeatability of CH_4 emission was slightly lower presumably because of the higher within cow variation. This is in line with earlier findings for dairy cows and sheep (Vlaming *et al.*, 2008, Pinares-Patino *et al.*, 2013). The repeatability of the CH_4 : CO_2 ratio in Holstein and Jersey cows was 0.37 and 0.33, respectively, which is considered to be a better measure of the CH_4 production than raw measures of CH_4 (Lassen *et al.*, 2012). Contrary to the present study, Pinares-Patiño *et al.* (2011) reported a very low repeatability (0.16) in sheep where the CH_4 was measured using chamber technique to rank the animals according to their emission rate. The repeatability was mentioned to be the upper boundary of the heritability of a particular trait (Lassen *et al.*, 2012). In this connection, it can be presumed that, the level of repeatability in this study would be a better indicator for selecting animals with low CH_4 emission.

A substantial variation in CH_4 (l/day) emission was observed among the individual cows over the two years. This variation was probably caused by the difference in the DMI and ECM between the two years. However, at a standardized ECM production, the CH_4 emission was strongly correlated between two years. This correlation can be referred to the phenotypic trait of CH_4 (l/day) which is probably related with genetic variation, i.e. the heritability of CH_4 production of the animals, previously been mentioned by Pinares-Patino *et al.* (2013). The same author stated that even after adjustment for feed intake or ECM the trait will be repeatable. The observed phenotypic correlation of CH_4 emission of the individual cows in the current study could be considered in CH_4 mitigation strategies, by selecting cows with low CH_4 emission during the breeding process. Finally, it should be acknowledged that when dealing with large number of animals for CH_4 measurements, there will always be some individuals (Figure 2b) different from others due to oestrus, lameness or any other problems which affect the normal feed intake physiology, body activity and metabolism and consequently the gas emission. This should therefore always be taken into consideration.

Diurnal variation

The variation of CH_4 emission (l/h) showed a very distinct diurnal pattern that is identical to the results described by Garnsworthy *et al.* (2012b). Moreover, the diurnal variation using chamber technique at the individual cow level was similar to Grainger *et al.* (2007). Some other methods of CH_4 estimation such as poly-tunnels grazing animals (Lockyer, 1997) and point source dispersion in grazing animals (McGinn *et al.*, 2011) showed a comparable diurnal pattern. The diurnal variation is most likely linked with the animal behaviour, digestive physiology and ambient condition (Garnsworthy *et al.*, 2012b), especially feeding behaviour. In the current study, although feed was always available to the cows, the daily allocation of feed was done at around 0700 h, which might lead to a synchronized feeding behaviour at a specific time. However, the milking time was very different for every cow in automatic milking system where milking was done throughout 24 - h for all cows. Therefore, the diurnal pattern might be more correlated with the time of feeding than with the milking time. The influence of milking time could be considered for other methods where milking is performed e.g. twice a day at a fixed time.

Conclusions

On herd average basis the daily CH_4 emission was significantly higher the second year due to a higher DM intake. The CH_4 emission per kg DMI was similar in the two years. The study indicates that the key factor of the variation in CH_4 output is the DMI which can also be described by the ECM production. When measuring for short period of time as, e.g. a visit in the AMS or a single day, the variation in CH_4 (l/day) emission between cows was lower than within cow. The diurnal pattern of CH_4 (l/h) production was probably influenced by the feeding behaviour of the cows and was lowest from 0000 to 0800 h. The repeatability of daily CH_4 output (l/day) ranged from 0.36 - 0.41 in the two years. Individual cow variation in seven days average shows a strong positive phenotypic relation, especially when the CH_4 production is standardized using ECM in both years. This between years phenotypic relation for individual cows show a potential opportunity for selection of cows with low CH_4 emission.

Acknowledgements
The authors wish to acknowledge all of the farm employees for their support during the experiment.

References

Bates DM and Sarkar D 2009. lme4: Linear mixed-effects models using S4 classes, R package version 0.99875-6. Retrieved on 17 April 2013, from http://cran.r-project.org/web/packages/lme4/index.html

Beauchemin K 2009. Dietary mitigation of enteric methane from cattle. CAB Reviews: Perspectives in Agriculture, Veterinary Science, Nutrition and Natural Resources 4, 1-18.

Bell MJ, Potterton SL, Craigon J, Saunders N, Wilcox RH, Hunter M, Goodman JR and Garnsworthy PC 2014. Variation in enteric methane emissions among cows on commercial dairy farms. Animal 8, 1540-1546.

Blaxter KL and Clapperton JL 1965. Prediction of the amount of methane produced by ruminants. Br J Nutr 19, 511-522.

Boadi DA and Wittenberg KM 2002. Methane production from dairy and beef heifers fed forages differing in nutrient density using the sulphur hexafluoride (SF6) tracer gas technique. Canadian journal of animal science. Ottawa ON 82, 201-206.

Boadi DA, Wittenberg KM and Kennedy AD 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.

CIGR 2002. Climatization of animal houses – heat and moisture production at animal and house level. In 4Th Report of CIGR Working Group (Eds. S Pedersen and K Sällvik) Research Centre Bygholm, Danish Institute of Agricultural Sciences, P.O Box 536, DK-8700 Horsens, Denmark.

Doreau M, van der Werf HM, Micol D, Dubroeucq H, Agabriel J, Rochette Y and Martin C 2011. Enteric methane production and greenhouse gases balance of diets differing in concentrate in the fattening phase of a beef production system. Journal of Animal Science 89, 2518-2528.

Ellis JL, Bannink A, France J, Kebreab E and Dijkstra J 2010. Evaluation of enteric methane prediction equations for dairy cows used in whole farm models. Global Change Biology 16, 3246-3256.

Garnsworthy PC, Craigon J, Hernandez-Medrano JH and Saunders N 2012a. On-farm methane measurements during milking correlate with total methane production by individual dairy cows. Journal of Dairy Science 95, 3166-3180.

Garnsworthy PC, Craigon J, Hernandez-Medrano JH and Saunders N 2012b. Variation among individual dairy cows in methane measurements made on farm during milking. Journal of Dairy Science 95, 3181-3189.

Gerber PJ, Steinfeld H, Henderson B, Mottet A, Opio C, Dijkman J, Falcucci A and 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), Rome, Italy.

Grainger C, Clarke T, McGinn SM, Auldist MJ, Beauchemin KA, Hannah MC, Waghorn GC, Clark H and Eckard RJ 2007. Methane emissions from dairy cows measured using the sulfur hexafluoride (SF6) tracer and chamber techniques. Journal of Dairy Science 90, 2755-2766.

Haque MN, Cornou C and Madsen J 2014. Estimation of methane emission using the CO2 method from dairy cows fed concentrate with different carbohydrate compositions in automatic milking system. Livestock Science 164, 57-66.

Hothorn T, Bretz F and Westfall P 2008. Simultaneous inference in general parametric models. Biometrical Journal 50, 346-363.

Johnson KA and Johnson DE 1995. Methane emissions from cattle. Journal of Animal Science 73, 2483-2492.

Kristensen VF and Ingvartsen KL 2003. Forudsigelse af foderoptagelsen hos malkekøer og ungdyr. In Kvægets ernæring og fysiologi. Bind 1 (Eds. T Hvelplund and P Nørgaard), pp. 511-564. Ministeriet for Fødevarer landbrug og fiskeri, Denmark, Næringsstofomsætning og fodervurdering: DJF rapport, Husdyrbrug.

Kuznetsova A, Brockhoff PB and Christensen RHB 2012. Tests for random and fixed effects for linear mixed effect models (lmer objects of lme4 package). Retrieved on 27 April 2013, from http://cran.r-project.org/web/packages/lmerTest/index.html.

Lassen J, Lovendahl P and Madsen J 2012. Accuracy of noninvasive breath methane measurements using Fourier transform infrared methods on individual cows. Journal of Dairy Science 95, 890-898.

Lassey KR, Ulyatt MJ, Martin RJ, Walker CF and Shelton ID 1997. Methane emissions measured directly from grazing livestock in New Zealand. Atmospheric Environment 31, 2905-2914.

Lockyer DR 1997. Methane emissions from grazing sheep and calves. Agriculture Ecosystems & Environment 66, 11-18.

Madsen J, Bjerg BS, Hvelplund T, Weisbjerg MR and Lund P 2010. Methane and carbon dioxide ratio in excreted air for quantification of the methane production from ruminants. Livest. Sci. 129, 223-227.

McGinn SM, Turner D, Tomkins N, Charmley E, Bishop-Hurley G and Chen D 2011. Methane Emissions from Grazing Cattle Using Point-Source Dispersion. Journal of Environmental Quality 40, 22-27.

McNaughton L, P. BD, H. C, C. PP, S. H and J. SR 2005. Factors affecting methane production in Friesian x Jersey dairy cattle. In Proceedings of the New Zealand Society of Animal Production, pp. 352-355. New Zealand Society of Animal Production, New Zealand.

Møller J, Thøgersen R, Kjeldsen AM, Weisbjerg MR, Søegaard K, Hvelplund T and Børsting CF 2000. Feedstuff Table. In Composition and feeding value of feedstuffs for cattle. Report 91, pp. 5-10. Danish Institute of Agricultural Science. The Danish Agricultural Advisory Centre.

Nakagawa S and Schielzeth H 2010. Repeatability for gaussian and non-gaussian data: A practical guide for biologists. Biological Reviews of the Cambridge Philosophical Society 85, 935-956.

NorFor 2011. The Nordic feed evaluation system. EAAP publication No. 130. Wageningen Academic Publishers, The Netherlands.

Pedersen S, Blanes-Vidal V, Joergensen H, Chwalibog A, Haeussermann A, Heetkamp MJW and Aarnink AJA 2008. Carbon dioxide production in animal houses: A literature review. In Agricultural Engineering International: CIGR Ejournal. Retrieved on 07 July 2014, from http://www.cigrjournal.org/index.php/Ejounral/article/viewFile/1205/1132.

Pinares-Patino CS, Hickey SM, Young EA, Dodds KG, MacLean S, Molano G, Sandoval E, Kjestrup H, Harland R, Hunt C, Pickering NK and McEwan JC 2013. Heritability estimates of methane emissions from sheep. Animal 7 Suppl 2, 316-321.

Pinares-Patiño CS, McEwan JC, Dodds KG, Cárdenas EA, Hegarty RS, Koolaard JP and Clark H 2011. Repeatability of methane emissions from sheep. Animal Feed Science and Technology 166-167, 210-218.

R Development Core Team 2013. R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. R version 3.0.0. Retrieved on 17 April 2013, from http://www.R-project.org.

Sjaunja LO, Baevre L, Junkkarinen L, Pedersen J and Setala J 1991. A Nordic proposal for an energy corrected milk (ECM) formula. In Performance Recording of Animals State of the Art. In EAAP Publication 50 (Eds. P Gaillon and Y Chabert), pp. 156-157. Centre for Agricultural Publishing and Documentation. Wageningen, The Netherlands.

Tamminga S, Bannink A, Dijkstra J and Zom R 2007. Feeding strategies to reduce methane loss in cattle. In Report: Animal Nutrition and Animal Sciences Group, Wageningen UR, Lelystad, The Netherlands.

Vlaming JB, Lopez-Villalobos N, Brookes IM, Hoskin SO and Clark H 2008. Within-and between-animal variance in methane emissions in non-lactating dairy cows. Aust J Exp Agric 48, 124-127.

Table 1 Feed allocation per cow and nutrient composition of the diet in two years.

Ingredients	1st year	2nd year
Total mixed ration (TMR, DM, Kg/day)		
Rapeseed cake	1.6	1.3
Soybean decorticated	1.4	1.0
Clover grass silage	3.4	2.9
Maize silage	9.0	10.7
Ryegrass straw	0.6	1.4
Urea	-	0.1
Beet pulp	-	0.9
Vitamin mineral premix	0.2	0.2
Concentrate supplied in AMS (DM, Kg/day)		
Concentrate	4.0	4.2
[2]Nutrient Intake		
Energy (MJ/kg DM)	7.6	6.3
NE (MJ/d)	153.0	146.0
AAT (g/MJ)	13.0	16.0
PBV (g/kg DM)	5.0	8.0
Fatty acid (g/kg DM)	35.0	28.0
NDF (g/kg DM)	-	342.1
Starch (g/kg DM)	212.3	199.1
Calcium total (g/d)	147.0	143.0
Total phosphorus (g/d)	84.6	78.3
Magnesium total (g/d)	58.2	56.0

[1]Nutrient and energy value was calculated using the Danish feed stuffs table (Møller *et al.*, 2000);
[2]Calculated according to The Nordic feed evaluation system (NorFor, 2011).

Table 2 Body weight, milk production and feed intake of the cows during two years of measurement.

Parameters	1st year	2nd year
BW (kg)	619.9±14.2	640.0±8.0
Milk yield (kg/day)	29.1±6.5	33.4±6.0
ECM (kg/day)	30.8±8.0	33.7±5.3
TMRI (DM, kg/day)	15.8±0.5	18.9±0.5
CI (DM, kg/day)	4.0±1.0	4.2±1.6
DMI (kg/day)	19.8±1.0	23.1±0.8

Values indicated arithmetic means and standard deviation [mean ± s.d.]
BW = body weight;
ECM = energy corrected milk;
TMRI = total mixed ration intake;
CI = concentrate intake;
DMI = dry matter intake.

Table 3 Variation and repeatability of the CH_4 production of the cows during two years.

Parameters	1st year				2nd year			
	Estimates[1]	CV_{bc} (%)	CV_{wc} (%)	R	Estimates[1]	CV_{bc} (%)	CV_{wc} (%)	R
CH_4 (l/day)	445.50	8.80	15.67	0.36	569.88	5.88	8.60	0.41
CH_4 (l/kg DMI)	23.73	9.12	15.70	0.37	23.70	6.01	8.57	0.41
CH_4 (l/kg ECM)	14.86	8.96	16.36	0.35	17.10	6.10	9.05	0.40
Ratio	0.08	8.38	15.94	0.34	0.09	6.22	8.78	0.41

[1] estimates from the model;
CV_{bc} = coefficient of variation between cow variation;
CV_{wc} = coefficient of variation within cow variation;
R = Repeatability.

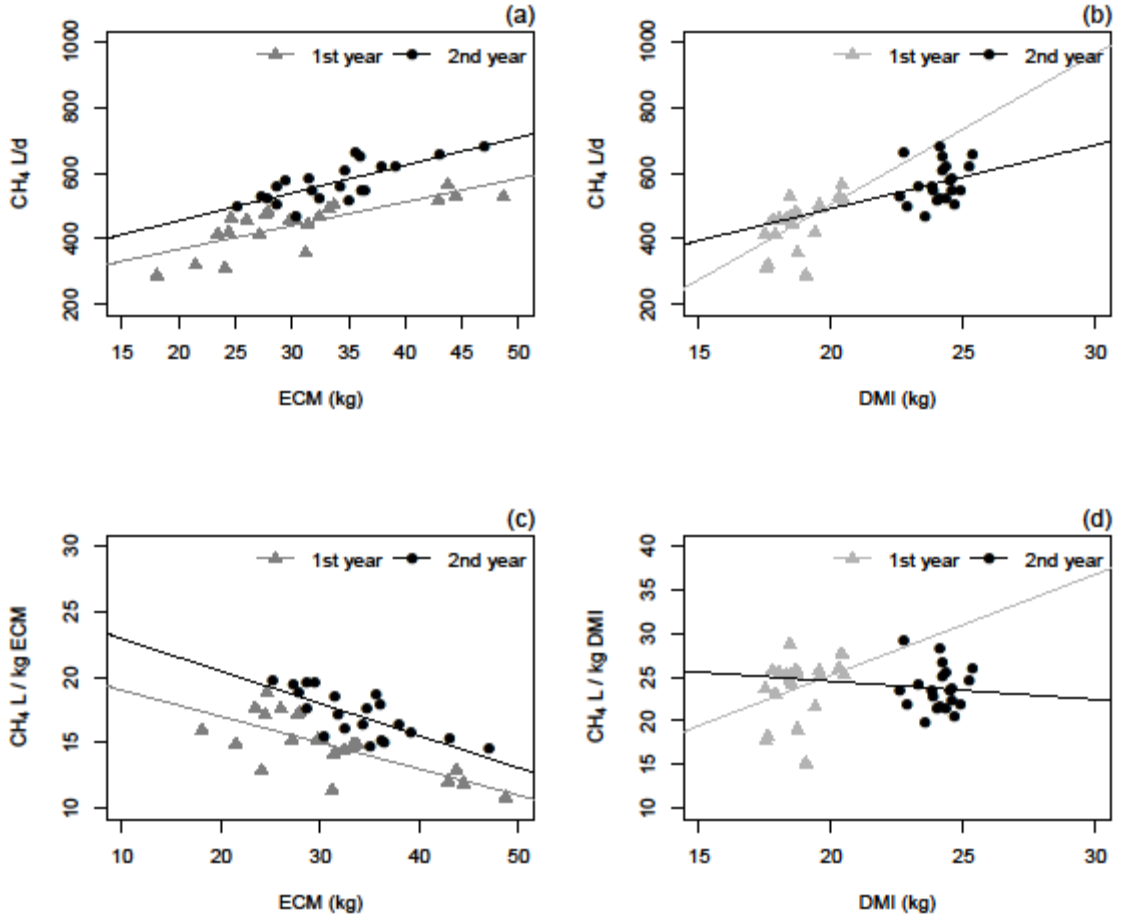


Figure. 1 Regression analysis of CH_4 emission based on ECM and DMI of individual cows in two years.

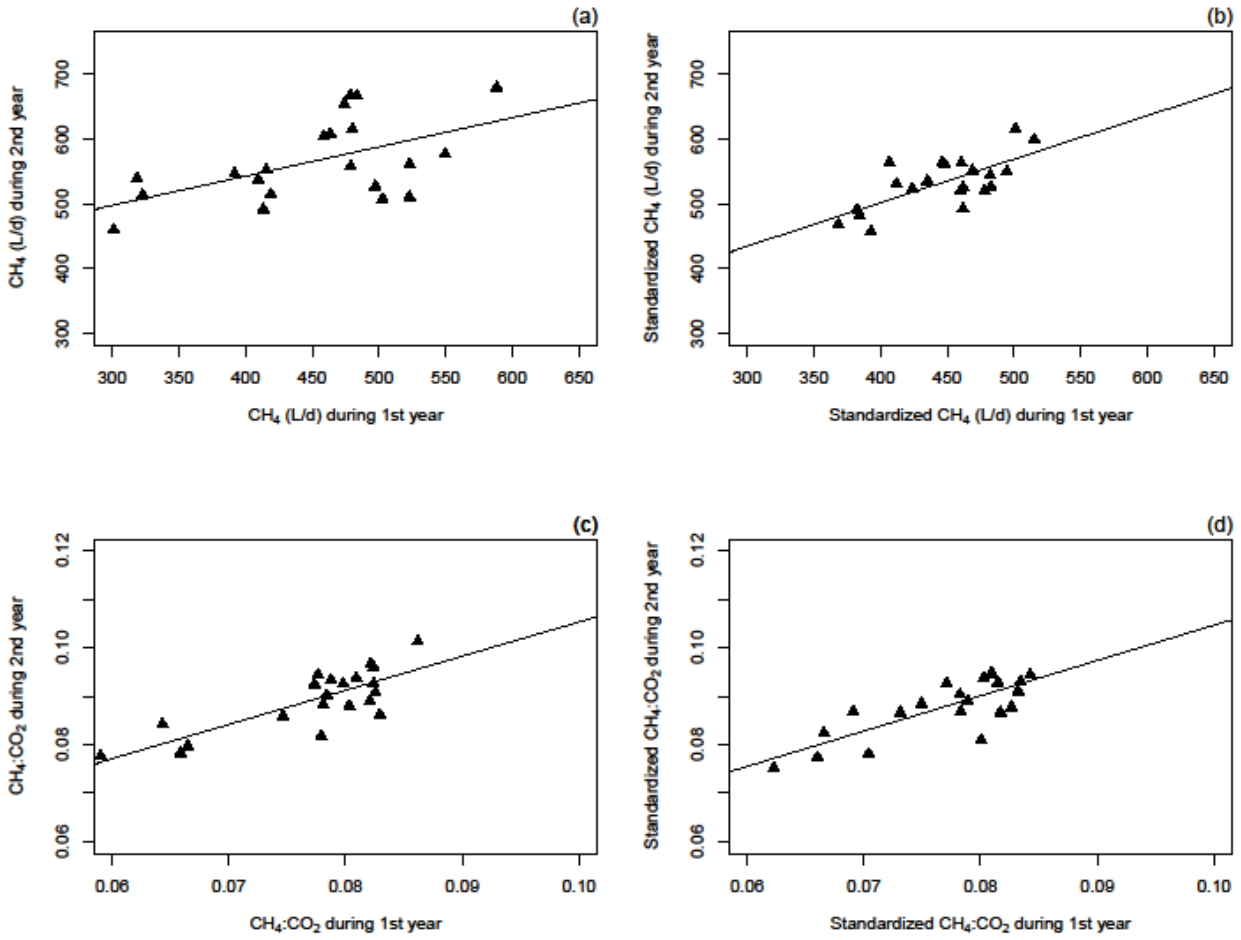


Figure. 2 Methane production and CH_4:CO_2 ratio of the individual cows in two years. The Figures shown on the right hand side (b and d) use the data corrected for ECM and expressed at a standardized ECM production (30 L/d).

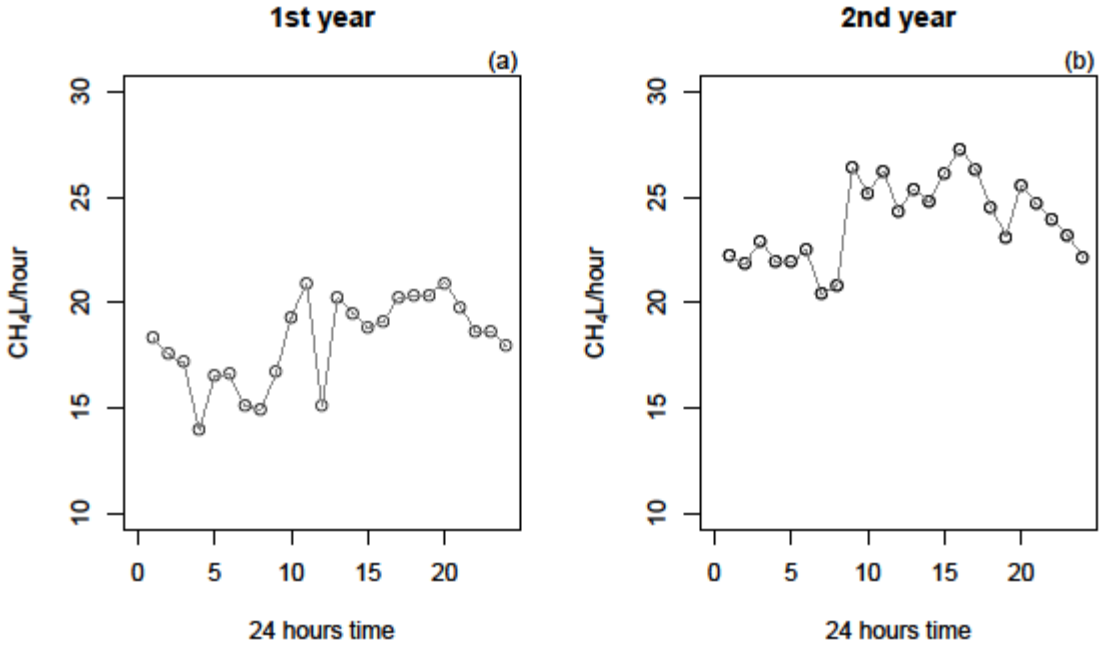


Figure. 3 Diurnal variation of CH_4 release (L/h) during the two years of measurements.

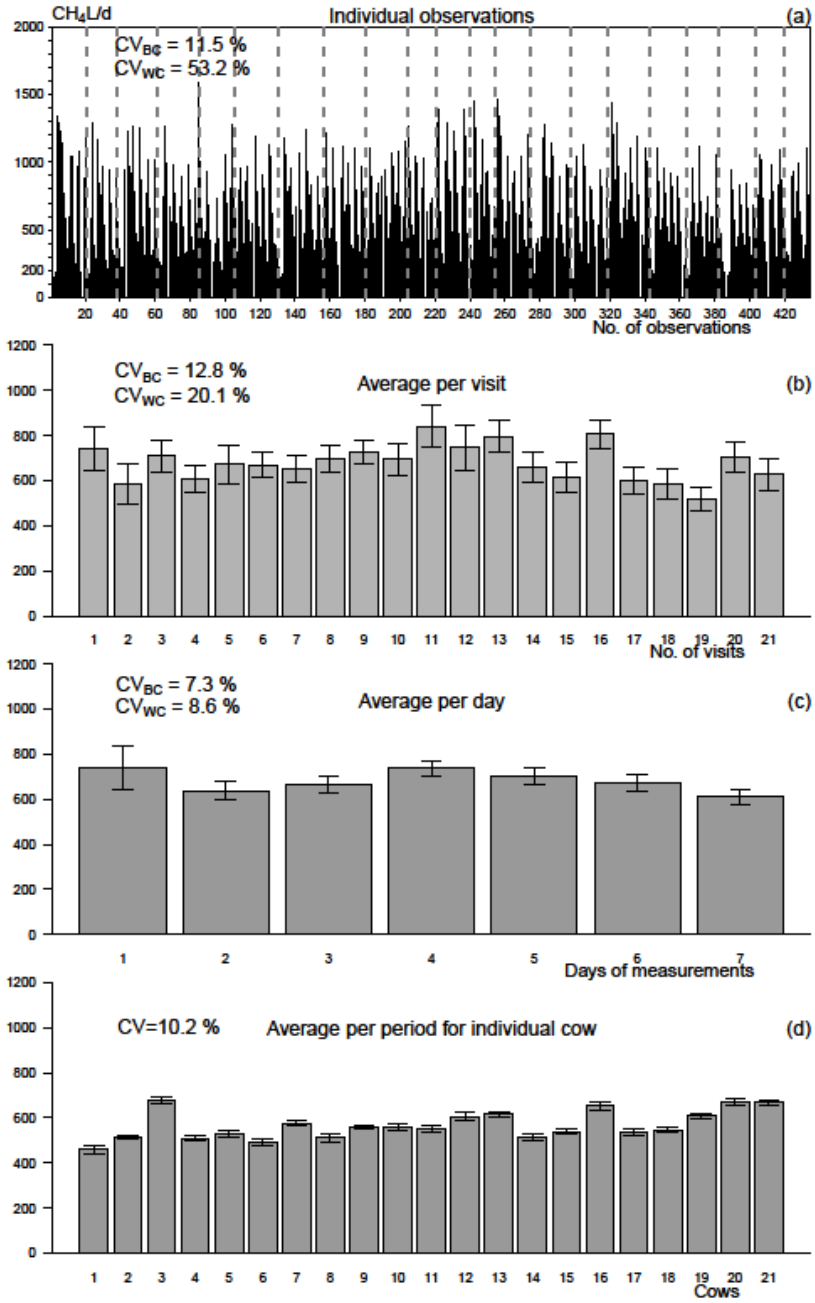


Figure. 4 Sources of variation during the breath samples measurements.(a) Individual breath concentration of CH_4 for a single cow, where the broken lines separate the visits to the AMS; (b) Average per visit of a single cow; (c) Average per day of a single cow; (d) Average emission of the 21 cows for the experimental period (7 days). The error bars indicate the standard error (±SE). .