Methane Production in Dairy Cows

Impact of Feed and Rumen Microbiota

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Abstract

Greenhouse gas emissions from the agricultural sector represent about 14.5% of total emissions related to human activity. Approximately 40% of agricultural sector emissions derive from enteric methane (CH_4) production by ruminants, due to their microbial digestion of feed. Level of CH_4 production varies according to feed type, feed intake and even among individual animals raised under similar conditions, but the underlying mechanism is not well known. This thesis investigated the effects of feed, feed additives, and rumen microbiota on CH_4 production within dairy cows and in a gas *in vitro* system. Effect of individual cow was stronger than effect of diet for both CH_4 production and methanogenic population when two different levels of forage proportions were fed. Dividing *Methanobrevibacter* species into two groups better explained the variation in CH_4 production. The effect of individual was evaluated in cows fed the same diet during mid-lactation. High, low and medium emitters were identified and selected for further studies on rumen microbiota. These revealed that CH_4 production was associated with archaeal and bacterial community structure. Differences were observed in volatile fatty acid proportions between communities, but not in fibre digestion or milk production.

Tests on feed additives, cashew nut shell extract (CNSE) and glycerol in a gas *in vitro* system for their ability to reduce CH_4 production showed that CNSE reduced CH_4 production by 18% and had a strong impact on microbiota, while glycerol increased CH_4 production by 12% and had less effect on microbiota compared with the control. Comparison of microbial composition in inoculum from the *in vitro* control and in inoculum from the donor cow before incubation revealed that the bacterial community was relatively similar, while relative abundance of some species changed for archaeal population. This effect of transfer into another system should be considered when evaluating *in vitro* data. Evaluation of the *in vitro* system by comparing predicted and observed CH_4 production on 49 test diets showed an overall good relationship, with small root mean square error for prediction (12.3% and 9.5% of observed mean for fixed and mixed models, respectively). However, the *in vitro* system had limitations in prediction of concentrate proportion.

Keywords: Dairy cattle, diet composition, cashew nut shell extract, glycerol, archaeal and bacterial community structure, methanogens, volatile fatty acids, *in vitro* gas production.

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Contents

List of Publications

This thesis is based on the work contained in the following papers, referred to by Roman numerals in the text:

I Danielsson, R., A. Schnürer, V. Arthurson and J. Bertilsson (2012). Methanogenic population and CH_4 production in Swedish dairy cows fed different levels of forage. *Applied and Environmental Microbiology* 78(17), 6172-6179.

II Danielsson R., J. Dicksved, L. Sun, B. Müller, H. Gonda, A. Schnürer and J. Bertilsson. Methane production in dairy cows correlates with rumen methanogenic and bacterial community structure (manuscript).

III Danielsson, R., A. Werner-Omazic, M. Ramin, A. Schnürer, M. Griinari, J. Dicksved, and J. Bertilsson (2014). Effects on enteric methane production and bacterial and archaeal communities by the addition of cashew nut shell extract or glycerol—An *in vitro* evaluation. *Journal of Dairy Science* 97(9), 5729-5741.

IV Danielsson, R., R. Mohammad, J. Bertilsson, P. Lund and P. Huhtanen. Evaluation of an *in vitro* system for predicting methane production *in vivo* (manuscript).

Abbreviations

DM	Dry matter
DMI	Dry matter intake
ECM	Energy corrected milk
GE	Gross energy
GEI	Gross energy intake
GHG	Greenhouse gases
NDF	Neutral detergent fibre
OM	Organic matter
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
qPCR	Quantitative polymerase chain reaction
T-RF	Terminal restriction fragment
T-RFLP	Terminal restriction fragment length polymorphism
VFA	Volatile fatty acid

1 Introduction

With an expected increase in the world's population from 7.4 billion today to 9.6 billion in 2050 and with an increasing middle class world-wide, there will be an increase in demand for animal products such as milk and meat (Gerber *et al.*, 2013). According to FAO (2011), demand for milk and meat is expected to increase by 73 and 58 %, respectively, from 2010 to 2050, but the natural resources for increasing the food supply are limited (Gerber *et al.*, 2013). Agriculture plays an important role in environmental issues, with a major impact on climate change, water pollution, land degradation and loss of biodiversity. A major challenge in livestock production is thus to increase productivity while at the same time reducing the environmental impact. In recent decades, increasing concentrations of greenhouse gases (GHG) in the atmosphere have led to rising global temperatures and climate change. Human activities (anthropogenic) are most likely responsible for causing the observed change to a warmer climate (IPCC, 2013). The main GHGs are water vapour, carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and ozone. Livestock sector is estimated to contribute to anthropogenic GHG emissions as follows: 5% of CO_2 emissions, 44% of CH_4 emissions and 53% of N_2O emissions (IPCC, 2007). To make comparisons between different GHGs, all gases are converted into CO_2 equivalents (CO_2-eq) according to their global warming potential (GWP). Measured over a 100-year period, CH_4 and N_2O have 28 and 265 times higher GWP than CO_2 (IPCC, 2013). Anyhow, it is important to be aware of that calculations in GHG emissions data from agricultural production contains a wide range of uncertainty due to the complexity of biological systems (Gerber *et al.*, 2013; IPCC, 2007).

Globally, there has been a major increase in GHGs since the pre-industrial era. According to IPCC (2014), total anthropogenic global GHG emissions in 2010 were 49 ± 4.5 Gt CO_2-eq, with the livestock sector accounting for around 7.1 Gt CO_2-eq per year (Gerber *et al.*, 2013). Within the livestock sector, enteric fermentation from ruminants is the largest source of GHG (40%), followed by manure management (Figure 1). Total CO_2-eq. from monogastric animals are much lower than from ruminants (Figure 2).

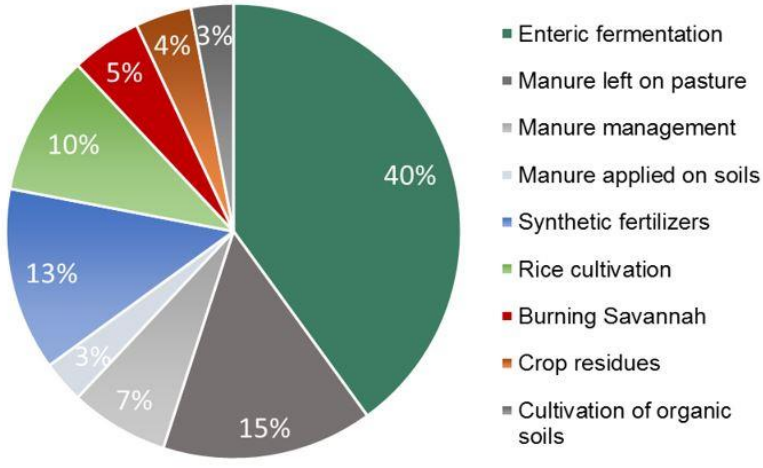

Figure 1. Agriculture emissions by subsector. Source; Gerber *et al.* (2013)

Swedish GHG emissions are about 54 Mt CO_2-eq per year, but decreased by 24% between 1990 and 2014 (Naturvårdsverket, 2016). The emissions from agriculture are about 7 Mt CO_2 -eq., which have also decreased since 1990, by around 11%. The reduction in emissions is mainly because of the decrease in livestock numbers, higher feed quality, increased production efficiency and reduced use of synthetic fertilisers (Naturvårdverket, 2016). On the other hand, food imports have increased dramatically since 1990 and GHG emissions from imported food are not accounted for as Swedish emissions (LRF, 2016). For example, imports of beef increased from 12 510 to 139 370 tonnes per year from 1990 to 2015 (Svensktkött, 2016).

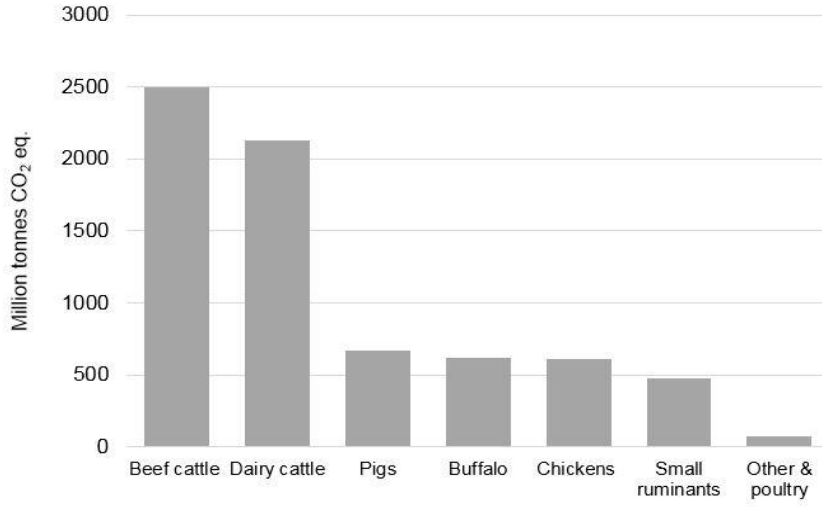

Figure 2. Global estimated emissions by species. Emissions are attributed to edible products and non-edible products such as draught power and wool. Beef cattle produce meat and non-edible outputs. Dairy cattle produce milk and meat, as well as non-edible outputs. Source: (GLEAM, Gerber *et al.,* 2013).

Methane emissions derive from several sources. Emissions can be divided into natural sources such as plants, wetlands, termites, ocean and hydrates, and anthropogenic sources such as rice fields, ruminants, landfills, sewage, biomass burning and fossil (Aronsson *et al.,* 2013). Increased concentration of CH_4 in the atmosphere over time is closely linked with human activities. Since pre-industrial times there has been an almost 2.5 fold increase in CH_4 concentration in the atmosphere. At the beginning of industrial revolution, around 1750, the concentration was about 750 parts per billion (ppb), which could be attributed to CH_4 production from natural sources. Today, with its high human activity, the concentration of CH_4 in the atmosphere is around 1800 ppb (IPCC, 2013). Since pre-industrial times there has also been a huge increase in the number of livestock. Even within this century, enteric CH_4 production has increased by 11%, from 1858 Mt CO_2-eq to 2071 Mt CO_2-eq between 2001 and 2011, with a major increase in developing countries (Tubiello *et al.*, 2014).

The potential for mitigation of CH_4 production from ruminants per unit of product, *e.g.* milk or meat, depends on a number of factors. Thus knowledge of CH_4 formation and of the factors that are important in the formation process is essential for any successive mitigation strategy. Methane production from ruminants occurs during microbial digestion of feed in the anaerobic environment in the rumen and hindgut. This fermentation process makes ruminants unique compared with humans and other monogastric animals.

Ruminants harbour a large number of microbes that have the capacity to convert feeds rich in fibre, which are non-valuable from a human perspective, into highly valuable products such as milk and meat. Level of CH_4 production depends on type of feed and host (Johnson & Johnson 1995). Furthermore, CH_4 production varies significantly between individual animals, even if they are raised under similar conditions (Pinares-Patiño *et al.*, 2013; Jami *et al*., 2012). The effect of animal may be influenced by physiological parameters such as chewing time, rumen size and passage rate from the rumen. These factors may benefit certain types of microbial community structure of microorganisms in the rumen, which in turn influences digestion of the feed into end-products that have an effect on both animal production and the environment (Jami *et al*., 2012). Due to the individual variation in CH_4 production, it might be possible to select cows with traits that seem to lower CH_4 production. However, it is essential that selection for low CH_4 production not affect other parameters negatively, as digestion of feed and milk or meat production.

As a first step to identify possible low CH_4 producers, diets or feed additives that contribute to lower CH_4 production and use of consistent measuring techniques are important. At present, several options with different approaches for reducing CH_4 production are available, all with their advantages and disadvantages, and most approaches lack long-term effect of inhibition.

For measuring CH_4 production *in vivo* several techniques can be used, all had their advantages and disadvantages that needs to be considered before choosing technique. In addition, *in vitro* measurements can be used as a first step to test the effect of feeds or feed additives on CH_4 production where many samples can be analysed at the same time. When CH_4 production has been measured, a further step is to examine parameters that may explain the variation in CH_4 production, such as different physiological parameters and the rumen microbial structure. Today, knowledge of rumen microbes is still somewhat limited and a better understanding of rumen microbiology is essential for strengthening the possibilities for improving feed utilisation or for manipulating the microbial community to reduce CH_4 production.

1.1 Aims of thesis

Reducing enteric CH_4 production from livestock is one of the main challenges in lowering the environmental impact from the agricultural sector. The overall aim of this thesis was to evaluate different factors with potential to reduce CH_4 production in dairy cows, *e.g.* feeds, feed additives and the rumen microbiota. In particular, the thesis investigated cows fed under Nordic feeding regimes, which are unique as agriculture is not practised at such high latitudes anywhere else in the world.

The specific aims of the studies described in **Paper I-IV** were to:

- Investigate correlations between methane emissions, microbial community structure and forage proportion (**Papers I-II**)
- Identify links between microbial community, feed digestion and fermentation product with in cows producing high and low levels of CH_4 (**Paper II)**
- Investigate the effects of the feed additives cashew nut shell liquid extract and glycerol on microbial community structure and CH_4 production (**Paper III**)
- Evaluate the ability of a gas *in vitro* system to predict CH_4 production and the effect on microbial community structure compared to *in vivo* studies (**Papers III-IV**).

2 Rumen fermentation and methane production

2.1 Rumen fermentation

In ruminants, the main digestion of the ingested feed occurs in the rumen, reticulum and omasum, which together are called reticulo-rumen. These three compartments of the four-compartment ruminant stomach contain different fractions of liquids and solids which all have different turnover times (Wolin, 1979). In the rumen, all three domains of microbial life (archaea, bacteria and eukarya (fungi and protozoa)) are present (Woese *et al*., 1990). When feed enters the rumen, primary fermenters, such as bacteria, fungi and protozoa, start to digest the feed macromolecules into monomers such as simple sugars and carbon skeletons. The effective degradation of fibrous feed in the rumen is due to fibrolytic enzymes produced by bacteria, protozoa and fungi, which include cellulases, xylanases, β-glucanases and pectinases. There are also other non-fibrolytic enzymes present in the rumen, such as amylase, protease and phytase (Wang & Mc Allister, 2002). To degrade the complicated structure of cellulose chains, several enzymes are needed: i) endoglucanase (cellulase) splitting the β-1,4 glyosidic bond within the chain, ii) exoglucanases (cellobiosidase or cellobiohyrolases) cleaving cellobiose from the end of the chain and iii) β-glycosidase (cellobiases) converting cellobiose into glucose (Lynd & Zhang, 2002). Several cellulolytic species are predominant in herbivores to optimise the utilisation of nutrients from cellulosic feed (Morrison & Miron, 2000). Carbohydrates are degraded to glucose equivalents, which are metabolised to pyruvate through the Emden-Meyerhof-Parnas (EMP) pathway (glycolytic pathway). In the pathway, NAD is reduced to NADH, which needs to be re-oxidised to NAD for complete sugar fermentation and during this re-oxidation hydrogen (H_2) can be produced (Moss *et al*., 2000). Pyruvate, the end-product of the EMP pathway, is then further fermented by microorganisms known as secondary fermenters to main end-products such as volatile fatty acids (VFA), mainly acetate, propionate and butyrate, H_2 and CO_2, but ethanol, formate, succinate, lactate and ammonia are also produced (Van Soest, 1994; Hungate, 1966). The amounts of the different end-products vary depending on diet composition, but when considering VFA, CO_2 and CH_4 as sole fermentation end-products the overall fermentation equation of hexoses can be summarised as:

57.5 Hexose → 115 Pyruvate → 65 Acetate + 20 Propionate + 15 Butyrate + 35 CH_4 + 60 CO_2 (Wolin, 1960)

The majority of the VFAs produced are rapidly absorbed through the rumen wall into the bloodstream and serve as major energy and carbon sources for the animal. Amino acids, peptides and ammonia, released during the degradation of proteins and non-protein N-compounds, can be taken up by the microbes and converted to microbial protein. Some of this microbial protein is degraded and utilised in the rumen, but the majority of protein, both microbial protein and undegraded feed protein, is further utilised in the small intestine. The fermentation in the rumen is dependent on syntrophic, and symbiotic relations between different microbes, including bacteria, archaea, fungi and protozoa. For instance, syntrophic interspecies H_2 transfer occurs in the rumen when some microbes produce H_2 that is then used by other microbes (Krause *et al.*, 2014). In the rumen, no single microbial species can ferment the substrate all the way and therefore other microbes with other substrate preferences are needed.

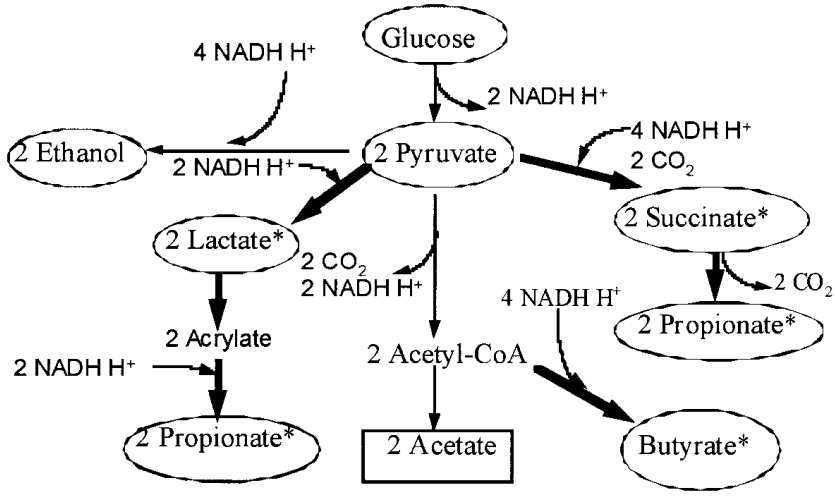

Figure 3. Metabolism of NADH H^+ and electron sink products*. Source: Moss *et al.* (2000)[1]

2.2 Methanogenesis

Depending on the amount and proportions of different VFAs produced, different amounts of CH_4 and CO_2 are also produced. When acetate is produced, re-oxidation of NADH occurs by production of H_2 that can be further used by methanogenic *Archaea* (methanogens) to reduce CO_2 to CH_4. In comparison,

during propionate production the re-oxidation of NADH occurs by production of succinate or lactate that is then fermented to propionate, giving no CH_4 formation (Figure 3). Methanogenesis, as the CH_4 production step is called, is where methanogens generate their energy in the form of ATP (Ferry & Kaestad, 2007). Methanogenesis is one of the important means to remove H_2 from the rumen, in order to maintain effective fermentation (Moss *et al.*, 2000). Hydrogen is the most common substrate for CH_4 production, but there are also other substrates that can be used by the methanogens, such as formate, methanol, methyl amines, methyl sulphides and the methyl group of acetate. Depending on the substrate used, the methanogens can be divided into three groups: i) hydrogenotrophic methanogens, which are most common in the rumen (Janssen & Kirs, 2008) and mainly use H_2, but some can also use formate to reduce CO_2 (Liu & Whitman, 2008; Thauer *et al*., 2008); ii) methylotrophic methanogens, which use methyl compounds from methanol, methylamines or methyl sulphides and oxidise them partly to CO_2 to produce electrons that can be used for further reduction of methyl groups to CH_4 (Lang *et al*., 2015; Janssen & Kirs, 2008); and iii) acetotrophic methanogens, which use the methyl group from acetate for dissimilation to CH_4 and CO_2 production (Janssen & Kirs, 2008). Furthermore, some of the methanogens are versatile and can use several substrates, while others are stricter and only use a certain substrate (Costa & Leigh, 2014). In the rumen, hydrogenotrophic and methylotrophic methanogenesis occur (Poulsen *et al.*, 2013; Hungate, 1970). Hydrogenotrophic methanogenesis, with H_2 as substrate, follows the reaction:

$$CO_2 + 8H \rightarrow CH_4 + 2H_2O$$

2.2.1 Alternative electron incorporating processes

There are other electron incorporating processes in the rumen than CH_4 formation and if this could be favoured, CH_4 production could be reduced. As mentioned above, in propionate production less H_2 is produced than in acetate production, giving less CH_4 production. Methane reduction is possible if the ratio of the VFAs is changed to favour a higher ratio of propionate (Johnson & Johnson, 1995). Increasing the proportion of concentrate in relation to forage usually increases the digestibility of the feed and leads to a higher propionate proportion, but this mainly has an impact when the diet contains >50% concentrate (Johnson & Johnson, 1995). However, this has not been observed with Nordic diets, where no effect has been found on propionate proportion with increasing starch proportion in the diet (Sveinbjörnsson *et al.*, 2006; Murphy *et al*., 1999). Another H_2-using process is biohydrogenation of unsaturated fatty acids, but this uses a very small proportion of H_2 compared with methanogenesis

(Johnson & Johnson, 1995). Hydrogenotrophic bacteria such as acetogens exist in the rumen and can reduce CO_2 to form acetate ($4H_2 + 2CO_2 \rightarrow CH_3COOH + 2H_2O$) by the Wood-Ljungdahl pathway (reductive acetogenesis). Acetogenic bacteria are the major utilisers of H_2 to reduce CO_2 to acetate in many environments. Acetogenesis, instead of methanogenesis from H_2, is favourable as it results in acetate, which is taken up by the animal and used as energy (Joblin, 1999). However, in the typical ruminal fermentation, methanogens drop H_2 pressure to a level at which reductive acetogenesis has been estimated to be thermodynamically unfeasible (Kohn & Boston, 2000) and as a result the process is believed not to occur to any significant extent, instead the methanogens have a strong advantage (Ellis *et al*., 2008; Le Van *et al*., 1998). If methanogenesis is inhibited acetogenesis will probably take place, as shown in a study by Fonty *et al*. (2007) where gnobiotic lambs were inoculated with a functional microflora but without methanogens. The lambs grew well and the acetogens developed in high numbers (10^6-10^7 cells/g), which also indicates that rumen fermentation seems able to cope with higher H_2 pressure.

There are other possible electron incorporating processes, such as reduction of nitrate and sulphate, which has an advantage in the reduction step (Table 1) compared with reduction of CO_2 to CH_4, due to higher energy yield that gives higher growth rate and that use of H_2 that occur at lower pressure (Ungerfeldt & Kohn, 2006). Sulphate and nitrate are not as common in the rumen and addition to the diet may pose a risk, as the intermediate product in conversion of nitrate to ammonia is nitrite, which is toxic (Lewis, 1951), while in conversion of sulphate hydrogen sulphide is produced and it is also toxic at a certain level (Gould, 1998). If these two additives could be fed in a safe way and within the recommended amounts, it would have a major impact in reducing CH_4 production (Van Zijderveld *et al*., 2010). However, the cost of these additives may make this unfeasible in practice. More detailed information on different diets and on factors that may inhibit CH_4 production or methanogenic activity is provided in Chapter 3.

Table 1. *Free energy yield from reduction of different possible hydrogen sinks in the rumen. ΔG_0 values from Ungerfeldt and Kohn (2006)*

Reduction	Gibbs free energy, (ΔG_0, kJ / mole hydrogen)
Nitrate → Nitrite	-130
Nitrite → Ammonia	-124
Sulphate → Hydrogen sulphide	-21.1
$CO_2 \rightarrow CH_4$	-16.9

3 Mitigation strategies for CH_4 production

Mitigation of CH_4 production is a central issue for reducing the climate impact from the agricultural sector. The goal is to reduce total CH_4 production from the sector in general, but also to reduce CH_4 production specifically per kg product (milk and meat). For the dairy industry, the main aim is to reduce CH_4 production per kg energy corrected milk (ECM). Increasing the efficiency of the animals by improved animal health and maintained high fertility has the main impact on CH_4 production per kg ECM. Furthermore, various strategies have been devised to improve feed digestion and optimise rumen functions, with the aim of reducing CH_4 emissions. Manipulating the ruminal microbiome through different dietary strategies may have a certain impact that can partly be additive to other reduction strategies. This chapter give some examples of possible strategies.

3.1 Intake level and composition of feed

3.1.1 Feeding level

Dry matter intake (DMI) is assumed to be one of the main factors that determines CH_4 production. Total CH_4 production (g/day) is positively correlated with DMI intake and hence high intake means more feed to ferment. Nevertheless, when DMI and gross energy intake (GEI) increase over maintenance level, CH_4 as a proportion of DMI or GEI generally decreases (Pinares-Patino *et al*., 2009; Moe & Tyrrell, 1979; Blaxter & Clapperton, 1965). This is most likely related to a decrease in DM digestibility changes in fermentation pattern and possible repartitioning of carbon between microbial cells and VFA production and associated increase in passage rate caused by the higher intake rate. An increase in productivity results in a reduction in CH_4 production per kg product, milk or meat, mainly due to dilution of the CH_4 that is always produced from the feed consumed to fulfil the animal's maintenance requirement. Gerber *et al.* (2011) showed how increasing milk production reduced total CO_2-eq per kg fat protein corrected milk (FCPM) from 12 kg CO_2-eq/kg FPCM at a very low level of production of around 300 kg FPCM per cow and year to 3 kg CO_2-eq/kg FPCM at a production level of 2000 kg FPCM per cow and year. Emissions were further reduced to 1.6-1.8 kg CO_2-eq/kg FPCM as yield increased to 6000 kg FPCM, after which they more or less stabilised. For CH_4 production only, the trend was very similar (Gerber *et al.*, 2011). In Sweden, the number of cows decreased from 525 000 in 1993 to 338 000 in 2014 (-36%), while total milk delivered to dairies only decreased marginally (SCB, 2016). This means that total CH_4

emissions decreased by more than 20%. However, in analyses using data on the linear relationship between DMI and CH_4 production, there is usually a high correlation between intake and CH_4 production. This high correlation is achieved with a wide range of DMI, while when considering a more narrow range of DMI and also increased feeding level the variation is much higher. The reason for this variation needs to be further investigated. Non-linear models has been used for predicting CH_4 in relation to DMI, the quadratic model of Axelsson (1949) predicts maximum CH_4 production at DMI of 12.5 kg/d and the declined values above that. In the study by Ramin and Huhtanen (2012a) non-linear models was more precise than linear DMI models, according to lower prediction error.

3.1.2 Concentrate inclusion

Type of diet has been shown to affect microbial community composition and microbial diversity, which in turn can impact on CH_4 production. In different cattle systems, the animals are fed forage-only diets or mixed diets with different proportions of forage and concentrate. Forage usually contains more fibrous material than concentrates. Concentrates are instead rich in starch. Reducing the proportion of forage and increasing the proportion of starch is widely assumed to be a useful strategy to reduce CH_4 production from ruminants, mainly with inclusion of more than 50% concentrate in the diet (Johnson & Johnson, 1995). According to IPCC (2006), CH_4 production as a percentage of gross energy intake (GEI) is 6.5% for dairy cows fed a mixed ration, compared with 3% for cattle in feedlots, which are commonly fed >90% concentrate. However, in the meta-analysis by Ramin and Huhtanen (2013), only diets with less than 75% concentrate as dietary DM were included. The results showed that the effect of forage to concentrate proportion was rather small up to 70-75% of dietary DM. Diets with concentrate comprising between 0-75 and 90% were suggested to probably rapidly change CH_4 production, but this may be difficult to predict (Ramin & Huhtanen, 2013). Digestion of starch-rich diets is faster than digestion of forage diets and this results in a rapid increase in the amount of VFAs, followed by a pH decrease (Krizan *et al.*, 2010; Cannas & Van Soest, 2000). It has been observed that on changing from a forage-rich diet to a high grain diet, the proportion of fibrolytic bacteria decreases and the proportion of starch-fermenting bacteria increases (Penner *et al.*, 2010). This type of diet shift has also been shown to result in a shift in VFA proportions as described above, with an increase in propionate and a decrease in CH_4 production (Penner *et al*., 2010). This effect has not been found in all cases however, as some studies report no effect (Popova *et al*., 2011) or the opposite effect (McGinn *et al*., 2006) on CH_4 production. In other studies with barley as the main starch source, an increase

has been observed in molar proportion of butyrate and a decrease in molar proportion of propionate with increasing barley proportion in the diet (Moss *et al*., 1995; Jakkola & Huhtanen, 1993). Furthermore, other studies show that the impact of concentrate has a variable effect on CH_4 production (Figure 4). This difference in CH_4 response with increasing concentrate level between different studies is most likely related to the different effects on fermentation pattern or feeding level depending on type of starch or concentrate used (Hristov *et al*., 2013; Moss *et al*., 1995).

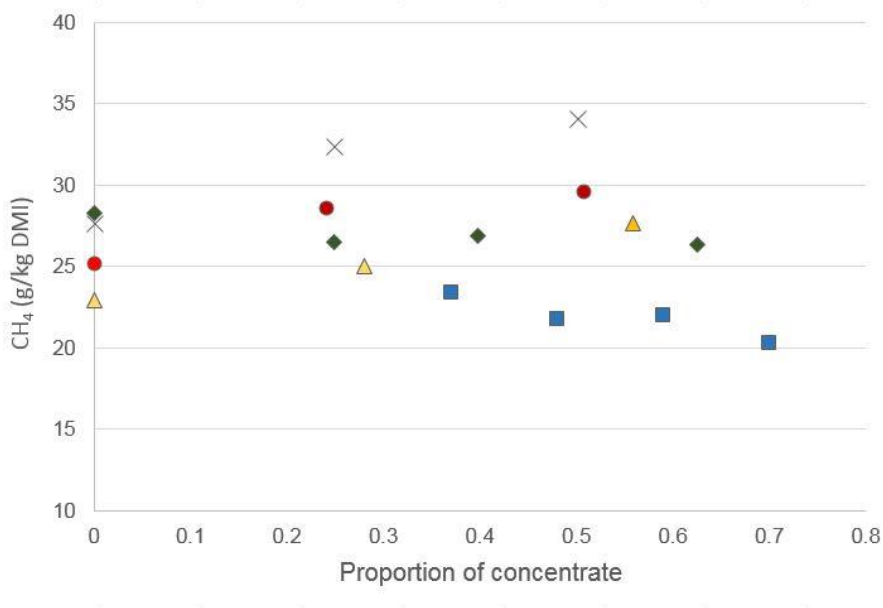

Figure 4. Results of five different studies showing different responses in terms of CH_4 production per kg dry matter intake (DMI) to proportion of concentrate in the total diet. Different colours and marks represent data from different studies. Red circles = Moss and Givens (2002), grey crosses = Moss *et al*. (1995), blue squares = Ferris *et al*. (1999), yellow triangles = Beever *et al*. (1988) and green diamonds = Kirkpatrick *et al*. (1997).

However, when changing to starch-rich diets, the advantage of ruminants' ability to degrade cellulose is decreased and instead there is greater competition for starch-rich feeds, such as cereals, that could be better used for monogastric animals or directly as human food. In addition, forage is crucial for the health and welfare of ruminants and too low a proportion of forage will decrease rumination and salivation. A decrease in salivation in turn reduces the capacity to buffer VFAs, which can cause the pH to fall too low for the microbes in the rumen (Van Soest, 1994). Instead, by increasing forage quality through

increasing its digestibility and its energy content, forage can constitute the majority of the diet fed to dairy cows. Swedish cows produce on average 9900 kg ECM/year, which is in the upper range of production levels in the world (FAOSTAT, 2015). In a study in Sweden by Patel (2012) it was shown that feeding cows with a forage:concentrate ratio of 70:30 ratio did not affect milk production compared with a 50:50 diet, provided the energy content of the silage was above 11 MJ ME and it contained 14-15% CP per kg DM. Furthermore, an increase in forage proportion in the diet of dairy cows will increase the demand for cultivation of leys compared with grain. This will have a positive impact on the environment, as there are several environmental benefits of ley cultivation, *e.g*. it is a perennial crop that needs less tillage and less energy from fossil fuels, less pesticide is used and it results in less eutrophication compared with grain (Flysjö *et al*., 2008).

3.1.3 Digestibility

Depending on type of forage, the NDF content increases in relation to maturity stage, which decreases the digestibility (Rinne *et al.*, 1997). High digestibility increases the utilisation of energy and increasing the level of energy intake gives only a small increase in CH_4 production, while milk and meat production increase dramatically (Hegarty *et al*., 2010). For example, for a lamb weighing 30 kg that consumes 900 g/day of forage, an increase in digestibility from 65 to 75 % would lead to an average increase in daily weight gain from 51 to 101 g, with only a slight increase in CH_4 output of less than 1 g CH_4/day. Thus this would give only half the emissions per unit average daily weight gain compared with the low digestibility diet (Hegarty *et al*., 2010). Dietary starch composition can vary widely between forage species, *e.g*. maize silage contains around 30% starch while the concentration in grass silage is very low (Rinne *et al*., 1997). High quality feeds with high digestibility and energy content can increase animal productivity by improving feed utilisation and also lower CH_4 production per kg product.

3.2 Feed supplements and additives

3.2.1 Fat

Fat inclusion seems to be one of the most promising strategies to reduce CH_4 production, but the effect varies between studies and seems to be influenced by type of diet, source of fat, type of fatty acid and level of inclusion (Beauchemin *et al*., 2008, 2007). In the review by Beauchemin *et al.* (2008), an analysis performed with results from 17 different studies showed that CH_4 (g/kg DMI)

was reduced by 5.6% per additional 1% of inclusion of fat in the diet compared with the control. A total reduction of 10-25% was proposed for a common commercial diet when fat inclusion was at most 6-7%, which is the maximum fat inclusion recommended in NRC (2001) feed recommendations (Beauchemin *et al*., 2008). The potential reduction in CH_4 production is due to several actions of the lipids. Lipids are not fermented in the rumen and thus the digestion of organic matter (OM) is less, giving less CH_4 production per kg OM (Martin *et al.*, 2010). Moreover, lipids have an anti-microbial action against methanogens and also affect protozoa, cellulolytic bacteria and other bacteria (Maia *et al.*, 2007; Doreau & Ferlay, 1995), which is promising for reducing the CH_4 production. This effect seems to be mainly due to different long-chain fatty acid (LCFA) sources, although CH_4 emissions are also somewhat lowered because of reduced fibre digestion (McGinn *et al*., 2004). The inhibition of cellulolytic microbes gives a shift in microbial population, which may increase propionate production (Martin *et al*., 2010). Moreover, as mentioned in section 2.2.1, biohydrogenation of unsaturated fatty acids can be an alternative source for use of the H_2, but incorporation of the H_2 produced is small compared with in methanogenesis (Czerwaski & Clapperton, 1984). In a study by Palmqvist and Jenkins (1980), addition of 3-5% fat was shown to have the best effect on milk production, without any negative effect on the microflora. Similar conclusions were drawn in a meta-analysis study by Huhtanen and Nousianen (2012), who found that 3-4% fat inclusion was most optimal with regard to milk production. Different sources of lipids can have a negative effect on DMI, which may affect milk production over longer periods of time, and it is therefore important to analyse the long-term effect of fat additives (Knapp *et al*., 2014; Beauchemin *et al*., 2008; Grainger *et al*., 2008). Furthermore, the economic aspect needs to be considered, as the cost of fat additives, especially refined oils, is usually higher than the cost of the fat source in the commonly used cereals, which makes use of fat additives unprofitable for commercial use. However, within Swedish dairy production there are small possibilities to make any gains by increasing fat feeding, as most common commercial feeds in Sweden already contain 5-10% crude fat.

3.2.2 Plant bioactive compounds

Plant bioactive compounds such as tannins and saponins may have CH_4 mitigating potential. Tannins, as feed supplements or as tanniferous plants, have frequently been shown to have potential for reducing CH_4 emissions by up to 20% (Mohammed *et al*., 2011; Waghorn *et al.*, 2002). The reduction in CH_4 is due to the inhibitory effect on methanogens, protozoa and other hydrogen-

producing microbes (Patra & Saxena, 2010; Tavendale *et al.*, 2005). At the same time, reduced digestibility is common for diets containing condensed tannins at high levels (Patra & Saxena, 2010; Waghorn, 2008). In addition, intake and animal health can be negatively affected if tannin inclusion rate is more than 50 g/kg feed (Mueller-Harvey, 2006). Temperate plants rich in tannins can replace other forages and in hot and arid regions many legumes are rich in tannins and represent a valuable feed resource. There is a large diversity within different types of tannins depending on chemical structure, which together with level of intake partly explains differences in mitigation potential for CH_4 production observed with different sources of tannins (Morgavi *et al*., 2013; Mueller Harvey, 2006). Tannins are also used in ruminant nutrition to increase protein utilisation. This effect is obtained though tannins binding to dietary proteins, which can then become 'rumen-escape' proteins that are further utilised in the intestine instead (McSweeney *et al*., 2001).

Saponins influence CH_4 production and protein metabolism in the rumen by their toxic effect on protozoa (Patra & Saxena, 2010; Jouany & Morgavi, 2007). In a meta-analysis by Goel and Makkar (2012), six of the nine studies investigated reported a decrease in CH_4 production from about 6 to 27% (per unit body weight (BW) or DMI). In sheep, decreases of 10-15% in CH_4 production have been reported with *Yucca schidigera* and *Quillaja saponaria* saponin sources (Wang *et al.*, 2009; Pen *et al.*, 2007) and similar results have been reported for tea saponins (Mohammed *et al*., 2011). The effect over time is unknown and it has been observed that there may be an inactivation of rumen bacterial populations (Newbold *et al.*, 1997), which may give a reduced effect over time.

3.3 Methane inhibitiors

Inhibitors such as bromochloromethane, 2-bromo-ethane sulfonate and chloroform have been shown to reduce CH_4 emissions, but with a harmful effect on the animal, which makes them unsuitable for use on commercial farms (McAllister & Newbold, 2008). Recently, the use of 3-nitrooxypropanol (3NP) was shown to reduce CH_4 emissions in dairy cows by 30 % without any effect on milk production or feed intake (Hristov *et al.*, 2015). However, in another study the effect was about 8% and no further reduction was obtained with increased inclusion (Reynolds *et al*., 2014). The difference in effect may be due to animal, diet and dosing method (Reynolds *et al*., 2014). In contrast to the above-mentioned inhibitors, the results indicate that 3NP shows no signs of toxic effects on the animal and no or a minor effect on DMI. The effect of 3NP is due

to blockage of CH_4 production by inhibition of the last step of methanogenesis (Haisan *et al.*, 2014).

3.4 Ionophores

Ionophores are lipid-soluble ion carriers that transfer ions over the cell membrane and thus disrupt the membrane potential, specifically in gram-positive bacteria, and as a consequence affect CH_4 production (Wolin & Miller, 2006). Monensin is the most commonly applied ionophore and it is routinely used in beef production and dairy cattle nutrition in North America to increase feed efficiency (Hristov *et al*., 2013a). It promotes the production of propionate at the expense of acetate and hydrogen (Johnson & Johnson, 1995). However, the use of monensin has been shown to cause a reduction in feed intake, which may explain part of the lowering effect on CH_4 through less feed being fermented (Hegarty, 1999; Johnson & Johnson, 1995). Monensin does not appear to have a consistent direct effect on CH_4 production in dairy or beef cattle, but due to the increase in production a reduction in CH_4 emissions per unit of meat (Goodrich *et al*., 1984) and milk (Duffield *et al*., 2008) may be obtained for a short period. However, ionophores are banned in the European Union due to the potential risk of antibiotic resistance. Furthermore, the image of milk as a 'natural product' could be affected negatively by using chemical additives.

3.5 Vaccination

Vaccination may be a possible strategy for inhibiting CH_4 production that is very attractive, as it can be applied in all types of animals and is a practical approach on farm level (Clark, 2013; Wedlock *et al*., 2013). A vaccine used for inhibition of CH_4 is thought to induce antibodies in saliva and this results in high levels of antibodies in the rumen, reducing the activity of the methanogens (Wedlock *et al*., 2013). However, no significant inhibition of CH_4 production has been observed with the vaccines tested to date (Williams *et al*., 2009; Wright *et al*., 2004). To succeed in reducing CH_4 production, an anti-methanogen vaccine needs to have broad specificity against common methanogens in the rumen. Identification of key antigens is needed and knowledge of the genome of methanogens will probably increase the potential to succeed with a vaccine (Wedlock *et al*., 2013). However, even if a potential vaccine is produced, there may be only a short-term effect as the adaptation of microbes and persistence of the effect are unknown.

3.6 Individual variation

Measurements on individual sheep in respiration chambers have shown that methane emissions from animals within the same group fed the same diet can vary significantly in terms of g CH_4 per kg DMI (Pinares-Patiño *et al.*, 2003)The mechanism behind the individual variation is not well known and researchers are trying to identify certain parameters that can have an impact. In a study by Goopy *et al.* (2014), a positive correlation was found between CH_4 production and rumen size and rumen retention time. Furthermore, the passage rate has been shown to be heritable (Smuts *et al*., 1995). The pH level in the rumen also varies and seems to be regulated by the individual through salivation, rumination and absorption of VFAs (Weimer *et al*., 2010). In addition, previous studies have shown host-specific interactions on the microbial flora, which could make it possible to breed for animals with a microbiota that promotes low CH_4 production (Hernandez-Sanabria *et al*., 2013; Guan *et al*., 2008). Breed of beef cattle has been shown to have an effect on microbial composition (Guan *et al*., 2008) and in a study by Hernandez-Sanabria *et al.* (2013) the sire had effect on the microbial composition in the offspring.

Recently, Roehe *et al.* (2016) analysed the links between microbial genes and CH_4 emissions according to sire effect and concluded that archaeal abundance is under host genetic control and that selection of low emitters would be possible. Another example showing that the microbiota is host-specific is provided by Weimer *et al.* (2010), who performed an almost total exchange of rumen content with differing VFA concentration and pH between two pairs of cows. The first pair showed recovery of pH and VFA within 24 hours and recovery of the bacterial community composition took 14 days for one cow and 61 days for the other. For the second pair of cows, one showed total recovery of pH and VFA within 24 h, while the other had a higher pH and lower VFA that was maintained during the test period of 62 days. The bacterial community for the second pair was different to the pre-change community, but was still more similar to the individuals' community pre-change than to the donor community. Weimer *et al.* (2010) therefore concluded that bacterial community composition is host-specific and is optimised according to the different ruminal conditions in different individuals. However, the differences in CH_4 production between animals may be related to the increased passage rate and also reduced fibre digestibility. Thus, before selecting low CH_4 emitters, it has to be confirmed that low CH_4 production is not associated with low fibre utilisation and less milk or meat production.

3.7 Other aspects to be considered

When analysing the effect of certain strategies, it is important to evaluate the long-term effect of the reduction potential and the overall effect of rumen function. The microbial community structure in the rumen seems to change according to dietary changes, but then there seem to be a return to the pre-change structure which may be only visible after a long period (Weimer *et al.*, 2010). Practical and economic issues are important in the evaluation of potential CH_4 production inhibitors for sustainable strategies at farm level. In low intensity systems, increased individual production by improving feed quality and feeding intensity level will significantly reduce CH_4 production per unit product through dilution of the CH_4 from maintenance. However, in intensive systems the mitigation potential is lower. In a study by Knapp *et al.* (2014), the effect of feed and feed additives in intensive dairy production systems was calculated to reduce CH_4 production/kg ECM by 2-15%. Combining feeds and additives with other strategies such as breeding and improved management leading to higher feed efficiency and higher lifetime production may reduce CH_4 production by 15-30%. When aiming for lower CH_4 production with certain strategies, it is important to avoid wastes in the whole chain from field to farm, so that a decrease in CH_4 production does not increase GHG in another part of the production chain.

4 Rumen microbiology

Rumen microbes have co-evolved with their hosts and perform specialist functions which are highly important for the health and nutrition of the ruminant (Morgavi *et al*., 2010). The rumen environment harbours microbes from all three domains: bacteria, archaea and eukaryotes. The microbial ecosystem in the rumen consist predominantly of bacteria, accounting for approximately 95% of the total amount of microorganisms. The numbers of the different group of microbes are around: 10^{10}-10^{11} bacteria/mL, 10^{4}-10^{6} protozoa/mL, 10^{3}-10^{7} fungi/mL, 10^{7}-10^{9} archaea/mL and 10^{9}-10^{10} viruses/mL (Wright & Klieve, 2011). However, less than 1% of rumen microbes have been cultured and identified (Amann *et al*., 1995). This lack of sufficient understanding of the ruminal microbiome is one of the major knowledge gaps hindering effective enhancement of rumen functions (Firkins & Yu, 2006). There are many factors that affect microbial community composition and function and it is important to get a better understanding of their interactions with environmental factors in order *e.g*. to succeed in attempts to redirect the fermentation pattern to increase fibre digestion and energy utilisation with reduced impact on the environment.

4.1 Bacteria

Within the rumen, about 70-75% of the bacteria are associated with feed particles (Craig *et al*., 1987). By attachment to feed particles, bacteria require a lower growth rate to remain in the rumen, compared with the higher passage rate and washout in the liquid phase (McAllister *et al*., 2004). The remaining 25-30% of bacteria are in the liquid phase, where they soon attach to feed particles or flow down to the lower tract, where they are utilised as feed protein for the host in the intestines (Legay-Carmier & Bauchart, 1989; Craig *et al*., 1987). There are also some bacteria that are attached to the epithelium of the rumen wall, but these represent just a small fraction of the total number of bacteria and seem to be more related to the metabolic signal to the host than other types of bacteria (Wallace *et al*., 1979). The bacterial community in the rumen is highly diverse and more than 200 species have been identified (McSweeney *et al*., 2005). The number of active bacteria depends on parameters such as animal, breed, type of feed, composition of feed and many other factors (Agarwal *et al*., 2015). Each microbial species has specialist substrate preferences and the rumen bacteria can be classified into cellulose, hemicellulose, pectin, starch and sugar digesters (Zhou *et al*., 2015). Cellulose, hemicellulose and pectin are the main components of plant cell wall polysaccharides, which can all be degraded with different types of enzymes from certain microbes. The dominant hemicellolytic bacteria

identified to date are *Butyrivibrio fibrosolvens*, *Ruminococcus flavefaciens*, *Ruminococcus albus and Prevotella ruminocola* (Dehority, 1994). Pectin and hemicellulose need to be degraded before it is possible to degrade the cellulose. Cellulolytic bacteria in the rumen are both gram-negative and gram-positive species. Many cellulolytic bacteria belong to the genera *Fibrobacter*, *Ruminococcus*, *Butyrivibrio*, *Prevotella* and *Eubacterium* (Koike & Kobayashi, 2009), with the most commonly found species present in almost all ruminants being *Fibrobacter succinogenes*, *Ruminococcus flavefaciens* and *Ruminococcus albus* (Weimer, 1992). These cellulolytic species have a very restricted range of growth substrate, as they can only utilise cellulose and its hydrolytic product glucose (Hungate, 1966). In forages, pectin represents a smaller percentage compared with hemicellulose and cellulose, but is fermented more rapidly (Chesson & Monro, 1982). The major pectin-degrading species have been identified as *Butyrivibrio fibrosolvens*, *Prevotella ruminocola*, *Fibrobacter succinogenes*, *Lachnospira multiparous* and *Succinovibrio dextrinosolvens* (Bryant & Small, 1956).

4.2 Archaea

Archaea are widespread in different anaerobic environments. Methanogenic archaea have been found in the rumen, lower intestinal tract of mammals, gut of termites, sewage, anaerobic digesters, landfills, rice paddies, freshwater sediments, marine sediments, geothermal systems and heartwood of trees (Liu & Whitman, 2008). In all these environments, methanogens form a large and diverse prokaryotic domain, not only ecologically but also phylogenetically (Cerosimo & Wright, 2015). Although the methanogens are strictly anaerobic, they are difficult to grow *in vitro* and consequently only a few methanogens have been cultured (Wright & Klieve, 2011). Compared with bacteria, rumen archaea are much less diverse (Henderson *et al*., 2015). Today there are seven known orders of methanogens: Methanobacteriales, Methanomicrobiales, Methanococcales, Methanocellales, Methanopyrales, Methanosarcinales and the recently discovered order Methanomassiliicoccales (Oren & Garrity, 2013). In ruminants, the genus *Methanobrevibacter*, which belongs to the order Methanobacteriales, is the most abundant methanogen (St Pierre & Wright, 2012; King *et al*., 2011; Jeyanathan *et al*., 2011; Wright *et al*., 2004). According to Cerosimo and Wright (2015), 120 species and 33 genera of methanogens have been identified in the rumen. Methanogens are not able to degrade complex molecules and thus need other microorganisms that provide them with their substrates. In the rumen, some methanogens live in a symbiotic relationship with other microbes, such as protozoa, and some are free-living. There is a synergistic

relationship between fermenting microbes producing H_2 and H_2-utilising methanogens, involving interspecies hydrogen transfer (Thiele *et al*., 1988). This synergistic relationship enhances the fermentation of feed by keeping the H_2 pressure low (Demeyer & Van Nevel, 1975). As mentioned in section 2.2, different methanogens utilise different substrates, and hydrogenotrophic and methylotrophic methanogens are most commonly found in the rumen. In general, species within the genus *Methanobrevibacter* utilise H_2 for the reduction of CO_2 to CH_4, but some species can also utilise formate. These include *Methanobrevibacter olleyae*, *Methanobrevibacter ruminantium*, *Methanobrevibacter millerae*, *Methanobrevibacter smithii* and *Methanobrevibacter woesei* (Ferry & Kaestad, 2007). Even though most methanogens in the rumen are hydrogenotrophs, some species also utilise methanol or/and methyl amines. *Methanosarcina barkeri* and *Methanosarcina mazei* utilise both methanol methyl amines (Ferry & Kaestad, 2007), whereas *Methanosphaera stadtmanae* only utilises methanol (Miller & Wolin, 1985). Recently, Poulsen *et al.* (2013) found that species within the recently discovered genus *Methanomassiliicoccus* also utilised methanol and methyl amines as substrate.

A feature in common for all methanogens is the use of methyl coenzyme M-reductase (MCR), an enzyme that is only present in methanogens (Luton *et al*., 2002). In the last step in methanogenesis, the methyl group in methyl coenzyme M is reduced to CH_4 by MCR reductase and coenzyme M is regenerated. There are different types of MCR enzymes which seem to be activated at different hydrogen pressures (Reeve *et al*., 1997). In general, hydrogenotrophic methanogens encode two of these MCR enzymes, Mcr I and Mcr II (Rospert *et al*., 1990). However, Leahy *et al.*, (2010) found that *M. ruminantium* M1 only encoded Mcr I, which is active at comparatively lower H_2 concentrations, which suggests that M1 has adapted to rumen conditions over time (Leahy *et al*., 2010).

4.3 Protozoa and fungi

In addition to archaea and bacteria, protozoa and fungi are other microbes found in the rumen that have an impact on feed digestion. Protozoa account for up to 50% of the microbial biomass in the rumen, although they are present in much smaller numbers than bacteria and may contribute to approximately one-third of feed degradation (Williams & Coleman, 1997; Hungate, 1966). Protozoa produce large quantities of H_2 via their hydrogenosome organelles containing hydrogenases (Yarlett *et al*., 1986) and can also live in a symbiotic relationship with hydrogen-consuming archaea (Williams & Coleman, 1997). Newbold *et al.*

(1995) concluded that 9-25% of rumen methanogens are associated with protozoa (living inside protozoa or in close contact). Up to 37% of rumen-derived CH_4 can be produced by protozoa-associated methanogens (Finlay *et al*., 1994). Protozoa are of two major types: holotrichs and entodiniomorphids. The differences between holotrich and entodiniomorphid protozoa seem to be related to substrate preference, O_2 consumption, H_2 production, growth rate and fermentation end-products (Ellis *et al*., 1989). It has been observed that holotrich protozoa seem to be more closely related to CH_4 production, as their presence increases CH_4 production compared with in defaunted (protozoa removed) sheep (Belanche *et al*., 2012). The symbiotic relationship between protozoa and methanogens has made the area of manipulation of protozoa very attractive as a strategy to reduce CH_4 production (Wright, 2015).

When fungi were first observed in the rumen, they were considered protozoa. It was not until 1975 that Orpin (1975) recognised these cells as fungi. Fungi in the rumen belong to the phylum Neocallimastigomycyota, which just comprises one family (Gruninger *et al*., 2014), with seven different genera (Callaghan *et al*., 2015). Fungi account for 5-20% of the microbial biomass in the rumen. It has been suggested that fungi are adapted to penetrate and disrupt plant tissues that cannot be degraded by other microorganisms, which further improves bacterial colonisation and degradation (Lowe *et al*., 1987). The fungi produce all the enzymes needed for biomass degradation, such as cellulases, xylanases, esterases, glucosidases and glucanases, but the level of their contribution to degradation of feed is not well known (Lee *et al*., 2000). More recently, fungi have been considered a key contributor in the degradation of lignocellulosic plant fibre (Gruninger *et al*., 2014). During fermentation, fungi produce H_2, CO_2, acetate, formate, lactate and ethanol as metabolic waste products (Gruninger *et al*., 2014). Similarly to the protozoans, H_2 is produced by hydrogenosomes. Furthermore, the wide range of enzymes that are produced by fungi and their ability to degrade lignified plant walls have made them interesting for different biotechnological methodologies. Studies on rumen fungi by next generation sequencing have observed a much higher variation between individuals within species and between species compared with bacteria and archaea (Kittelman *et al*., 2013).

4.4 Core rumen microbiome

A common set of microbes that are shared by individual samples may contribute to basic rumen function (Henderson *et al*., 2015). To define the core rumen microbiome, knowledge of basic microbial structure and function is important to further identify temporary changes (Jami *et al*., 2012). A meta-analysis by

Henderson *et al.* (2015) in which rumen fluid was sampled from different ruminants from 35 different countries suggested that there is a core microbiome of some dominant groups within ruminants. The seven most abundant groups identified in that study were: Prevotella, *Butyrivibrio*, *Ruminococcus* unclassified Lachnospiraceae, Ruminococcaceae, Bacteriodales and Clostridales, representing 67.1% of total sequence data. Henderson *et al.* (2015) concluded that it is not likely that any new dominant species will be found. In a study by Jami *et al*. (2012), Bacteroidetes and Firmicutes mainly dominated at phylum level, but with a high variation in relative abundance between individuals, while at genus level 32 genera were shared across all samples. Although there was high variation in abundance of each genus between samples and some genera were present in very low abundance, they were still shared between all individuals and clearly have a key role in rumen function (Jami *et al*., 2012). In the study by Henderson *et al.* (2015), the archaea were dominated by methanogens. Clades of *Methanobrevibacter gottschalkii* and *Methanobrevibacter ruminantium* were found in almost all samples. These clades were represented by closely related species with 99% identity, with the *M. gottschalkii* clade being represented by the species *M. gottschalkii*, *M. millerae* and *M. thaueri* and the *M. ruminantium* clade by *M. ruminantium* and *M. olleyae* (Seedorf *et al*., 2015). Both clades accounted for 74% of all archaea (Henderson *et al*., 2015). *Methanosphaera* sp. and two Methanomassiliicoccaceae-affiliated groups were also widely found and, together with both the *Methanobrevibacter* clades, represented 89.2% of the archaeal communities. Similar results were observed in the study by Seedorf *et al.* (2015), where *Methanobrevibacter ruminatium*, *Methanobrevibacter gottschalkii*, *Methanospharea* spp. and *Methanomassiliicoccales* spp. represented 99.98% of all archaeal sequences.

5 Methods used to study CH_4 production and rumen microbiota

Reliable methods are important when estimating the amount of CH_4 produced from individuals and/or from different types of diets or added supplements. The same applies when studying rumen microbial population. Today there are many different methods for measuring CH_4 and for investigating rumen microbiota, all with their advantages and disadvantages. The choice of method affects the results, which is important to take into consideration when evaluating the results and before comparisons between different studies can be made. The choice of method depends on the purpose of the study and is usually a balance of pros and cons. This chapter mainly discusses methods used to study CH_4 production and rumen microbiota in the different papers in this thesis. A summary of methods used for measuring CH_4 production and microbial populations in Papers I-IV is presented in Table 2.

5.1 Measuring CH_4 production

The choice of method among those that are available today for measuring CH_4 production *in vivo* depends on the purpose of the study. In general, the choice is between high accuracy in CH_4 production from few animals, as with the chamber technique, and/or CH_4 measurements in many animals with a higher variability compared with the chamber technique. Cost in relation to the quality of the results from a certain method is another important aspect to take into account. Methane production can also be measured *in vitro*, although *in vivo* methods are more accurate because they measure directly on the animal. The *in vitro* approach is a way to mimic the *in vivo* situation, with certain limitations. Advantages with *in vitro* applications are that they are cheaper and have no effect on the animal.

5.1.1 Chamber technique

The respiration chamber is the most consistent technique and is usually used as a reference method for evaluation of other CH_4 measurement techniques (**Paper IV**; Yan *et al*., 2010; Johnson & Johnson, 1995). In brief, the animal is placed in the chamber for some hours or days, with ventilation for inlet and exhaust air. Production of CH_4 is calculated from gas flow and gas concentrations from the chamber. All CH_4 production from the animal is taken into account, even rectal. The main disadvantage of the chamber technique is the controlled environment, which is unnatural for the animal and can have an impact on intake behaviour (Johnson & Johnson, 1995). However, a recent study in which the chamber was

covered with transparent polycarbonate to allow the test animals to see other animals found no impact on DMI (Hellwing *et al*., 2012). Other disadvantages are the cost of the equipment and the high labour requirement.

5.1.2 Sulphur hexafluoride (SF_6)

The SF_6 technique was used for measuring CH_4 production by the cows in **Paper I.** The technique is described in full in Johnson *et al.* (1994) and has been used in a number of studies (Hammond *et al*., 2011; Pinares-Patiño *et al*., 2003; Boadi & Wittenberg, 2002). It is based on the known release of the tracer gas SF_6 and the ratio between SF_6 and CH_4 concentration. A brass tube with a permeable membrane containing the SF_6 gas, with known release rate, is placed in the rumen. Cows are fitted with a PVC yoke which is pre-evacuated so there is a constant draw of air into the yoke (Figure 5). Methane and SF_6 gas are collected, usually during 24 hours, and samples from the yokes are then analysed for concentrations of both gases. Release rate and concentration of SF_6 are related to the concentration of CH_4. Background SF_6 and CH_4 concentrations are also measured and subtracted from the final CH_4 calculation. Enteric measurements reported by Patel *et al.* (2011) were the first measurements of CH_4 production on dairy cows in Sweden. The major advantage of this technique is that it can be used on cows in their 'natural' environment and can also be used on grazing cows. The disadvantages are high labour requirement and high variability between samples, much higher than with chambers (Pinares-Patiño *et al*., 2011).

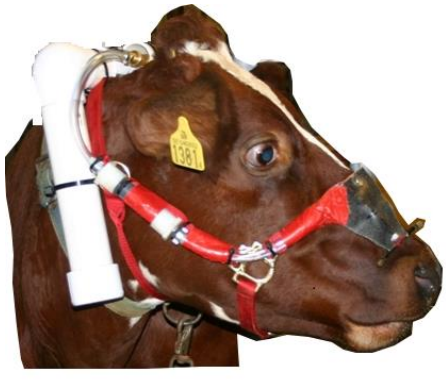

Figure 5. Cow 1381 equipped with halter with inlet tube connected to a PVC yoke.

5.1.3 Spot sampling

Measuring CH_4 production on many animals in chambers or by SF_6 would be very laborious and costly. Therefore other techniques have been developed in

recent years to make representative measurements of CH_4 on many individuals. Today there are several types of so-called spot sampling techniques, which are generally based on measuring CH_4 production in the air while the cow is eating during milking or in concentrate feeders. These measurements are repeated several times during the day and over a period of days, weeks or months. One of the spot techniques that used in **Paper II** was initially described by Garnsworthy *et al.* (2012). The equipment is set up in a feeding bin, usually in a robotic milking system, and CH_4 is measured each time a cow makes a visit. Calculation of CH_4 concentration per cow and day is then based on CH_4 eructation frequency and CH_4 concentration per unit eructation at milking. CH_4 concentration is measured by an infrared technique. This technique has been used in several studies and permits repeated analysis of CH_4 production in a large number of cows during periods of weeks and/or whole lactations (**Paper II;** Bell *et al.*, 2014; Garnsworthy *et al.*, 2012). There are other similar spot sampling techniques, such as the method described by Madsen *et al.* (2010) for measuring CH_4 and CO_2 in the breath when a cow is milked or fed from a feeding bin. Predicted CH_4 is calculated based on predicted CO_2 production (according to intake of metabolisable energy (ME) or heat-producing units) and the measured CH_4:CO_2 ratio. When CO_2 production is predicted, this is based on the assumption that there is no difference in feed utilisation efficiency between cows, which may not be the reality. The main disadvantage with the spot sampling techniques is higher variability than with respiration chambers (Huhtanen *et al.*, 2015).

Another spot sampling technique is a gas flux quantification method called the GreenFeed system (C-Lock Inc., Rapid City, SD). This method uses a similar technique to the respiration chamber, with a constant airflow through the system and continuous analysis of CH_4 and CO_2 concentration in the air and gas flow. The GreenFeed system is equipped with head position sensors and data are only used when the head is in the right position, which is one of the main advantages compared with the spot techniques described by Garnsworthy *et al.* (2012) and Madsen *et al.* (2010) (Huhtanen *et al.*, 2015).

5.1.4 In vitro

In vivo studies are expensive and in order to reduce costs and reduce the impact on the animal, *in vitro* systems have been developed as an alternative way to analyse CH_4 emissions. Continuous culture experiments as described by Czerkawski and Breckenridge (1977) and batch culture experiments as reported by Van Nevel and Demeyer (1981) are commonly used for evaluating the effects of diets and additives on enteric CH_4 production. *In vitro* studies can also be used

for screening a large number of substrates/diets or as a first step in evaluating possible CH_4 inhibitors. The gas *in vitro* technique used in **Papers III** and **IV** was developed by Ramin and Huhtanen (2012). This method predicts CH_4 production in the cow rumen using kinetic parameters obtained from an automated *in vitro* gas production system and a two-compartment rumen model. This approach takes into account rumen dynamics of digestion and passage kinetics in the rumen, which may have advantages compared with single timepoint batch culture systems. In this batch culture system (Figure 6), samples of gas are collected from each bottle during incubation at different times, *e.g*. 2, 4, 8, 24, 32, and 48 h. Based on the kinetic data on CH_4 production obtained from the *in vitro* gas production, CH_4 production can be predicted *in vivo* using the modelling approach described in detail by Ramin and Huhtanen (2012). A disadvantage with the *in vitro* system is the artificial environment used to mimic the *in vivo* system. The batch system used is closed and there is no outflow of VFA produced, which may have an impact on CH_4 production and on development of the microbiota. A continuous *in vitro* system that has been used in many studies is the Rusitec semi-continuous rumen simulation system (Czerkawski & Breckenridge, 1977). In Rusitec, the fermentation can continue for several weeks. However, loss of protozoa and probably some bacteria has been observed in the Rusitec system, which may make a difference to the *in vivo* environment (Prevot *et al*., 1994). *In vitro* techniques are used to explain what happens *in vivo* and it is therefore important that the techniques are reliable and well validated. It is also important to be aware of the differences from the *in vivo* state and the limitations of *in vitro* systems when evaluating *in vitro* results.

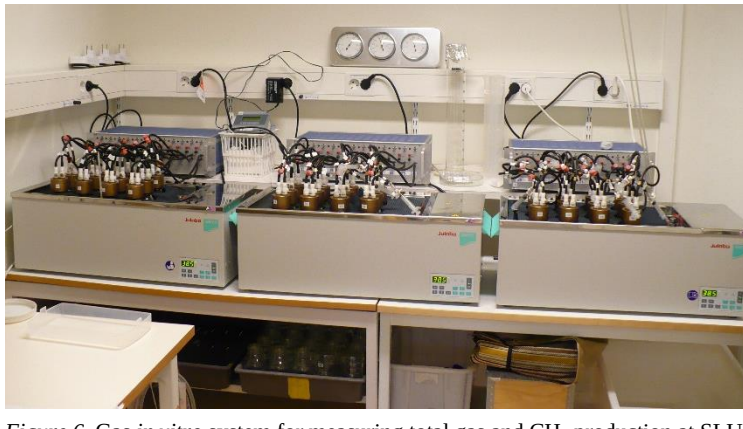

Figure 6. Gas *in vitro* system for measuring total gas and CH_4 production at SLU Umeå

5.2 Methods for measuring archaeal and bacterial communities in rumen

The rumen microbial environment has been investigated mainly with cultivation techniques in the past. Robert Hungate, a pioneer in microbial ecology, developed the first techniques for culturing anaerobic microbes by using agar layers in roll tubes (Hungate, 1960, 1969). This technique was applied in his studies of the bovine rumen and has thereafter contributed to rapid progress in microbiological studies in other anaerobic environments (Tajima & Aminov, 2015). The advantage with cultivation is that the isolates can be studied as regards metabolic properties and other physiological parameters. However, cultivation has several disadvantages; it is labour- and time-consuming and does not provide a fair picture of the whole community structure. Although microorganisms are dependent on interplay with other microorganisms in the community, functions related to interaction and or competition are difficult to determine on isolated microorganisms (Vanwonterghem *et al.*, 2014). Uncultured microorganisms represent the majority of the microorganisms in the rumen, while less than 15% have been isolated and identified (Henderson *et al.*, 2015; Wright and Klieve, 2011). Based on genomic data, a variety of culture-independent methods have been developed during recent years. These culture-independent methods can be used for further understanding the complex microbial diversity and function of ruminal microbes. Characterisation and taxonomic assignment of microbial rumen communities by culture-independent methods usually includes small subunits of 16S ribosomal RNA gene (Leahy *et al*., 2013; Woese, 1987). The 16S rRNA gene, which is found within all prokaryotes, archaea and bacteria, contains conserved, variable and hypervariable regions that make it possible to differentiate between organisms (Juste *et al*., 2008). Databases such as Greengenes, a ribosomal database project with sequence information on the 16S rRNA gene, are well established. Moreover, the functional gene methyl coenzyme A (*mcrA*) (encoding the α subunit of methyl co-enzyme reductase) has also a general application for studying methanogens due to its specific occurrence and involvement in methanogenesis within methanogens (Sirohi *et al.*, 2013).

5.2.1 qPCR

For quantification of certain species or groups of species, real-time quantitative PCR (qPCR) is a commonly used culture-independent method. Quantitative PCR has been used in many studies on rumen microbiota to compare differences between individuals, diets and effects of feed additives (**Papers I** and **II;** Wallace *et al*., 2015; Zijderveld *et al*., 2010; Denman *et al*., 2007). Compared

with end-point PCR, which displays the amplification product at the end, real-time qPCR displays the amplification process in real time. The detection of PCR products is possible due to inclusion of a fluorescent reporter molecule, *e.g*. an intercalating dye such as SYBR green, which fluoresces at double-stranded DNA. The concentration of double-stranded DNA increases after each amplification cycle, which subsequently increases the fluorescent signal. A standard curve is made by dilution of a known amount of target DNA cloned into a cloning vector. By comparing the signal against the standard curve, absolute amount of the gene of interest can be calculated. To decrease the risk of false positive signals, such as primer dimer and amplification errors, a melt curve analysis is performed during the programme (Van Guilder, 2008). The limitations of the method relate to PCR artefacts such as chimeras generated during the amplification step (Wintzingerode *et al*., 1997). It is also known that there is overestimation within genomes according to heterogeneity (copies of 16S rRNA within a sequence) and different target regions within 16S rRNA have more or less heterogeneity (Sun *et al*., 2013). A PCR bias is also related to different programmes based on temperature, time for elongation and number of cycles. In this thesis, qPCR was used in both **Papers I** and **II** to quantify certain groups of archaea and bacteria based on 16s rRNA gene. In **Paper I**, primers were used for quantifying total numbers of archaea and species within the genera *Methanomicrobiales* and *Methanobrevibacter*. In **Paper II,** primers were designed for the group of species within the genus *Methanobrevibacter*, which was shown in **Paper I** to be related to low CH_4 production.

5.2.2 Fingerprinting techniques

Fingerprinting techniques can be used for comparison between treatments or other types of changes to the study environment in order to reveal shifts in the community. Terminal restriction length polymorphism (T-RFLP) has been used in several rumen studies for profiling microbial communities (**Paper I**; Frey *et al*., 2010; Fernando *et al*., 2010). T-RFLP is based on a target gene using PCR, where at least one primer in the PCR reaction is labelled with a florescent dye, such as 6-carboxyfluorescein (FAM). The PCR-amplified product is digested with a restriction enzyme that is suitable for the desired sequence. The digested products, which are called fluorescently labelled terminal restriction fragments (T-RFs), are separated by capillary electrophoresis and detected based on their fluorescence by an automated sequencer. T-RFLP profile is presented as relative abundance of each T-RF at a specific length. Limitations of the technique are that there is no identification of sequences and that it is unable to give a comprehensive view of the structure and function of the microbiota. T-RFLP was used in **Paper I** to get an overview of the methanogenic community

structure in cows fed different proportions of concentrate. For identification of species that contribute to certain TRFs, a clone library can be constructed (**Paper I**).

5.2.3 Clone library

A clone library is used for investigation of DNA extracted from an environmental sample by cloning and following sequencing (Chouari *et al.*, 2005). The clone library approach was used in **Paper I** for identification of specific methanogenic species in combination with T-RFLP. Clone libraries can be constructed from PCR amplicons, such as 16S gene amplicons, which are subsequently ligated into cloning vectors. Vectors are transformed into competent cells (a vector/cell) and by cultivating competent cells individual cloning vectors with different inserts can be isolated and further sequenced. Quality-checked sequences are matched against a database for identification or alignment for phylogenetic construction. Cloning is mainly used in small-scale projects as it is very time- and labour-consuming in relation to the number of sequences obtained. It has largely been replaced by next generation sequencing, especially in large-scale analyses.

5.2.4 Next generation sequencing

Next generation sequencing (NGS), such as 454-pyrosequencing and Illumina sequencing, are cost-effective massive parallel sequencing technologies that can be applied on environmental samples. These techniques enable sequencing of PCR products without an extra clone step, which eliminates clone bias and gives an opportunity for evaluation of the phylogenetic relationship within a community (McCann *et al*., 2014). Barcodes (a string of nucleotides) added onto primer ends enable processing of large numbers of samples at the same time. These NGS techniques have inspired research regarding ruminal microbial diversity, where most studies have applied 454-pyrosequencing to study the rumen microbiota (**Paper III**; Zened *et al*., 2013; Jami *et al*., 2012; Castro-Carrera *et al.*, 2014), because of longer reads compared with Illumina (McCann *et al*., 2014). However, the use of Illumina sequencing has increased due to substantially higher throughput and lower costs compared with 454-pyrosequencing (**Paper II**; Ross *et al*., 2013; Hess *et al*., 2011). The limitations of NGS mainly relate to short reads and the fact that these techniques are only semi-quantitative, generally providing only information on relative abundance. Depending on the sequencing depth, the coverage of the microbial diversity varies, but it usually covers dominant microbes. However, low abundance microbes may be missed and these might still have a key role within the microbiota (Zarraonaindia *et al.*, 2013).

5.2.5 Some other modern culture independent techniques used for studying rumen microbiota

Whole genome sequencing determines the complete DNA sequence of an organism, which enables understanding of its function. The first rumen bacterial genome sequenced was that of *Fibrobacter succinogenes* S85, which revealed genes involved in plant cell degradation (Jun *et al*., 2007). From that first attempt, genome sequencing has increased continuously. Leahy *et al.* (2010) sequenced the whole genome of the important methanogen *Methanobrevibacter ruminatium* M1 and provided new information on the cellular processes and the lifestyle of this certain rumen methanogen. This information can potentially increase the rate of success in development of a vaccine for inhibition of methanogenesis, as it includes identification of methanogen-specific adhesion enzymes and also specific components of the cell envelope (Leahy *et al*., 2010). Hungate 1000 (http://www.hungate1000.org.nz/) is a database project that aims to produce a reference set of 1000 rumen microbial genome sequences, in order to get a better understanding of rumen function. This approach opens up new ways for targeting genes central in ruminant nutrition and CH_4 production FibRumBa (Fibrolytic Ruminal Bacteria, (http://jcvi.org/rumenomics) is a database that provides genetic information on the dominant species of fibrolytic ruminal bacteria, with the aim of increasing knowledge on rumen microbes.

Transcriptomics

Metagenomics has expanded the understanding of rumen microbial diversity and function, but this method mainly reveals whether a gene is present or not (Kumar & Pitta, 2015). Transcriptomics has recently been applied for the expression of genes and it is likely that coming research will focus more on expressed genes in a microbiome using RNA, instead of studying the functions present using DNA sequencing (Kumar & Pitta, 2015; McCann, 2015). Deep metagenome and metatranscriptome sequencing was recently used by Shi *et al.* (2014) to analyse the relationship between microbial population in rumen content and CH_4 production. The relative abundance of methanogens did not differ between high and low CH_4 emitting sheep, but the transcripts showing expression of the genes involved in methanogenesis were higher in high CH_4 emitting sheep than in low emitting sheep (Shi *et al*., 2014).

5.2.6 .and back to culturing.

Even though the culture independent techniques have open new ways for understanding the complex microbial diversity Edwards *et al.* (2004) highlight the need of combination of classical culture-based rumen microbiology methods

with molecular ecological methods to define the metabolic role of uncultivated species in rumen. Isolation and cultivation of microorganisms is an important stage and available pure culture makes the development of molecular tools possible based on genomic information. Anyhow, the information of overall structure of microbial communities and genomic structures that is obtained by culture-independent methods may improve the rate of success for isolation and identification by culture methods for studying the morphology, physiology and genetics of specific microorganisms (Zhou *et al.*, 2015). For instance, in the study by Pope *et al.*, (2011) it was possible to isolate the bacterial species WG-1 (from family Succinovibrionacea) by reconstruction of the bacterium's metabolism based on information from metagenomics datasets reported in the literature from similar bacterial species.

5.3 Variations in sampling procedure between studies

There are a number of factors that can affect the results and are important to keep in mind when comparing results between different studies and laboratory experiments. In rumen studies, it is important to be aware of how the sampling was performed, *e.g*. the rumen contents can be sampled via a ruminal fistula (**Paper I**) or through stomach tubing (**Paper II**; Shingfield *et al*., 2002). When using stomach tubing, it is more difficult to control where the sample is taken, while samples taken through the fistula sample can be taken from different sites in the rumen, such as the liquid or solid phase (Pitta *et al*., 2010; Geishauser & Gitzel, 1996). However, Tapio *et al.* (2016) did not find any significant differences in the taxa present in buccal samples compared to rumen fistula samples, but some species relative abundance varies. Similar results were found in a comparison in post-weaned calves, where no changes were observed in molar proportion of VFA or bacterial population (Terré *et al.*, 2013).

Extraction of the DNA is the first processing step of the sample and also another step with a bias effect. There are many different extraction kits, all producing different results in terms of yield and purity (Henderson *et al*., 2013). In most studies the kit has in some way been modified for optimisation of the procedure (McCann *et al*., 2014). In order to compare results between studies and laboratories, standardisation of the sampling procedure and DNA extraction is important. This is discussed by Henderson *et al*. (2013), who evaluated several different extraction kits used for rumen populations and found high variation in community composition.

Choice of primer has an impact, as there are differences in primer binding energy. Primers can be universal or specific. Universal primers are designed to analyse as many species as possible, but no one primer can target all

bacteria or archaea, so some species are probably not covered (Klindworth *et al.*, 2013).

Considering these bias effects, it is important to be aware when comparing results between studies where different sampling techniques, extraction procedures, analytical methods and primers have been used.

Table 2. *Summary of objective and main results in Paper I-IV and methods used for measuring CH_4 production and microbial population*

Paper	General objective	No. of cows and diets	CH_4 method	Microbial population	Molecular techniques	Main results
I	Investigate CH_4 production, methanogenic population structure and yield for diets with high-quality forage in two forage/ concentrate ratios (900/100 and 500/500) fed to high-producing Swedish Red dairy cows	5 cows, 2 diets, cross-over design	Sulphur hexa-fluoride (SF_6) tracer technique	Methanogenic population	T-RFLP, clone library, qPCR.	Forage proportion had different effects on methanogenic community in individual cows. Dividing *Methanobrevibacter* spp. into two groups better explained the variation in CH_4 production
II	Identify high and low CH_4 emitters and investigate the correlation to microbial population, fermentation pattern, feed intake, digestibility and milk production.	73 cows in mid lactation, 21 cows selected for microbial analyses	Spot sampling, Garnsworthy IR technique	Archaea and bacteria	Illumina, qPCR	Methane production was associated with microbial community structure and fermentation pattern. No effect was found on fibre digestion or milk production.
III	Investigate the effects of cashew nut shell extract (CNSE) and glycerol on *in vitro* production of CH_4 and VFA and investigate effects of these feed additives on the archaeal and bacterial community structures.	5 treatments; Control, CNSE low (5 mg), high (10 mg) and Glycerol low (15 mmol) and high (30 mmol)	*In vitro*	Archaea and bacteria	454-pyro-sequencing	CH_4 production was reduced with CNSE treatment by at most 18%, and there was a shift in microbial communities. CH_4 production increased with glycerol up to 12%, with no direct effect on microbial population.
IV	Evaluate *in vitro* system and possibilities to rank diets compared with chamber technique	49 diets selected from *in vivo* studies	*In vitro*	No analyses included		The *in vitro* system seems to predict CH_4 production with reasonable accuracy and precision, but has limitations in evaluating the effect of concentrate on CH_4 production.

6 Results and discussion

6.1 Impact of forage proportion on CH_4 production

In **Paper I**, CH_4 production from five cows included in the study by Patel (2012) was further investigated. The results showed no significant differences between diets, although there was a numerical difference in CH_4 production for the 50:50 and 90:10 diets (16.9 and 20.2 g CH_4/kg DMI). Due to the low number of cows in the study and the high variation within cows between days, it was perhaps not possible to find significant differences. The SF_6 technique used may have had an influence in the high variability in CH_4 production between days within animals, as has been shown in previous studies (Hammond *et al*., 2011; Pinares-Patiño *et al*., 2008). Therefore this may not be the best technique for a low number of animals. However, the non-significant difference in CH_4 measurements was supported by the results on concentration and proportions of VFA. There was no difference in total VFA concentration and a difference was only seen for butyrate proportion, which was higher in relation to total VFA for the 50:50 diet (11.7%) than the 90:10 diet (9.9%). This small difference may be related to a relatively higher number of protozoa (producing butyrate) in the diet with higher concentrate level (Jakkola & Huhtanen 1993).

6.1.1 Methanogenic population

To further investigate the effect of forage proportion on CH_4 production, in **Paper I** the methanogenic population was investigated in rumen fluid samples which was the first study on methanogenic population in dairy cows in relation to CH_4 production in Sweden. There was a significant difference in numbers of total methanogens and in the dominant group Methanobacteriales, which were both present in higher total copy numbers in the 50:50 diet compared with the 90:10 diet. However, this did not have any significant effect on CH_4 production, suggesting that the composition or activity of methanogens rather than their absolute numbers is of higher importance for CH_4 production. The relationship between numbers of methanogens and amount of CH_4 produced has been debated elsewhere and it has been suggested that the amount of CH_4 produced relates to the species that are present, rather than the total number of methanogens (Shi *et al*., 2014; Zhou *et al*., 2011). However, others claim that there should be a proportional relationship between the number of methanogens and the level of CH_4 produced, as this is the only way for methanogens to gain energy by ATP (Wallace *et al*., 2014). According to the T-FRLP analysis on the archaeal population in **Paper I**, diet composition had no clear effect on population structure. In fact, the response on population level appears to be

individual, as different cows responded differently to changes in the feed. The clone library related to the TRFs revealed that the two dominant TRFs, obtained with two different restriction enzymes (*Hha I* and *Hae III*) were ≥97% related to *Methanobrevibacter* species. This dominance of methanogens belonging to the genus *Methanobrevibacter* confirms previous findings on cattle in other countries (Seedorf *et al*., 2015; St-Pierre *et al.*, 2013; King *et al*., 2011; Hook *et al*., 2009) and in our other studies on Swedish cows (**Papers II** and **III**). Species within the *Methanobrevibacter* genus are assumed to represent core members of the microbiome in the rumen (Henderson *et al*., 2015; Jami *et al*., 2012).

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6.1.2 Dividing *Methanobrevibacter* into two groups

The dominant TRFs in the study investigating the effect of different levels of forage (**Paper I**) showed that the methanogens present were ≥98% related to species belonging to *Methanobrevibacter ruminantium* and *Methanobrevibacter olleyae* or ≥98% related to species belonging to *Methanobrevibacter smithii, Methanobrevibacter gottschalkii* or *Methanobrevibacter thaueri.* Based on the phylogenetic distribution of *Methanobrevibacter*-related 16S rRNA sequences, two major clades have been observed previously (King *et al*., 2011). One group comprises sequences similar to *Methanobrevibacter ruminantium* and *Methanobrevibacter olleyae*, called the RO group, and the other group has sequences similar to *Methanobrevibacter smithii*, *Methanobrevibacter gottschalkii*, *Methanobrevibacter millerae* or *Methanobrevibacter thaueri*, called the SGMT group (King *et al*., 2011). Several studies have shown that methanogenic community composition in a host seems to be dominated by either one of these groups, also depending on breed and/or geographical location (Seedorf *et al*., 2015; King *et al*., 2011). Moreover, **Papers I-III** demonstrated a relationship between individual animal, methanogenic clade and total amount of CH_4 production. Cows with a methanogenic community dominated by the SGMT group, also called the *M. gottschalkii* clade, were associated with higher CH_4 production compared with cows with a methanogenic community dominated by the RO group or *M. ruminantium* clade. The relationship between high CH_4 production and *M. gottschalkii* clade was also observed by Shi *et al.* (2014). Setting these two different groups in relation to each other apparently helps to identify changes in the methanogenic population associated with levels of CH_4 production. However, when studying abundance at genus level, no differences may be observed or they may be difficult to statistically correlate to changes in CH_4 production. The relationship between host and type of methanogenic group may represent a genetic influence, or may indicate that methanogenic groups thrive in different environments according to pH, passage

rate or type of substrate coming from the fermentation by other microbes (St-Pierre *et al*., 2015). Differences between the two clades of *Methanobrevibacter* spp. would be interesting to investigate further to increase understanding of the mechanism behind their presence and their connection to CH_4 emissions. *Methanobrevibacter ruminantium* uses both formate and H_2 for the reduction of CO_2, while *M. gottschalkii* only uses H_2. This may give an advantage for *M. ruminatium* if there is restricted availability of H_2. On the other hand, other members of the SGMT group, such as *M. thaueri* and *M. smithii*, can both utilise H_2 and formate (Miller & Lin 2002; Miller & Lin 1982). *Methanobrevibacter ruminatium* also seems to express only methyl CoM reductase Mcr I, which is used at lower H_2 pressures, while *M. gottschalkii* has the capacity to express both Mcr I or Mcr II at low and high H_2 pressure (Leahy *et al*., 2010).

6.2 Linkage between microbial community structure and CH_4 production

The results in **Paper I,** where the CH_4 emissions seemed to be related to individuals rather than diet, and those in other studies, where an individual variation in CH_4 production has been shown (Yan *et al*., 2009; Ellis *et al*., 2007), prompted further investigations of the relationship between CH_4 production and individual microbiota (**Paper II**). This was performed by selecting animals with persistent low or high CH_4 production over the study period (three months) and by reducing the impact of certain physiological and dietary parameters by choosing cows in the same lactation stage (mid-lactation) and fed the same diet. The microbial community structure in rumen fluid was assessed by sequencing the 16S rRNA gene. The results showed that the microbial flora from individual cows was clearly divided into one of two clusters of bacterial OTUs and a similar, but less segregated, pattern was shown for the archaea. Here, cluster L correlated with comparatively higher CH_4 production than cluster H. Moreover, different molar proportions of VFAs such as propionate were higher in cluster L, whereas the molar proportion of butyrate was higher in cluster H, which probably partly explains the different levels of CH_4 production. Similar results on different 'ruminotypes' have been reported by Kittelmann *et al.* (2014), who suggested that the difference in CH_4 production was attributable to host selection of different microbial communities which produce different amounts of H_2, giving more or less CH_4. In **Paper II,** the microbiota of cows with high and low CH_4 emissions were investigated. The results showed that the bacterial community was mainly represented by *Prevotella*, which is one of the most commonly found bacterial genera in the rumen (Henderson *et al*., 2015; Stevenson & Weimer, 2007). Different species within the genus *Prevotella* have

high metabolic versatility and ferment cellulose, protein and starch, which makes it difficult to fully understand their function looking only at genus level. *Prevotella* as a genus did not contribute to separation into clusters related to CH_4 production in **Paper II**, but different OTUs of *Prevotella* were related to each of the clusters. Only few species of *Prevotella* have been isolated from the rumen and cultured and it has been shown that these species only represent a small part of the genus *Prevotella* (Bekele *et al*., 2010; Stevenson & Weimer, 1997). The individual effect was clear, as community profiles of both bacteria and archaea within animals co-occurred in the same cluster in all three measuring periods over the three-month study (Figure 2 and 4). Moreover, the microbiota in one cow from each CH_4 group appeared in the 'opposite' cluster, which was true both for bacteria and archaea, giving a robust correlation between the bacterial and archaeal community.

6.2.1 Fermentation pattern and feed digestibility

The individual composition of the community structure could be due to factors such as rumen size, intake and chewing behaviour. As mentioned above Goopy *et al*. (2013) observed that high emitter sheep had a larger rumen and a lower passage rate than low emitter sheep. Therefore, when the effect of individual properties on the level of CH_4 production is being evaluated, it is important to investigate whether a reduction in CH_4 production is an effect of decreased fibre digestion, which may also give less milk or meat production. There are few studies on rumen microbial populations associated with differences in CH_4 between individuals that further analyse factors related to digestion of feed and production of milk or meat. Due to the risk of reduced digestion with low CH_4 production, many parameters, such as intake, digestibility of feed, feed digestion, milk production, rumen fermentation products and microbiota, were analysed in **Paper II**. However, differences were mainly found for fermentation products, with an increase in the proportion of propionate in cluster L and an increase in the proportion of butyrate in cluster H, which may explain the association with CH_4 production. There was difference in weight between cluster which may also mean that rumen size were bigger in cluster H. There was no effect on fibre digestion or milk production, which is in accordance with findings by Goopy *et al.* (2013) that the difference in apparent digestibility in total tract between sheep with different rumen size was not significant. Even when there are differences in rumen size and passage rate, the reduced digestion in the rumen may perhaps be compensated for by increased digestion in the hindgut (Goopy *et al*., 2013). If the digestion increases in the hindgut compared with the rumen, it can be speculated that less CH_4 is produced in the hindgut due

acetogenesis seem to occur there (Ramin *et al.*, 2015; Demeyer *et al.*, 1996). Furthermore, the differences in microbial population and perhaps degradation of feed in the rumen may not be totally compensated for by hindgut fermentation. In studies analysing feed efficiency, it has been observed that cows with high residual feed intake (RFI) and lower degradation efficiency have differing methanogenic community structure in the rumen. High RFI is related to presence of higher abundance of *Methanobrevibacter smithii* (Carberry *et al*., 2014; Zhou *et al*., 2010). Moreover, *Methanosphaera stadtmanae* and *Methanobrevibacter* sp. strain AbM4 have been shown to be related to high-RFI animals (Zhou *et al*., 2009). Interestingly, *M. smithii*, a member of the SGMT group, is related to high RFI and high CH_4 production, suggesting that individuals with higher abundance of RO group may be both more efficient in utilising their feed for milk or meat production and produce less CH_4.

6.3 Impact of feed additives on CH_4 production in an *in vitro* system

For increasing efficiency in feed utilisation and/or inhibition of CH_4 formation, some microbial communities need to be favourable or the microbial fermentation needs to be redirected. Certain substrates may have potential for this redirection by an inhibitory effect of H_2-producing bacteria and methanogens or enhancement of non-H_2 producing fibrolytic microbes, without decreased forage degradability. To investigate the effect of a certain substrate or inhibitor, tests in an *in vitro* system can be a first step, to reduce the need for animal trials. *In vitro* systems cost less than *in vivo* studies, which makes it possible to test several treatments at the same time. Based on the results from *in vitro* studies, the most promising substrate/diet can then further be tested *in vivo.* In an *in vitro* study, Watanabe *et al.* (2010) tested a promising substrate that can be obtained from a waste product from the cashew nut industry, cashew nut shell liquid (CNSL), which contains cardanol, cardol and anacardic acid. Anacardic acid is a compound shown to inhibit gram-positive bacteria and has been tested as a feed supplement (Shinkai *et al*., 2012). CNSL was shown to have a dramatic impact on certain species in the rumen microbiome (Watanabe *et al.*, 2010). To further reveal possible CH_4 inhibition by CNSL and to investigate effects on rumen archaea and bacteria communities, cashew nut shell extract (CNSE) was tested *in vitro* in **Paper III.** CNSE was added at two different levels, 5 and 10 mg, to a 60 mL inoculum mixed with a forage:concentrate (60:40) substrate. The addition resulted in a reduction in CH_4 of up to 18% and the relative abundance of unclassified *Bacteriodales* clearly decreased compared with the control.

However, in two previous *in vivo* studies, feeding cows with CNSL (4 g/100 kg BW) in the diet resulted in a reduction in CH_4 production, but caused decreased DM digestibility in only one of the two studies (Shinkai *et al.*, 2012). The observed inhibition in **Paper III** with CNSE could be due to the observed shift in bacterial population, possibly resulting in decreased production of hydrogen. Otherwise, the reduction could be explained by a shift in the methanogenic community. In that study too, reduced CH_4 production was related to higher abundance of *Methanobrevibacter* species belonging to the RO group, whereas the control had higher abundance of *Methanobrevibacter* belonging to the SGMT group. Besides, a recent *in vivo* study found no effect on CH_4 production when technical cashew nut shell liquid (TCNSL) without anacardic acid was added to the diet, which indicates that the main effect on CH_4 production shown *in vitro* and *in vivo* is due to anacardic acid (Branco *et al*., 2015). Extract from cashew nut shell may be an alternative to achieve inhibition of CH_4 production if it can be used in an appropriate formulation.

In **Paper III**, the effect of glycerol was also evaluated, with the hypothesis that glycerol with an estimated value of 16.2 MJ ME/kg of DM for ruminants (Mach *et al.*, 2009) will enhance the energy content of the diet and thus also result in increased propionate production and consequently a reduction in CH_4 production. Glycerol was added at two different levels, 15 and 30 mmol, to a 60 mL inoculum mixed with a forage:concentrate (60:40) substrate. Glycerol treatment with 30 mmol addition increased CH_4 production by 12% and no effect was observed on archaea communities compared with the control. For bacteria, glycerol gave an increase in the relative abundance of unclassified Ruminococcaceae and Anaerovibrio. The increase in CH_4 production was probably due an increase in total VFA levels, thus giving an increase in total CH_4 production. However, the response *in vivo* may be different from that *in vivo*. It has been observed that 70% of glycerol added to an empty rumen is absorbed directly through the rumen wall (Omazic *et al*., 2014). If this high absorption occurs, a smaller proportion of OM in the total diet is fermented in the rumen, giving less CH_4 production. Overall, however, the results seem to vary between studies; Piantoni and Allen (2015) infused glycerol into the abomasum or reticulo-rumen of cows fed a commercial diet and found that this increased blood glucose more than infusion into the rumen, suggesting that more of the glycerol in the rumen is degraded to non-glucogenic end-products by microbes. However, a smaller proportion may be used for microbial digestion in the rumen compared to in *in vitro* systems where all glycerol is used for microbial digestion. The non-inhibitory effect of glycerol on CH_4 production *in vitro* is consistent with findings by Avila *et al*. (2011) and Avila-Stagno *et al*. (2013).

6.4 Evaluation and effect of *in vitro* method

When using an *in vitro* system, it is crucial to be confident that the technique is reliable in relation to what happens *in vivo* in the rumen. Evaluation of the system is thus needed and as there are several factors that can affect the predictions *in vitro*, it is important to be aware of the different biases and differences compared with *in vivo*. A recently developed *in vitro* method for prediction of *in vivo* CH_4 production that takes into account rumen dynamics in a model for prediction (Ramin & Huhtanen, 2012) was evaluated in **Paper IV**. The evaluation was performed by comparing predicted CH_4 production with observed CH_4 production from several *in vivo* studies. The test diets had differing dietary composition in terms of: feeding levels, proportion of concentrate, carbohydrate composition of concentrates, protein and fat supplementation, forage type and maturity of forage. Overall, the system-predicted values were well correlated to observed values *in vivo*, but there were also some weaknesses in the system. The gas *in vitro* system did not work for analysing the effect of increasing level of concentrate. Observed values showed variable response to CH_4 depending on the increase in concentrate level, but this was not possible to show in the *in vitro* system as overall predicted CH_4 values increased with increasing level of concentrate. This emphasises the importance of keeping in mind that effects obtained in the *vitro* environment may not always be the same as in the *in vivo* environment and *vice versa*. Furthermore, the inaccurate prediction of concentrate level could be an effect related to the different pools linked to the model for prediction. It was also shown that the system mainly under-predicted CH_4 production slightly at high feeding level. Based on this, it is recommended that the *in vitro* system be used to measure CH_4 production related to maintenance feeding level. After screening different treatments *in vitro*, the most promising additives could then be evaluated *in vivo* at maintenance level and, if still promising, tested at production level.

6.4.1 In vitro versus *in vivo*

One of the biases in the *in vitro* system is the inoculum. The individual donor animal has an impact on the digestion of the feed depending on the individual microbiota. As a way to reduce the effect of inoculum, pre-adaptation to the feed is recommended. In this way the microbiota has time to adapt to the substrate before transfer to the *in vitro* system (Broudiscou *et al*., 2014). In **Paper III**, where CNSE and glycerol were tested, the donor cows were already adapted to the same diet, 60:40 forage:concentrate ratio, as was used *in vitro*. The effect of the additives may still vary *in vivo* compared with *in vitro*. In **Paper IV** the rumen fluid used as inoculum was taken from donor cows given the 60:40 forage:concentrate ratio and all diets evaluated in the *in vitro* test were different

to this feed. This may have had an impact on the results and better predictions might have been possible if similar diets had been fed to the donor cows.

The rumen is a heterogeneous system where differences in fermentation pattern occur all the time depending on feed type, feed level, microbial interaction and passage rate. *In vitro* fermentation is principally a type of enrichment culture, where various parameters such as pH, type and concentration of substrate type affect the growth of some microbes and inhibit others which may differ from original inoculum. To further evaluate the effect of the *in vitro* system, one aim in **Paper III** was to compare how the microbial population developed in controls (with no feed additive) *in vitro* and *in vivo.* Bacterial and archaeal community structure were compared between inoculum in the *in vitro* control and rumen fluid from *in vivo* (**Paper III).** For bacteria the community structure was similar in the *in vitro* control with no feed additive compared with *in vivo* sample, thus indicating that the transfer of the rumen fluid to the *in vitro* system had little impact on the bacterial community structure. An effect of time was shown in a decrease in relative abundance of *e.g. Prevotella* in the control in both treatments. This is in agreement with findings by Mateos *et al.* (2015) that the diversity of bacteria was lower in a batch culture *in vitro* system than *in vivo* in sheep. This indicates that there is selection of some bacteria species over time. However, the overall fermentation effect of forage was similar *in vivo* and *in vitro* in the sheep studied by Mateos *et al*. (2015). For the archaeal community there was an effect of transfer to the batch system. The effect was mainly clear for the dominant species *M. olleyae* and *M. thaueri.* The relative abundance of *M. olleyae* decreased, while the relative abundance of *M. thaueri* increased (Figure 7). It can be speculated that this reflects an increase in H_2 pressure as discussed above, which may favour *M. thaueri in vitro.* For the CNSE treatment the effect on bacteria community was more similar to *in vivo*. If there is a negative effect of CNSE on H_2-producing bacteria, the resulting lowering of H_2 pressure might give an advantage for *M. olleyae.* More specific studies are needed on the impact on rumen inoculum in the *in vitro* system and whether certain parameters can be optimised.

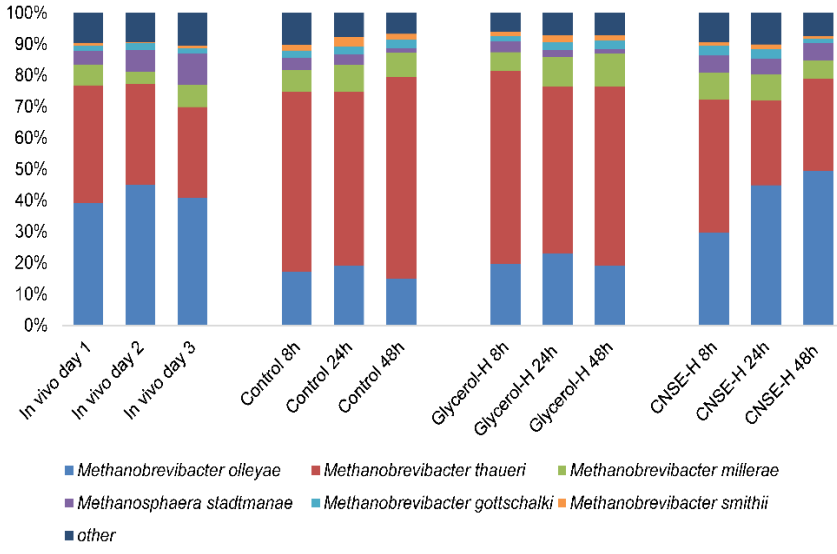

Figure 7. Relative abundance at OTU level of the archaeal population for *in vivo* treatments at day 1, 2 and 3 and for *in vitro* treatments at 8, 24 and 48 h of incubation. OTUs were compared with sequences in BLAST and described at species level. OTUs representing the same species (>97% identity) have been pooled together.

7 Conclusions

- The effect of individual animal on CH_4 production is stronger than the effect of diet
- Increasing the level of highly digestible forage in the diet fed to dairy cows has no significant effect of CH_4 production, but has a minor effect on fermentation end-products and the methanogenic population
- Individual differences in CH_4 production in cows cannot be explained by reduced fibre digestion or reduced milk production, but are rather associated with different microbial community structures
- The gas *in vitro* system can be used for screening diets and additives before testing *in vivo*, with some limitations regarding concentrate inclusion
- Division of rumen *Methanobrevibacter* species into two groups reveals a correlation to CH_4 production. The group consisting of *M. smithii, M. gottschalkii, M. millerae and M. thaueri* is correlated to high CH_4 production, while the group comprising *M. ruminantium* and *M. olleyae* is correlated to low CH_4 production
- Cashew nut shell extract can reduce CH_4 production, based on *in vitro* data
- Glycerol addition may increase CH_4 production, based on *in vitro* data
- Results from *in vitro* studies need to be verified *in vivo.*

8 Implications of findings and future perspective

- It is essential that studies on rumen microbiology, digestion and milk production are combined, as the correlations between these are complex and gains in one aspect might easily affect other parameters negatively
- To reduce the bias effect of inoculum, it is recommended that donor animals be fed similar diets will be tested for adaptation of microbes.

Future research to increase knowledge on the possibility to reduce CH_4 production from dairy cows should focus on:

- Host effect on community structure, together with measurements on CH_4 production, feed digestibility and milk production
- Microbial analyses on rumen microbiota to investigate co-occurrence of different microbes in relation to CH_4 production and milk production
- Use of genomics and transcriptomics to characterise methanogenesis and identify methanogens with upregulated genes in high CH_4 emitter animals
- Identify parameters that differ between farms that are most and less effective regarding emissions in relation to production
- Increase milk productivity, particularly in developing countries, by improved feed quality
- Effects on CH_4 production and milk production of increased proportion of high quality forages rather than concentrate.
- Effects on CH_4 production and milk production using potential inhibitory CH_4 additives from waste or by-products.

9 Svensk sammanfattning

Med en ständigt växande befolkning och en ökande medelklass så ökar efterfrågan på mjölk och kött. En ökad produktion av animala livsmedel bidrar dock till en ökad miljöbelastning. De negativa aspekterna måste begränsas samtidigt som produktionen ökar.

Globalt sett står jordbruket för ca 14 % av de antropogena (beror på mänsklig aktivitet) växthusgasutsläppen. I Sverige har växthusgaserna från jordbruket minskat med 11 % sedan 1990. Detta beror främst på minskningen av antalet djur, men också högre foderkvalitet, ökad produktionseffektivitet och minskad användning av konstgödsel. Dock har köttkonsumtionen i Sverige ökat under samma period, vilket lett till dramatiskt ökad livsmedelsimport. Siffrorna är därför något vilseledande då utsläppen istället hamnar i det land djuret produceras. Kor pekas idag ut som de största miljöbovarna från lantbruket på grund av deras metanproduktion. Metan från kor utgör ca 40 % av totala utsläppen inom jordbruket. Kor spelar dock en stor roll i ett hållbart jordbruk och att endast se till metanutsläppen ger inte en rättvis bild.

Idisslarnas största mage, våmmen, har ett mycket komplext ekossystem bestående av många olika mikrobiella grupper varav en av grupperna är de metanbildande metanogenerna (tillhör Arkeér). Vid nedbrytning av cellulosa (ex. gräs) bildas koldioxid och vätgas, som metanogenerna i sin tur omvandlar till slutprodukten metan. För att minska metanproduktionen görs försök att reducera antalet metanogener, minska deras aktivitet eller främja alternativa vägar som gör att metan inte bildas som biprodukt.

Det övergripande syftet med denna avhandling var att utvärdera olika faktorer med potential att minska metanproduktion hos mjölkkor genom foder eller fodertillsatser, samt hur den individuella mikrobfloran påverkar metanproduktionen. I avhandlingen har kor som utfodrats under nordiska förhållanden undersökts.

9.1 Sammanfattning av studierna och resultat

Metanproduktion och arkeépopulation i våmmen hos mjölkkor utfodrade med olika grovfoderandelar

Idag utfodras mjölkkor med foderstater som innehåller en stor andel kraftfoder. I Sverige utgör kraftfoderandelen i konventionella foderstater oftast mer än 50 % av TS (torrsubstans). Önskvärt vore att bättre utnyttja kons potential att bryta ner grovfoder (ex. gräs), istället för att utfodra kraftfoder som enkelmagade djur eller människor kan utnyttja istället. I den första studien undersöktes metanproduktionen och arkeépopulationen i våmmen när olika proportioner av grovfoder utfodrades. Hypotesen var att metanproduktionen ökar med ökad andel grovfoder. Fem kor utfodrades i två olika perioder med en foderstat av grovfoder och kraftfoder i olika proportioner, 50:50 och 90:10. Mängden metan uppmättes med en spårgasteknik som kallas svavelhexafluorid (SF_6). Metanogenerna i våmmen undersöktes med olika molekylära metoder. Resultaten visade att varken metanproduktionen eller totala antalet metanogener skiljde sig signifikant mellan de olika foderstaterna med olika grovfoderandelar. Den individuella kon visade sig ha större påverkan på metanproduktionen jämfört med typ av foderstat. Resultaten visade också att genom att dela upp olika arter av *Methanobrevibacter* i två grupper så sågs en koppling till högre metanproduktion med ena gruppen och mindre metanproduktion med andra gruppen.

Kor med låg eller hög metanproduktion

För att vidare undersöka effekten av individen studerades metanproduktion och sammansättningen av mikroorganismer i våmmen, samt foderintag och mjölkproduktion. För att minska påverkan av vissa fysiologiska och foderrelaterade parametrar undersöktes kor i samma laktationsstadie utfodrade med samma foderstat. Utifrån totalt 73 kor valdes kor ut som producerade låg, medel eller hög mängd metan. Metanproduktionen uppmättes från kons utandningsluft i samband med mjölkning. Den mikrobiella samhällsstrukturen i våmvätska studerades genom sekvensering av 16S rRNA-genen från bakterier och arkeér. Resultaten från denna studie visade att den mikrobiella floran från enskilda kor var tydligt uppdelad i en av två grupper (kluster) av bakterier, liknande kluster uppstod även för arkeépopulationen. I kluster hög (H) fanns kor med högre metanproduktion jämfört med kor i kluster låg (L). Det fanns ingen skillnad i nedbrytningen av fiber mellan kor i de olika klustren. Metanproduktionen per kilo mjölk var lägre för de kor som var i kluster L.

Proportionerna av fermenteringsprodukter såsom flyktiga fettsyror skiljde också mellan klustren. Proportionen av propionat var högre i kluster L och proportionerna av butyrat var högre i kluster H, vilket delvis kan förklara de olika nivåerna av metanproduktion. Samma uppdelning av *Methanobrevibacter* arter som i studie ett visade även här tydliga kopplingar till metanproduktion.

Utvärdering av fodertillsatsers effekt på metanproduktion och mikroorganismer i våmmen

Som ett första steg i att utvärdera olika foderstater eller fodertillsatser så kan tester utföras i olika labb-system, så kallade *in vitro* system. Test *in vitro* gör att färre tester på djur, *in vivo*, behövs samt att det är en lägre kostnad. Dock skiljer sig *in vitro* och *in vivo* åt i vissa avseenden. Foderstater eller tillsatser som resulterat i sänkt metanproduktion *in vitro* måste vidare utvärderas *in vivo* innan en effekt kan fastställas. I studie tre undersöktes metanproduktionen vid tillsats av två olika potentiella metanhämmande fodertillsatser i ett gas *in vitro* system. Båda substraten, glycerol och extrakt från cashewnötskal, är rest- eller biprodukter från olika industriella processer. Substraten tillsattes till en mix av grovfoder och kraftfoder (60:40) och inkuberades tillsammans med våmvätska i gas *in vitro* systemet under 48 timmar. Metankoncentrationen mättes vid sex olika tillfällen och med hjälp av en teoretisk våm-modell beräknades mängden metan som producerats. Vid olika tidpunkter (8, 24 och 48 timmar) togs prover för analys av bildningen av flyktiga fettsyror samt för vidare studier av mikroorganismstrukturen (mikrobiota). Resultaten visade att metanproduktionen minskade med extrakt från cashewnötskal men ökade när glycerol tillsattes jämfört med kontrollen (inget tillfört). Effekter på mikrobiotan var tydlig med extrakt från cashewnötskal, andelen mellan grupperna av *Methanobrevibacter* ändrades, med en lägre andel av den grupp som kopplats till hög metanproduktion jämfört med kontrollen. För glycerol var sammansättningen av mikroorganismer liknande den i kontrollen. Koncentrationen av flyktiga fettsyror var klart högre när glycerol tillsattes vilket förmodligen förklarar metanökningen.

Utvärdering av gas in vitro systemet

I den fjärde studien utvärderades gas *in vitro* systemet. Genom att mäta metan på samma eller liknande foderstater som tidigare utfodrats till kor eller får där metanmätningar utförts direkt, *in vivo*, på djuren kunde de olika mätvärdena jämföras. Fyrtionio olika dieter valdes ut för att utvärdera olika typer av foderkomponenter så som; andel kraftfoder, nivå av utfodring utöver

underhållsbehovet, kolhydratsammansättning, fett, protein, fodertyp, samt grovfoder skördat tidigt och sent. Metanvärdena från gas *in vitro* systemet stämde överens med de observerade värdena *in vivo*, vilket visades med ett ganska litet skattningsfel (9,5 %). Systemet kan därmed användas för att utvärdera effekt av olika fodertillsatser, exempelvis fett. Det fanns dock vissa brister med systemet, det fungerade inte att analysera effekten av ökande andel kraftfoder i totala foderstaten. Direkta uppmätta *in vivo* värden visade varierande metanvärden när kraftfoderandelen ökade, men detta var inte möjligt att visa i gas *in vitro* systemet då metan alltid ökade vid ökad kraftfoderandel. Detta understryker vikten av att effekter som erhållits i *in vitro* miljön inte alltid är densamma i *in vivo* miljön och vice versa.

9.2 Slutsatser

Sammanfattningsvis visar resultaten i denna avhandling att individuella skillnader i metanproduktion var större än effekt av foder. Ökad nivå av grovfoder i foderstaten till mjölkkor hade ingen signifikant effekt på metanproduktionen eller den metanogena populationen. Genom att dela upp *Methanobrevibacter* arter i två grupper sågs en korrelation till metanproduktion som tyder på att vissa miljöer främjar de olika grupperna. Beroende på våm-miljö bildas mer eller mindre metan, men ingen effekt visades för varken mjölkproduktion eller foderutnyttjande i studien med kor med låg och hög metanproduktion. Tillsats av glycerol *in vitro* ökade metanproduktionen medan tillsats av extrakt av cashewnötskal resulterade i minskad metanproduktion, resultat från *in vitro* måste även verifieras *in vivo*. Det utvärderade gas *in vitro*-systemet kan användas för screening av foderstater och tillsatser innan de testas *in vivo*, med vissa begränsningar när det gäller effekt av kraftfoderandel i foderstaten. Ökad kunskap om vad som egentligen sker i våmmen skulle ge ett bättre underlag om vad som kan göras för att reducera metanproduktionen.

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