Methane Production in Dairy Cows

Individual Cow Variability in Methane Production

Edward Hernando Cabezas Garcia
Faculty of Veterinary Medicine and Animal Science
Department of Agricultural Research for Northern Sweden
Umeå

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Abstract

Enteric methane (CH_4) emissions vary between individual cows, and this variation is attributed to both animal and dietary factors. In addition, measurement technique of *in vivo* CH_4 emissions from individual animals still represents a major challenge for successful emissions mitigation strategies. This thesis investigated the contribution of different factors to between-animal variation in CH_4 production, in order to improve the current knowledge of its biological basis. In a study comparing on-farm systems for measuring CH_4 emissions from large numbers of animals and the variation between individual animals, the GreenFeed system was used as the normal set-up (flux method) or modified to mimic gas analysers systems based on CH_4 concentrations (sniffer system) to measure CH_4 emissions. Measurements taken by the GreenFeed system proved be more reliable than those made by the simulated sniffer method. The GreenFeed data were consistent with literature values determined in respiration chambers, while the sniffer method were poorly correlated to flux method values and were not significantly related to either feed intake or milk yield. Despite GreenFeed being a spot sampling method, it proved to be a promising tool for ranking cows as high and low CH_4 emitters. A meta-analysis based on an individual cow dataset investigating the effects of between-cow variation and related animal variables on predicted CH_4 emissions from dairy cows. Between-cow variation in fermentation pattern are not likely be the major factor influencing predicted *in vivo* CH_4 emissions. Variation and repeatability for volatile fatty acid concentrations were greater for ruminal concentrations than molar proportions, indicating strong control by the individual cow. Digestion kinetics variables were more repeatable than rumen fermentation or microbial synthesis, as a result of variations in passage rate. In studies in which late-cut silage and rolled barley were gradually replaced with early-cut silage in the diet of dairy cows, production responses and *in vivo* CH_4 emissions were studied in 16 intact lactating cows and possible physiological mechanisms were assessed in four rumen-cannulated cows. Improvements in forage quality by graded addition of early-cut silage was an effective strategy to reduce concentrate supplementation, without compromising performance or increasing CH_4 emissions in lactating dairy cows. Differences in intake between treatments were partly compensated by differences in silage digestibility.

Keywords: Dairy cattle, methane yield, between-cow variation, repeatability.

Author's address: Edward Hernando Cabezas Garcia, SLU, Department of Agricultural Research for Northern Sweden, P.O. Box, 901 83 Umeå, Sweden
E-mail: edward.cabezas.garcia@slu.se; ecabezasg@hotmail.com

Dedication

To my beloved family for your unconditional support during the moments of uncertainties.

To all of science teachers whom I had the chance to share with since I was a child for their encouragement, example and guidance through my scientific journey.

The ruminant animal: *"A small fermentation unit which gathers the raw material, transfers it to the fermentation chamber, and regulates its further passage, continuously absorbs the fermentation products, and transforms them into a few valuable substances such as meat and milk. To these advantages must be added the crowning adaptation: the unit replicates itself"*

Robert E. Hungate, 1950

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 Huhtanen, P., E. H. Cabezas-Garcia, S. Utsumi, and S. Zimmerman (2015). Comparison of methods to determine methane emissions from dairy cows in farm conditions. *Journal of Dairy Science* 98, 3394–3409.

II Cabezas-Garcia, E. H., S. J. Krizsan, K. J. Shingfield, and P. Huhtanen. (2017). Between-cow variation in digestion and rumen fermentation variables associated with methane production. *Journal of Dairy Science* 100, 1–16 (In Press).

III Cabezas-Garcia, E. H., S. J. Krizsan, K. J. Shingfield, and P. Huhtanen. (2017). Effects of replacement of late-harvested grass silage and barley with early-harvested silage on milk production and methane emissions. *Journal of Dairy Science* (In Press).

IV Cabezas-Garcia, E. H., S. J. Krizsan, K. J. Shingfield, X. Dai, and P. Huhtanen. (2017). Effects of replacement of late-harvested grass silage and barley with early-harvested silage on ruminal digestion efficiency in dairy cows (Manuscript).

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The contribution of Edward Hernando Cabezas Garcia to the papers included in this thesis was as follows:

I Planned the research with the first author and performed the field experiment at Röbäcksdalen research barn including data collection and statistical analysis. Participation in writing the manuscript jointly with the rest of co-authors.

II Planned the research jointly with the main supervisor, collected the dataset in collaboration with the co-authors, analysed the data and wrote the manuscript.

III Planned the experiment in collaboration with the co-authors, performed the experiment, and analysed the data together with co-authors and wrote the manuscript.

IV Planned the experiment in collaboration with the co-authors, performed the experiment, processed experimental samples, and analysed the data together with co-authors and wrote the manuscript.

Abbreviations

CH_4VFA	Stoichiometric methane
CH_4/DMI	Methane yield
CH_4/ECM	Methane intensity
CV	Coefficient of variation
DMI	Dry matter intake
ECM	Energy corrected milk
GE	Gross energy
GEI	Gross energy intake
GHG	Greenhouse gases
iNDF	Indigestible neutral detergent fibre
k_d	Fractional digestion rate
k_p	Fractional passage rate
NDF	Neutral detergent fibre
OM	Organic matter
Rep	Repeatability
VFA	Volatile fatty acid

1 Introduction

1.1 The role of ruminants in methane and greenhouse gas emissions

Population growth is challenging for agricultural systems around the world because it means producing more food to support an increasing population in a more efficient manner within the constraints of available natural resources, without compromising the future of coming generations. The future demand for livestock products in a scenario where most of the population lives in large cities, rather than in rural areas, will dictate consumption trends in coming years (Kearney, 2010; FAO, 2016). The demand for livestock products will more than double by 2050 compared with 2000 (Steinfeld *et al*., 2006; FAO, 2011). In developing countries, the demand will increase as a consequence of increasing population and net income (FAO, 2011, 2016), which are usually associated with increases in demand for animal products. On the other hand, in developed countries the demand will increase slower than in developing countries and socio-cultural values will be more relevant in people's food choices (FAO, 2011).

Livestock production has been criticised in recent decades for reasons such as: use of agriculture land that could be used for human food production, water consumption, deforestation, environmental pollution, animal welfare, human health concerns from eating animal products *etc*. Since feedstuff production is what links livestock production to land use, both directly via grazing and indirectly via traded grain or forage, environmental sustainability is an issue of major importance in the feed industry (Herrero *et al*., 2013). Livestock supply 13% of the energy in human diets, consume around 50% the world's production of grains (Smith *et al*., 2013) and, at the same time, are responsible for about 14.5% of total anthropogenic greenhouse gas emissions (7.1 Gt CO_2-equivalents per year) (Gerber *et al*., 2013).

Ruminants have the unique ability of transforming roughages which are not used by monogastric animals into human food (*e.g.* milk, meat) and could therefore reduce the competition for arable land between animal feed production and food production for humans. Comparing total and human-edible efficiency for different livestock production systems *e.g.* in the USA (Table 1), it can be seen that in terms of conversion of feed resources into human-edible products, ruminants are more efficient than monogastric animals despite inefficiencies in total terms, as in the case of beef production. Milk production is advantageous compared with beef meat production when an efficiency perspective is considered. Monogastric animals in intensive production systems are fed high levels of grain with high quality protein supplements in the diet, whereas forage is the main component of rations for dairy cattle. In addition, concentrate supplementation can be reduced significantly in grazing-based systems and even more when agricultural by-products are included in the diet. Intensive feedlot production systems for beef cattle usually use very high levels of concentrate in the diet (>90%) and, despite the advantages in terms of faster production returns, this type of production system represents direct competition for food with humans. In extensive production systems (*i.e*. tropical conditions), despite low animal productivity per hectare human-edible efficiency should be considered, since ruminants also represent economic status and wellbeing in developing countries.

Table 1. *Comparative efficiency of different livestock production systems in the USA (adapted from Gill et al., 2010)*

	Energy efficiency		Protein efficiency	
Product	Total[1]	Human-edible[2]	Total[1]	Human-edible[2]
Milk	0.25	1.07	0.21	2.08
Beef	0.07	0.65	0.08	1.19
Pigs	0.21	0.30	0.19	0.29
Poultry meat	0.19	0.28	0.31	0.62

[1]Total efficiency calculated as outputs of human-edible energy and protein divided by total energy and protein inputs.
[2]Human-edible efficiency calculated as outputs of human-edible energy and protein divided by human-edible inputs.

Methane (CH_4) has 28-fold higher greater global warming potential than carbon dioxide (CO_2) (IPCC, 2007). Methane emissions to the atmosphere derive from both natural sources, *e.g*. natural wetlands, termites, ocean and hydrates, and anthropogenic sources, *e.g.* rice fields, ruminants, landfills, biomass burning and fossil fuels (Moss *et al*., 2000; Aronson *et al*., 2013). The

concentration of CH_4 in the atmosphere has increased from 750 ppb during pre-industrial times to about 1800 ppb today as a consequence of human activities (IPCC, 2013). In such a scenario, the contribution of the livestock sector to the total anthropogenic CH_4 emissions is important. Within the livestock sector, it is clear that cattle (beef and dairy) have the highest total GHG emissions (CO_2 equivalents) compared with monogastric animals (Figure 1). Most of the GHG contribution of ruminants per unit of edible product (>40%) comes from enteric CH_4 fermentation (Gerber *et al*., 2013).

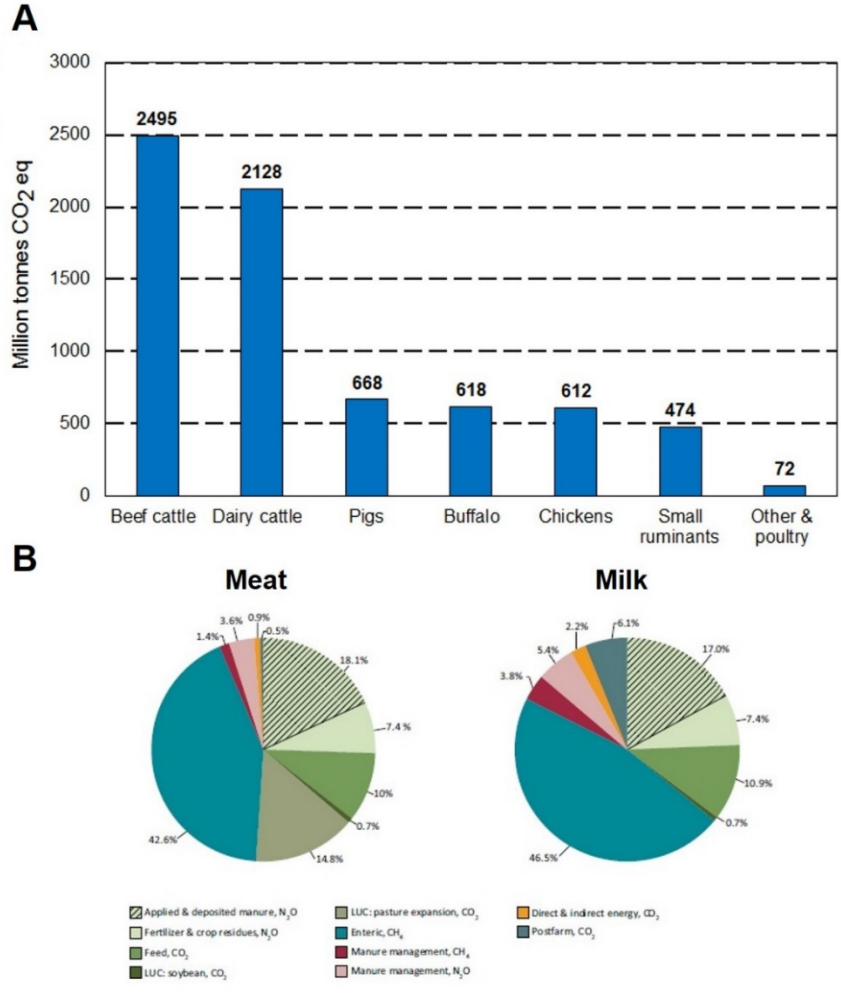

Figure 1. A) Global estimated emissions by species. Emissions are attributed to edible products and non-edible products. B) Emissions from cattle milk and beef supply chains. Source: GLEAM. Modified from Gerber *et al*. (2013).

In a global perspective, CH_4 emissions from ruminants are a function of ruminant population size, productivity, diet composition and associated manure management systems (Knapp *et al*., 2014). Methane production also represents an energy loss to the animal, which can vary from 2 to 12 % of gross energy intake depending on intake level and diet composition (Johnson and Johnson, 1995). On the other hand, it also could be assumed that by increasing feed efficiency, the amount of feed and waste material (*e.g*. manure, CH_4 emissions) produced per unit of product can be reduced. However, in the near future the major challenge will not only be to increase the feed efficiency of animals, but also to mitigate the impact of the livestock industry on the environment (Godfray *et al*., 2010), especially in developing countries (*e.g*. Latin America), where it is predicted that cattle production will continue to expand because agriculture is still a major source of income (McAllister *et al*., 2011). Improvements in animal productivity per hectare by strategic animal feeding practices using local feeds would contribute significantly to lowering CH_4 emissions in those countries. It is worth mentioning that in tropical regions, ruminant animals raised in extensive conditions are not only used as food, but also for cultural purposes, draft power and financial security.

Modern dairy cows are not the same animals as the old phenotypes. As a consequence of genetic selection and improvements in feeding and management practices, modern dairy cows have increased their production performance and consequently their dry matter intake (DMI). The productivity in the US dairy herd has increased considerably (milk yield per cow was 2074 kg/year in 1944, compared with 9193 kg in 2007) and these improvements have been accompanied by a substantial reduction in the number of cows (Capper *et al*., 2009). At individual animal level, this means that the total energy requirement per kg of milk produced is reduced by decreasing the energy requirement for maintenance, and hence the cows are more efficient in feed conversion. Capper *et al*. (2009) also calculated that a cow (650 kg; 3.69% milk fat) yielding 29 kg/day needs only 4.6 MJ net energy (NE) per kg of milk, compared with 9.2 MJ NE/kg milk when the production level is only 7 kg/day. Despite this, the carbon footprint per cow increased two-fold between 1944 and 2007, although it decreased when expressed per kg milk (from 3.66 to 1.35 CO_2-eq/kg milk). This change was also reflected in substantial reductions in nitrogen, phosphorus and CH_4 emissions from manure per unit of edible product. The Swedish Board of Agriculture has estimated that enteric CH_4 from ruminants in 2011 constituted one-third of the total emissions (2.6 million tons CO_2-equivalents) from Swedish agriculture (Naturvårsverket, 2008).

Methane production from enteric fermentation decreased by about 12% over the period 1990-2011 (Naturvårsverket, 2013), mainly due to a reduction in the dairy cattle population (from 525,000 to 338,000 head between 1993 and 2014), whereas the total amount of milk delivered to dairies decreased only marginally (SCB, 2016). At individual animal level, milk production increased from 9100 kg milk/year/head in 2014, compared with 7060 kg milk/year/head in 1990.

Global warming potential from ruminants cannot be neglected, but statistics sometimes exaggerate its contribution. It is also important to standardise the criteria for measuring GHG emissions, since there is great variation in current estimates. For instance, in some cases such exaggerated estimates detract from the major cause of climate change, which is mainly associated with combustion of fossil fuels (Herrero *et al.* 2011). Ruminants have the advantage of transforming carbon from photosynthesis into human-edible food and, from an ecological point of view, CH_4 emissions from ruminants can be considered recyclable carbon.

1.2 Methane production in ruminants

1.2.1 Rumen fermentation

Enteric CH_4 is produced from anaerobic fermentation of feeds, which takes place mainly in the rumen with a minor contribution from the hindgut. No single microbial species is responsible for complete degradation of substrate in the rumen. Instead, a complex succession of organisms takes part in the cooperative catabolism of substrates and the production of fermentation end-products. The diversity, size and activity of the microbial population in the rumen are largely determined by the diet composition (Van Soest, 1994), but are also influenced by animal-related factors such as saliva production, rumen volume and rates of intake and passage (Pinares-Patiño *et al.*, 2003; Hegarty, 2004) and management factors such as inclusion of essential oils in the diet (Patra and Yu, 2012).

Three separate factors that affect CH_4 emissions per unit intake can be identified: the rate of degradation of organic matter, the efficiency of microbial growth and the type of volatile fatty acids (VFA) produced from the fermentation of organic matter (Czerkawski, 1986; Van Soest, 1994). The fermentation of carbohydrates is by far the most important source of energy for rumen microbes (Ørskov, 1990) and bacteria are the principal organism fermenting carbohydrates in the rumen (Hungate, 1966).

The main pathways of carbohydrate metabolism in the rumen and relevant rumen microbes involved are shown in Figure 2. Feed entering the rumen is

primarily digested by bacteria, fungi and protozoa (primary fermenters), which digest feed components to simple monomers (McAllister *et al*., 1996). The breakdown of carbohydrates performed by the rumen microbiota can be divided into two stages (McDonald *et al*., 2011; Morgavi *et al*., 2010). The first stage involves the hydrolysis of complex carbohydrates to glucose equivalents and is performed by primary fermenters such as *Fibrobacter succinogenes* for the cell wall carbohydrates.

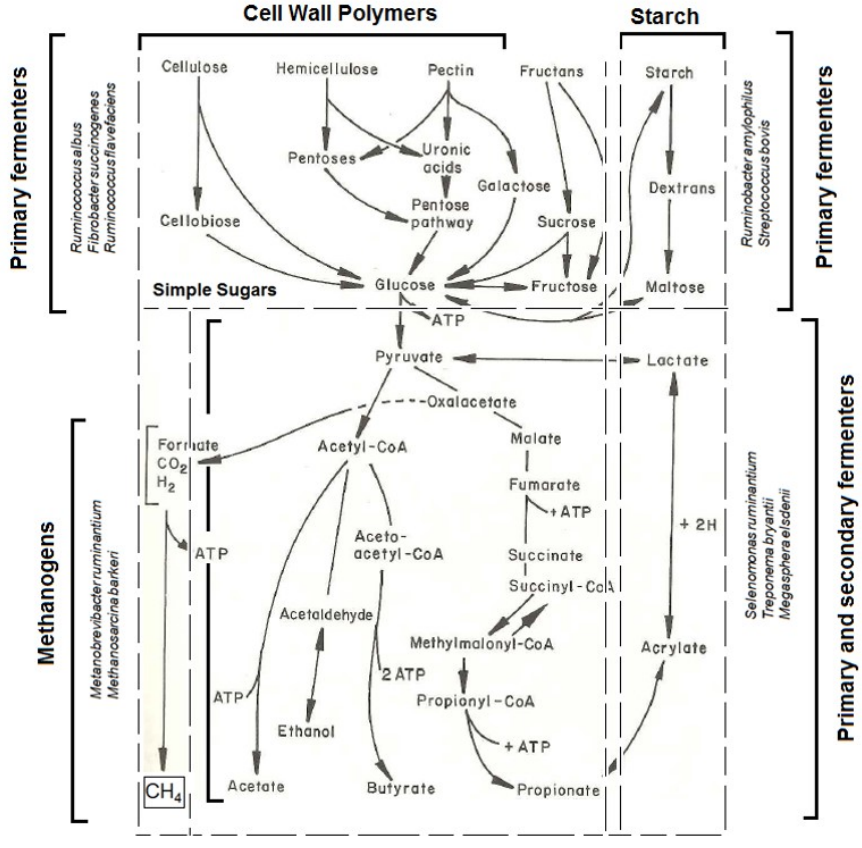

Figure 2. Overview of carbohydrate metabolism in the rumen and examples of microbiome species involved with substrate fermentation and methane (CH_4) production. Adapted from Van Soest (1994) and McAllister *et al*. (1996).

The hydrolysis of carbohydrates in the rumen is briefly described by Mc Donald *et al*. (2011) as follows: Cellulose is decomposed by β-1,4-glucosidades to cellobiose, which is then converted either to glucose or, through the action of a phosphorylase, to glucose-1-phosphate. Starch and

dextrin are first converted by amylases to maltose and isomaltose and further converted to glucose or glucose-1-phosphate by maltose phosphorylases and 1,6-glucosidases. Fructans are hydrolysed by enzymes attacking 2,1- and 2,6-linkages to release fructose units, which may also be produced together with glucose by the digestion of sucrose. Pentoses are the major product of hemicellulose hydrolysis, which is brought about by enzymes attacking the β-1,4 linkages xylose and uronic acids. The most common pathway of hexose metabolism in the rumen is glycolysis, which produces two equivalents of pyruvate, ATP and NADH.

The second stage (microbial fermentation) of carbohydrate digestion involves the conversion of pyruvate, a 3-carbon simple molecule, to different fermentation end-products through metabolic pathways that produce metabolic hydrogen and reducing equivalents (Moss *et al*., 2000; McDonald *et al*., 2011). Because the rumen is an anaerobic habitat, substrates are only partially oxidised and reducing equivalent disposal (*e.g*. NADH) is a critical feature for fermentation (Russell, 2002). Primary and secondary fermenters are involved in the degradation of simple sugars to the main products of rumen fermentation, such as main VFAs (acetate, propionate and butyrate), hydrogen gas (H_2) and CO_2, whereas CH_4 is produced in the final stage by methanogens (*e.g*. *Methanobrevibacter ruminantium*), using H_2 (80%) or formate ($HCOO^-$; 18 %) together with CO_2 as the main substrates (McAllister *et al*., 1996). When considering VFA, CO_2 and CH_4 as sole fermentation end-products using stoichiometry principles (Wolin, 1960), the fermentation equation for hexoses is to produce 100 units of VFA in the ratio 60:20:15:

57.5 Hexose → 115 Pyruvate → 65 Acetate + 20 Propionate + 15 Butyrate + 35 CH_4 + 60 CO_2

The fermentation pattern of the main VFAs in the rumen varies depending on diet composition and interval since feeding. Commonly, the molar ratio of acetate to propionate to butyrate is found to vary between 75:15:10 and 40:40:20 (Bergman, 1990). Acetate is produced from pyruvate following the loss of one carbon as CO_2, whereas butyrate is formed by the condensation of two molecules of acetyl-CoA. The reactions involved in the formation of acetate and butyrate from pyruvate are interrelated and all proceed through acetyl-CoA (Bergman, 1990). As a result of acetate formation, re-oxidation of NADH occurs and H^+ is produced, and methanogens use it to reduce CO_2 to CH_4 that is subsequently utilised for their maintenance and growth (McAllister and Newbold, 2008).

The metabolism of oxaloacetate to succinate is the main route used by rumen organisms to produce propionate, but the pathway through lactate and acrylate is favoured in the rumen of animals fed a high-concentrate diet (Van Soest, 1994). Propionate is the only VFA that makes a significant contribution to glucose synthesis, and is quantitatively the most important single precursor of glucose. Additional VFAs are also formed in smaller quantities by the deamination of amino acids such as isobutyrate from valine, isovalerate from leucine and 2-methyl butyrate from isoleucine. Their production is important, since they are growth factors for many cellulolytic organisms. 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. In ruminants such as sheep and cattle, the contribution of VFAs to the energy requirement can be as high as 70% (Bergman, 1990), whereas CH_4 production represents an energy loss to the animal.

1.2.2 Hydrogen production and H^+ sinks in the rumen

As a consequence of the lack of oxygen and the excess of reduced cofactors in the rumen, it is necessary to have sinks for disposing of H_2 produced during microbial fermentation. Hydrogen is produced during enzymatic oxidation of the NADH formed during glycolysis to NAD^+ (Czerkawski, 1986). Accumulated metabolic H_2 has to be removed, since otherwise it inhibits the re-oxidation of NADH, microbial growth and fibre degradation (Wolin *et al*., 1997; Joblin, 1999; McAllister and Newbold, 2008). Syntrophic (cross-feeding) interspecies H_2 transfer occurs when some microbes produce H_2 that is then further used by other microbes (Krause *et al*., 2013). The amount of specific VFAs produced is the major determinant of the amount of H_2 produced in the rumen (Table 2).

Compounds with negative oxidation values act as H_2 sinks. According to stoichiometric principles, CH_4 has the lowest (-2) possible oxidation state per unit of carbon compared with VFAs and CO_2 (highest +2). Therefore, the conversion of H+ and CO_2 to CH_4 and H_2O is the most important H_2 sink in the rumen (8H) compared with other pathways such as CH_4 conversion from formic acid (6H) or propionate production (2H) (Wolin, 1960; Van Soest, 1994). In addition to methanogenesis performed by archaea, other H_2 sinks can also be promoted under special conditions such as the addition of nitrates to the diet. An overview of different H_2 sinks in rumen conditions is presented in Figure 3.

Table 2. *Theoretical stoichiometric carbon-hydrogen balance equations describing conversion of glucose in the rumen*

glucose → 2 acetate + 2 CO_2 + 8 H
glucose → butyrate + 2 CO_2 + 4 H
glucose + 4 H → 2 propionate
CO_2 + 8 H → CH_4 + 2 H_2O
Net 3 glucose → 2 acetate + butyrate + 2 propionate + 3 CO_2 + CH_4 + 2 H_2O
Acetate production increases threefold and propionate and butyrate are unchanged:
3 glucose → 6 acetate + 6 CO_2 + 24 H
glucose → butyrate + 2 CO_2 + 4 H
glucose + 4 H → 2 propionate
3 CO_2 + 24 H → 3 CH_4 + 6 H_2O
Net 5 glucose + 6 acetate + butyrate + 2 propionate + 5 CO_2 + 3 CH_4 + 6 H_2O

Note: In Case 1, the acetate-to-propionate ratio is 1:1 and the methane-to-glucose ratio is 1:3; in Case 2, acetate-to-propionate ratio is 3:1, and methane-to-glucose is 3:5 (Source: Van Soest, 1994).

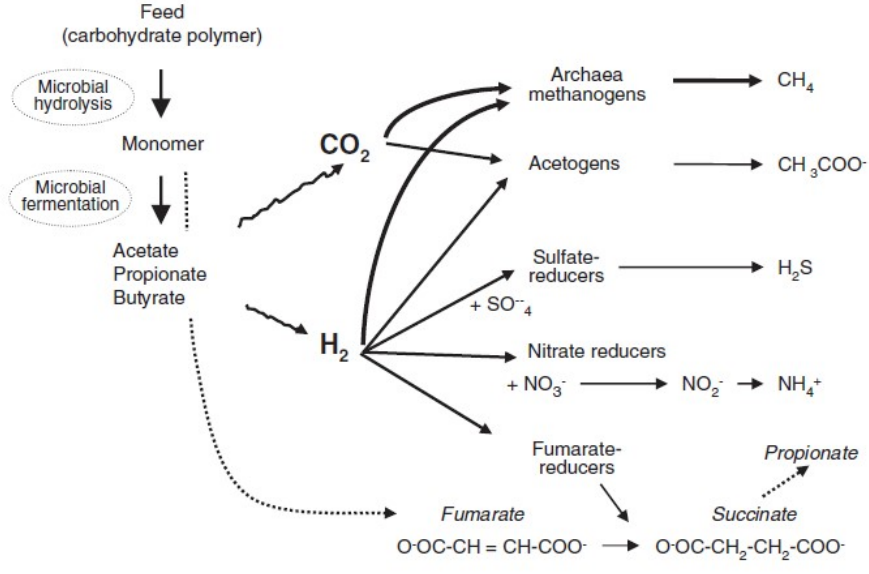

Figure 3. Schematic microbial fermentation of feed polysaccharides and H_2 reduction pathways in the rumen (Morgavi *et al*., 2010).

1.3 Strategies to reduce methane emissions

Different strategies have been proposed to mitigate CH_4 emissions from ruminants and thus reduce their impact on climate change. The effectiveness of a particular strategy depends upon the level at which it is evaluated (*e.g*. individual animal, farm, country) and its impact not only in the short term, but also in the long term. Effective mitigation strategies have to consider two major issues: i) improving rumen fermentation efficiency and ii) increasing animal productivity. In practice, profitability is often the most important decision-making factor in cattle production systems and it determines the adoption of a particular mitigation strategy at farm level, since farmers are unlikely to adopt practices that have no economic benefit or are not mandatory or supported by governmental subsidies (Hristov *et al*., 2013).

Many additives which have showed promising results in reducing CH_4 production *in vitro* have failed to produce similar results *in vivo* (McAllister, 2011). When *in vivo* reductions in CH_4 production have been observed, they have often been accompanied by a decrease in feed intake, digestibility and/or productivity. Therefore *in vitro* results have to be interpreted with caution if they are not tested in *in vivo* conditions.

1.3.1 Diet manipulation (feeding level and digestibility)

Intake and diet composition are the main factors affecting enteric CH_4 production in ruminants. Higher feed intake is associated with shorter retention time of feed particles in the rumen and thus rumen microorganisms have less time to digest the available substrate. In addition, the efficiency of microbial cell synthesis increases with increased passage rate, which partitions less fermented carbon to gases and volatile fatty acids. Conversely, higher digestibility usually increases CH_4 emissions per unit intake (Blaxter and Clapperton, 1965), but emissions per unit digested intake decrease and emissions per unit of product most likely decrease with improved diet digestibility. Digested neutral detergent fibre (NDF) produces more CH_4 than digested neutral detergent solubles, due to changes in fermentation pattern in the rumen (Jentsch *et al.*, 2007).

1.3.2 Increased level of concentrate

The reported effects of the level of concentrate feeding on CH_4 emissions are not consistent. In feedlot-type diets (>90% concentrate on DM basis), it is clear that increased addition of starch in the diet promotes propionate fermentation in the rumen (Johnson and Johnson, 1995). Sauvant and Giger-Reverdin (2009) reported a quadratic effect of the proportion of concentrate on CH_4 emissions, with a maximum at 35% concentrates on a dry matter (DM) basis. Starch

supplementation tends to increase butyrate, rather than propionate, in dairy cattle diets based on grass silage containing up to 70-75% concentrates on a DM basis (Jaakkola and Huhtanen, 1993). In addition, high levels of starch in the diet can compromise animal performance by decreasing fibre digestibility and increasing the incidence of acidosis.

1.3.3 Fat supplementation

Ruminant diets are low in dietary lipids due to the low contents in forages (Van Soest, 1994). Fat clearly decreases CH_4 emissions, as shown in *in vivo* studies (Jentsch *et al*., 2007; Beauchemin *et al*., 2008), and also in meta-analysis approaches (Ramin and Huhtanen, 2013). In addition, and especially at high level of supplementation, fat reduces feed intake and diet digestibility (Jenkins, 1993). At least four mechanisms are involved in the inhibitory effect of fat on CH_4 production: i) Fat is not a fermentable substrate in the rumen, ii) the bio-hydrogenation of fatty acids acts as an alternative H_2 sink in the rumen, iii) fat promotes increases in propionate concentrations in the rumen and iv) fat may decrease protozoal numbers (Van Soest, 1994).

However, fat supplementation in dairy cows above economic optimum increases feed costs and can reduce milk protein content (NRC, 2001). Fat supplementation has also been suggested to decrease fibre digestibility (Jenkins, 1993) but, according to a recent meta-analysis (Weld and Armentano, 2017), these effects are observed only with medium-chain and unsaturated fatty acids. In dairy cows diets, maximum recommended inclusion rate in ruminant diets is 6 to 7% (total fat) of dietary DM (Hristov *et al*., 2013).

1.3.4 Additives

The main objective of additives as a mitigation strategy is to improve rumen fermentation efficiency (Hristov *et al*., 2013). McAllister and Newbold (2008) defined two general mechanisms by which these substances act to reduce CH_4 emissions: i) by reducing the supply of metabolic H^+ for methanogens (*e.g*. defaunation, acetogenesis) and ii) by direct inhibition of methanogens (*e.g.* plant extracts). A summary of the main additives used to mitigate CH_4 emissions is provided below.

Inhibitors

Methane inhibition alters the microbial community, H_2 production and fermentation response in the rumen of cattle (Martinez-Fernandez, 2016). Different CH_4 inhibitors have been studied due to their specific inhibitory effect on rumen archaea. These include: bromochloromethane (BCM), 2-bromoethane sulfonate, chloroform and cyclodextrin. However, while these

compounds have been found to be effective in reducing CH_4 emissions (by up to 50%; Hristov *et al.*, 2013), they have a harmful effect on the animal (McAllister and Newbold, 2008). Adaptation of the rumen ecosystem compromises the effectiveness of using BCM as a mitigation strategy, since it is transitory (McAllister and Newbold, 2008). Recently, 3-nitrooxypropanol has been suggested as a promising compound in mitigating CH_4 emissions from ruminants, since it is not toxic for the animal and it has minor effects on dry matter intake. In dairy cows fed a diet containing 3-nitrooxypropanol, reductions of up to 30% in CH_4 emissions have been reported, without negative effects on feed intake or milk production, in a long-term (12-week) study (Hristov *et al.*, 2015a).

Ionophores

Ionophores are highly lipophilic ion carriers that modify ion transport through biological membranes. Monensin is the most studied ionophore and was originally marketed as a coccidiostat (anti-protozoan) for chickens. Nowadays, it is routinely used in North America, but banned in the European Union for use as a feed additive. Monensin acts on the cell wall of the Gram-positive bacteria that produce H^+ and interferes with ion flux, which results in decreasing acetate to propionate ratio in the rumen and thus a reduction in enteric CH_4 emissions. Hristov *et al.* (2013) concluded that ionophores are likely to have a moderate CH_4 mitigating effect, but their effect appears to be inconsistent. Some studies suggest a stronger anti-methanogenic effect in beef steers than in dairy cows (mostly fed forage-based diets), *e.g.* Guan *et al.* (2008) reported up to 30% reduction in enteric CH_4 production in beef cattle and up to 9% reduction in dairy cattle Van Vugt *et al.* (2005). The effects in dairy cows can be improved by dietary modifications and increasing monensin dose, as reported by Appuhamy *et al.* (2013).

Electron receptors

Nitrates/nitrites have shown promising results in decreasing CH_4 production (Van Zijderveld *et al.*, 2010). An important issue as regards using nitrates in the diet is the potential for increased ammonia production and potential toxicity from intermediate products (*e.g.* nitrite; Leng, 2008). The best option to reduce CH_4 emissions using nitrates is to replace urea as a non-protein nitrogen source in the diet to meet the microbial requirements for rumen-degradable N. However, when the supply of degradable N is sufficient, nitrate supplementation will increase nitrogen losses to the environment.

Adding sulphate to the diet of sheep has been found to reduce CH_4 production and, when both nitrate and sulphate are added, the effects on CH_4

production have been shown to be additive (Van Zijderveld *et al*., 2010). Distiller's grain contains high levels of sulphate, which has resulted in intensive research on the effect of high-sulphate diets (also in combination with high-sulphate drinking water). However, high-sulphate diets induce polioencephalomalacia (Gould, 2000), which is caused by excessive production of hydrogen sulphide (H_2S) in the rumen.

Organic acids such as fumaric and malic acids have also been studied as alternative hydrogen sinks in the rumen (Molano *et al*., 2008). The mitigating potential of fumarate has been questioned (Ungerfeld *et al*., 2007), because it is generally lower than that of nitrates and results have been inconsistent.

Plant compounds

Plant-bioactive compounds form a large and heterogeneous group and vary in chemical structure. Tannins, saponins and essential oils have been reported as the main compounds with anti-methanogenic activity in ruminants (Waghorn *et al*., 2002; Hristov *et al*. 2013).

Tannins are polyphenolic substances widely distributed in plants which are characterised by their ability to bind proteins in aqueous solutions. Tannin-protein complexes involve both hydrogen-bonding and hydrophobic interactions, causing a reduction in protein degradation in rumen conditions. Tannins are anti-nutritional factors when dietary crude protein concentrations are limiting production, because they reduce absorption of amino acids (Waghorn, 2008). The anti-methanogenic effect of hydrolysed tannins is caused by their inhibition of the rumen archaea, whereas condensed tannins act indirectly by inhibition of fibre digestion (Goel and Makkar, 2012). In a review by Hristov *et al*. (2013), tannins were reported to show good potential for reducing CH_4 emissions, by up to 20%.

Saponins are glycoside compounds present in many plants in which the sugars units are linked to a triterpene or steroidal aglycone moiety. They modify ruminal fermentation by their toxic effect on ruminal protozoa. Therefore, saponins have the potential to enhance flow of microbial protein, which is an alternative H_2 sink in the rumen, and in this way increase the efficiency of feed utilisation and reduce enteric CH_4 production. However, their effect is not always consistent, since it has been reported that saponins can be inactivated by rumen bacterial populations and the saliva of adapted animals (Newbold *et al*., 1997). The potential of essential oils as inhibitors of CH_4 production has been studied extensively in *in vitro* experiments (Calsamiglia *et al*., 2008; Bodas *et al*., 2008; Benchaar *et al*., 2011). These substances have an antimicrobial activity against rumen archaea by reducing

H_2 availability. However, it is likely that the doses required for any substantial mitigations *in vivo* are not economically feasible.

1.3.5 Manipulation of microbes

Defaunation refers to the removal of rumen protozoa. Rumen protozoa share a symbiotic relationship with methanogens, participating in interspecies hydrogen transfer. The literature reports contradictory results regarding defaunation (McAllister and Newbold, 2008). However, some studies indicate that defaunation may lower the amount of hydrogen in the system and thereby reduce CH_4 production (Vermorel and Jouany, 1989; Morgavi *et al*., 2010). According to a review by Morgavi *et al.* (2010) defaunation decreases CH_4 emissions by on average 10.5%. Their review also found that methane production per mole of VFA, calculated according to Wolin (1960) using data from Eugène *et al.* (2004) was 6.9% greater in faunated than in defaunated animals (116-118 comparison) and digestibility of OM was 15 g/kg (2.2%) higher in faunated animals. In addition, the efficiency of microbial N synthesis was higher in faunated animals, which repartition fermented carbon from VFA and gas production to microbial cells (Morgavi *et al*., 2010). In conclusion, it seems that the lower CH_4 emissions in defaunated animals can be entirely attributed to changes in diet digestion and rumen fermentation pattern.

Significant efforts have been devoted to suppressing archaea and/or promoting acetogenic bacteria in the rumen (Hristov *et al*., 2013). Vaccines trigger the immune system of ruminants by a continuous supply of antibodies against archaea to the rumen through saliva. Since the rumen methanogen population present can differ based on diet and geographical location of the host, applying a single-targeted approach it could be expected difficult its implementation in *in vivo* conditions.

New approaches are under investigation, one of which involves identifying genes encoding specific membrane-located protein as antigens to vaccinate sheep (Buddle *et al*., 2011). Another involves generation of antisera against subcellular fractions of this microorganism in *in vitro* conditions, reducing microbial growth and CH_4 production (Wedlock *et al*., 2013).

Reductive acetogenic bacteria reduce two moles of CO_2 to acetate by oxidation of H_2 (Joblin, 1999). However, they are less efficient than archaea populations, as H_2 sinks in the rumen under normal ruminal conditions (Fievez *et al*., 1999). Acetogenesis could take place more actively in the rumen when methanogenesis is inhibited at increased hydrogen concentrations if dissolved hydrogen concentrations increased as a result of suppressed CH_4 production (Le Van *et al*., 1998). In summary, *in vitro* approaches testing CH_4 inhibitors (see Figure 4) are useful for screening purposes (*e.g.* doses), but are still rather

far from explaining observed CH_4 emissions from ruminants in *in vivo* conditions. Long term in vivo studies are required to confirm results obtained in in vitro conditions.

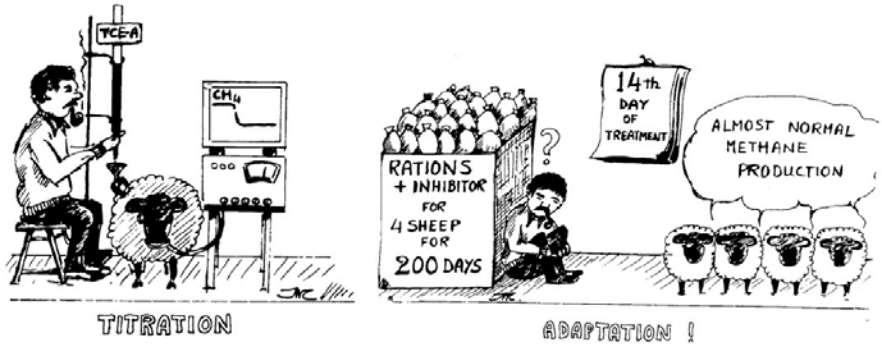

Figure 4. Cartoon showing the *in vivo* side-effects of dietary additives to inhibit CH_4 production. Reprinted from: *An Introduction to Rumen Studies* by J.W. Czerkawski, page 106. Copyright © (1986).

1.4 Techniques to measure methane emissions *in vivo*

1.4.1 Respiration chambers

Respiration chambers have been favoured for their accuracy and low coefficient of variation (Blaxter and Clapperton, 1965; Grainger *et al*., 2007) compared with other methods for measuring CH_4 in *in vivo* conditions and have been used widely to measure differences between diets (Figure 5). Indeed, respiration chamber experiments have been performed for over 100 years and provide the basis for our current understanding of energy metabolism in farm animals (McLean and Tobin, 1987).

The principle of this technique is to collect all exhaled breath from the animal and to measure gas concentrations (*e.g.* CH_4). Gas concentrations are corrected by continuous airflow to adjust the background concentrations. The animals are kept in closed chambers for about 2-4 days with ventilation for intake and exhaust air. The chamber gives an inflow and outflow of gas concentrations (CO_2, O_2, and CH_4) and therefore is possible to calculate the energy balance of the animal. Methane flux (L/day) is calculated as CH_4 flow = Air flow $\times 10^6 \times$ [CH_4 Outflow (ppm) - CH_4 Inflow (ppm)]. This technique has been criticised for the fact that the animal inside the chamber is not experiencing natural conditions and that the restriction could have consequences for animal behaviour, especially feed intake, and could lower heat production owing to the reduction in physical activity. Among practical

considerations, the technique is expensive, labour-intensive and not designed for measuring a large number of animals simultaneously. Recently, in the large scale EU project RuminOmics (www.ruminomics.eu), *in vivo* CH_4 individual data from 100 dairy cows was collected in respiration chambers at LUKE (Natural Resources Institute Finland).

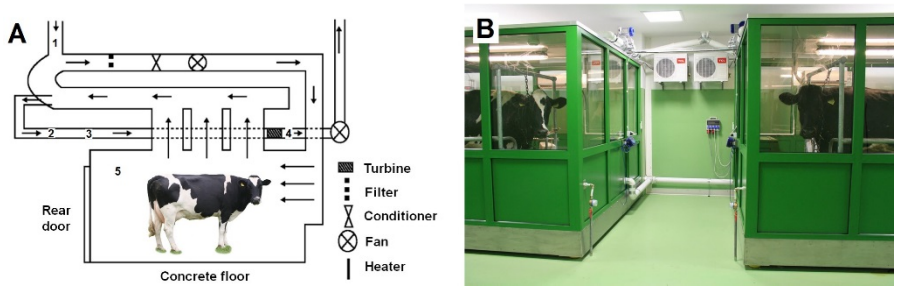

Figure 5. Respiration chambers. A) Schematic diagram of the open-circuit respiration chamber showing air fluxes (adapted from Grainger *et al.* (2007). B) Research facilities at Poznan University of Life Sciences, Poland (Source: http://globalresearchalliance.org/country/poland/ Accessed: 22 March 2017).

1.4.2 Spot sampling methods

GreenFeed system

The GreenFeed system (C-Lock Inc., Rapid City, South Dakota, USA) is a spot gas sampling method which can be attached either to a concentrate feeder station or an automatic milking system in farm conditions (Figure 6). It records both CH_4 and CO_2 fluxes on an individual animal basis from the exhaled air during breathing by the animal when eating small amounts of concentrate feed released into a tray in a semi-enclosed hood (http://www.c-lockinc.com/). GreenFeed is highly dependent on high frequency of animal visits per day, which increases repeatability and certainty in estimating daily CH_4 and CO_2 emissions from individual animals. The duration of individual visits is especially important for CH_4 measurements, because most CH_4 is eructated at 40- to 120-s intervals (Hammond *et al*. 2016). The system recognises the individual animal by interfacing with an attached tag reader and the data are stored online for further calculations.

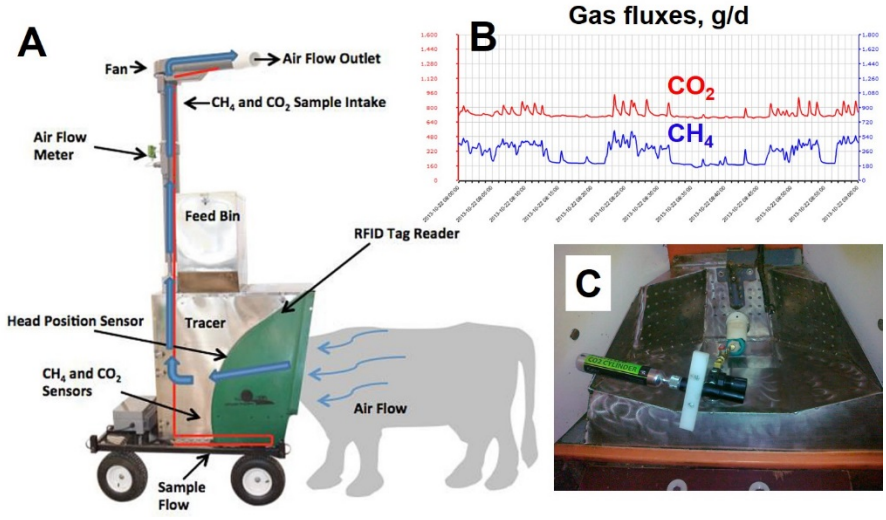

Figure 6. The GreenFeed system (C-Lock Inc., Rapid City, South Dakota, USA). A) General layout in the stand-alone concentrate feeder (Source: Hristov *et al.*, 2015b). B) Online user interface for visualizing methane (CH_4) and carbon dioxide (CO_2) fluxes. C) Carbon dioxide recovery test for system calibration purposes.

The GreenFeed device uses a similar principle for measuring gas emissions as in respiration chambers, where an active airflow is induced to capture emitted air by integrating measurements of air flow, gas concentrations, and detection of muzzle position (Zimmerman, 2011; Huhtanen *et al.*, 2015b). The ideal gas law is then used to convert the data in terms of mass fluxes and the values obtained are adjusted for head position relative to the airflow.

Sniffer methods

Concentrations of both CH_4 and CO_2 in air released by eructation are recorded by gas analysers throughout individual milking in robotic stations. Analysis of changes in CH_4 concentration provides information on frequency of eructation and average CH_4 release per eructation. The product of these variables provides an estimate of CH_4 emission rate for each milking.

Daily means are calculated, allowing for within-herd diurnal variation to be taken into account, if necessary, for at least 7 days, as recommended by Garnsworthy *et al.* (2012a), and combined into an overall mean for each cow. Individual mean CH_4 emission rates are then converted into daily CH_4 output using calibrations against chamber measurements (Garnsworthy *et al.*, 2012a). Thus, this technique does not measure CH_4 emissions directly. Despite the technique having been used to measure CH_4 emissions on very large numbers of animals in farm conditions, there are concerns over the accuracy,

repeatability and precision of the data obtained, which constrains the sensitivity of the device to detect treatment differences in CH_4 emissions (Hammond *et al*., 2016). The sniffer methods are also highly dependent on the muzzle position of the animal (Huhtanen *et al*., 2015b), an issue that significantly increases the variation and compromises the reliability.

1.4.3 Tracer gas methods

Both sulphur hexafluoride (SF_6) and CO_2 techniques are based on the concentration of a tracer gas for their measurements. One major requirement for any tracer gas is that concentrations in the environment should be very low, relative to the concentration of the tracer in collected samples, with background gas concentrations accounted for (Berndt *et al*., 2014).

The SF_6 technique

The SF_6 technique is especially useful for measuring CH_4 emissions in free-ranging animals. General aspects of the SF_6 technique are presented in Figure 7. Sulphur hexafluoride as a tracer gas is released from a bolus placed in the rumen, gas samples are continuously collected from exhaled air in a canister and the concentrations of SF_6 and CH_4 in the collected gas are analysed by chromatographic methods. When SF_6 release rate and gas concentration (corrected for background) are known, CH_4 flux can be calculated. Background gas concentrations can be a problem in indoor conditions and therefore is preferable to use this technique in grazing experiments (Hristov *et al*., 2016; Dorich *et al*., 2015). Moreover, use of the SF_6 technique to measure CH_4 emissions in cannulated animals is not recommended because cannulation introduces more variability into the SF_6 technique with its head canister (Beauchemin *et al*., 2012).

Although the SF_6 procedure has been used for large-scale genetic and nutritional evaluations, it remains labour-intensive, expensive and dependent on technical specialists for operation and analysis (Hristov *et al*., 2013). The use of SF_6 has also been criticised since this chemical compound has a greenhouse effect in the atmosphere (Berndt *et al*., 2014). Recent modifications of the SF_6 procedure have improved the accuracy of the technique (Deighton *et al*., 2013).

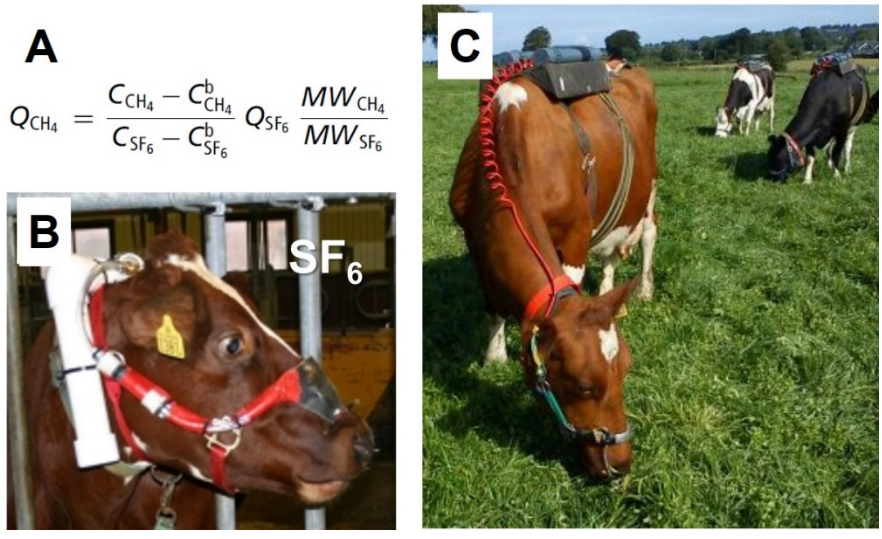

Figure 7. The sulphur hexafluoride (SF_6) tracer technique. A) Formula used in the calculations according to Johnson *et al*. (1994). B) Sample collection apparatus (SLU, Uppsala, Sweden). C) The SF_6 technique under grazing conditions – back-mounting system for collection vessels (Berndt *et al*., 2014).

The CO_2 technique

Carbon dioxide is calculated as a function of heat production (heat calculated from maintenance and production requirements) and CH_4 emissions determined by the product of the multiplication of CH_4/CO_2 ratio (ppm/ppm) by CO_2 production (L/day), as shown by Madsen *et al*. (2010). This technique basically assumes that there is no variation in the efficiency of metabolisable energy utilisation between animals for maintenance and production which not makes biological sense. For instance high CH_4/CO_2 ratio can be as a result of high emissions (more CH_4) or high production efficiency (less CO_2 per unit of product). Therefore CH_4/CO_2 ratio cannot be a reliable indicator of CH_4 fluxes for individual animals. In the discussion section, the implications of CH_4/CO_2 ratio are discussed in detail.

1.4.4 The laser technique

A review by Chagunda (2013) summarises the potential of laser systems for CH_4 detection in dairy cows. Laser methane detection (LMD) equipment is based on infrared absorption spectroscopy, using a semiconductor laser as a collimated excitation source and using the second harmonic detection of wavelength modulation spectroscopy to establish a CH_4 concentration measurement in parts per million-metre (ppm-m). The LMD equipment is able to detect CH_4 concentrations within a mix of gases in the environment.

Physical activity of the animal has a strong influence on CH_4 emissions by this technique and it has to be considered at individual animal level.

Despite of the fact that this novel technique is a non-invasive method, it is still not widely used. However, a comparison study conducted in dairy cows fed a diet of grass silage with 0.3 or 0.7 w/w of a concentrate supplement demonstrated a high and positive correlation between measurements from the LMD and the indirect open-circuit respiration calorimetric chamber ($r = 0.8$, $P<0.001$) (Chagunda and Yan, 2011). However, the range in data was six-fold higher, which increased the correlation coefficient. Within the practical range for dairy cows (max 2-fold) the relationship was rather poor.

1.4.5 Proxies to measure *in vivo* methane

Novel non-invasive methods have been proposed to account for *in vivo* CH_4 emissions in ruminants. Negussie *et al*. (2017) discussed the current potential of available proxies as effective mitigation strategies. These methods can be classified according to the chronological progression of nutrients through the animal: (i) feed intake and feeding behaviour; (ii) rumen function, metabolites, and microbiome; (iii) milk production and composition; (iv) hindgut and faeces; and (v) measurements at the level of the whole animal (Negussie *et al*., 2017). The authors concluded that most of proxies tend to be accurate only for the production system and the environmental conditions under which they were developed. As a result, the greatest shortcoming today is the lack of robustness in their general applicability.

Both laser technique as the different proxies to measure *in vivo* CH_4 in dairy cows are not further discussed since they are beyond of the scope of the present thesis.

2 Objectives

The overall aim of the studies presented in this thesis was to investigate the contribution of different sources of variation to *in vivo* CH_4 emissions from dairy cows. Between-cow variation in CH_4 emissions were further explored by studying the effects of the measurement technique, animal-related factors and diet effects. Specific objectives were to:

1. Compare two spot-sampling methods, i) the sniffer method and ii) the flux method, for determining *in vivo* emissions from loose-housed dairy cows.
2. Evaluate between-cow variability in different digestion and rumen fermentation variables related to CH_4 production and their contribution to the observed individual animal variation.
3. Study the effects of graded replacement of late-harvested grass silage and barley by highly digestible grass silage (early-harvested) on milk production, CH_4 and CO_2 emissions and N efficiency.
4. Examine in depth the effects of graded replacement of late-harvested grass silage and barley by highly digestible grass silage (early-harvested) on the efficiency of ruminal and total tract digestion and nutrient supply, in order to explain production responses in dairy cows.

3 Materials and methods

A general overview of the sources of variation contributing to between-cow differences in CH_4 emissions studied in this thesis is presented in Figure 8.

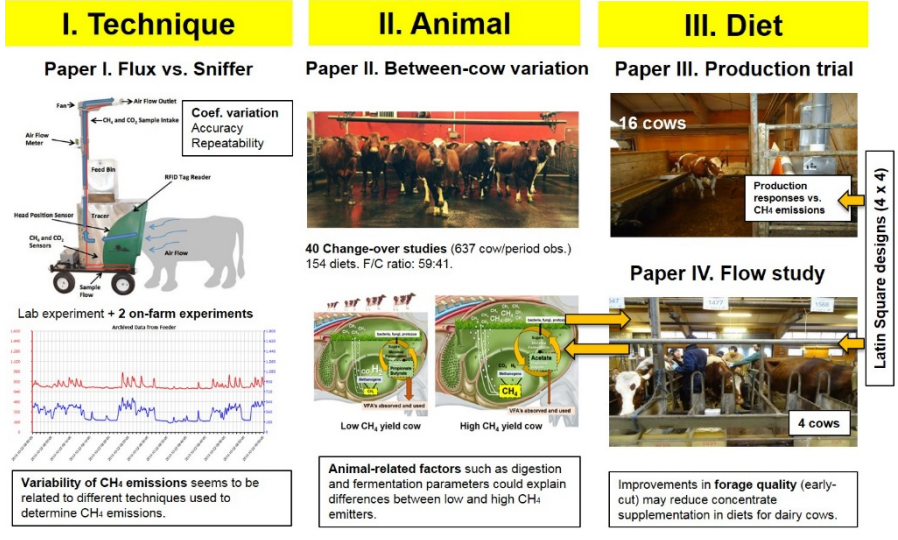

Figure 8. General layout showing sources of variation in CH_4 emissions from dairy cows studied in this thesis. Results from the flow study (**Paper IV**) are also included in the meta-analysis based on an individual cow dataset (**Paper II**).

3.1 Paper I

Two spot-sampling methods for measuring CH_4 emissions in cattle were compared in dairy farm conditions. The gas emissions were measured using portable units of the GreenFeed system (C-Lock Inc., Rapid City, SD) attached either to a concentrate feeder or automatic milking system which was set up in two different configurations (methods). In the first method (sniffer method),

both and CO_2 concentrations were measured in close proximity to the muzzle of the animal, and average concentrations or CH_4/CO_2 ratio were calculated. In the second method (flux method), measurements of CH_4 and CO_2 concentrations were combined with an active airflow inside the feed troughs to capture emitted gases coming from the animal. The flux method is the normal set-up of the GreenFeed system and the purpose of the sniffer method was to mimic the mechanism of commercially available gas analysers (Garnsworthy *et al*., 2012a; Lassen *et al*., 2012). A muzzle sensor was used, allowing data to be filtered according to the proximity to the cow's head, allowing better estimates of gas emissions for both methods. The proximity to the head adjustments for each method was assessed by a study conducted in laboratory conditions using a model cow's head that emitted CO_2 at a constant rate, by simulating different cow head positions with respect to the manifold inlet.

The methods were compared in two on-farm studies conducted using either 32 (experiment 1) or 59 (experiment 2) cows in a switch-back design of 5 five (experiment 1) or four (experiment 2) periods for replicate comparisons between methods. In experiment 1, the experimental design was a cyclic changeover with four blocks of eight mid-lactation Nordic Red cows, eight diets and three experimental periods of 21 days. The eight treatments were allocated to a 2 × 4 factorial arrangement consisting of two forages (mixtures of grass and red clover silages), and increasing levels of CP in the diet by gradually replacing ensiled barley grain with rapeseed expeller. Details of that experiment are reported by Gidlund *et al*. (2017). Gas emissions for both methods were recorded by two GreenFeed units attached to concentrate feeders, which were switched to either sniffer or flux methods in the middle and at the end of each experimental period. The cows were allowed to visit the GreenFeed units every 7 h, and they were given eight 50 g servings of a commercial concentrate at 40-s intervals during each visit.

Experiment 2, performed on Holstein-Friesian cows, lasted 40 days and comprised four periods of 10 days. For method comparison purposes, the herd was divided into two groups that were assigned to one of the two automatic milking system units (AMS), which were retrofitted with both sniffer and flux-method equipment and set up as follows: flux–sniffer–flux–sniffer (AMS 1) and sniffer–flux–sniffer–flux (AMS 2). Cows were given unrestricted amounts of total mixed ration (TMR; 60% forage, 40% concentrates on a DM basis) and fed three times per day. In addition, the cows received a commercial concentrate pellet during milking in the automatic system at a rate of 1 kg of pellet per 7 kg of milk. Data obtained by each method were adjusted based on filtering of muzzle position data as described previously.

Data (CH_4 and CO_2 or CH_4/CO_2 ratio and corresponding fluxes) from the on-farm studies were analysed with linear mixed models (PROC MIXED; SAS Institute, 2008), taking into account the fixed effects of period, diet (experiment 1), DMI (experiment 1) and the random effect of cow. Repeatability was calculated as: $Rep = \delta^2_{cow} / (\delta^2_{cow} + \delta^2_{residual})$. The relationships between concentrations of CH_4 and CO_2 or CH_4/CO_2 ratio and corresponding fluxes were estimated by linear regression using least squares means for each cow.

3.2 Paper II

In **Paper II**, a meta-analysis based on an individual cow dataset was conducted to investigate the effects of between-cow variation and animal variables related to CH_4 emissions from dairy cows. Data were collected from 40 change-over studies comprising a total of 637 cow/period observations. Animal production and rumen fermentation characteristics were measured for 154 diets in 40 studies; diet digestibility in the total tract was measured for 135 diets in 34 studies, digesta flow was measured for 103 diets in 26 studies, and ruminal digestion kinetics was measured for 56 diets in 15 studies. The experimental diets were based on silages (mainly grass with some legume and whole-crop silage), with cereal grains or by-products as energy supplements, and soybean or rapeseed meal as protein supplements. Average forage: concentrate ratio across all diets on DM basis was 59:41. The diets were fed *ad libitum* either as TMR or fixed amounts of concentrate with forage *ad libitum*. Finnish feed tables values (LUKE, 2016) were used when starch and fat content in concentrate ingredients was not reported.

Apparent diet digestibility was determined by total faeces collection (27 studies) or by faeces spot sampling (seven studies) using either acid-insoluble ash (Van Keulen and Young, 1977) or indigestible neutral detergent fibre (NDF) (Huhtanen *et al*., 1994) as internal markers. Digesta flow was assessed using the omasal sampling technique (Ahvenjärvi *et al*., 2000) with the triple-marker system (France and Siddons, 1986). Microbial N synthesis was determined using ^{15}N as a microbial marker except in two studies, where purine-based derivatives were used. Rumen pool size was determined by rumen evacuation and digestion and passage kinetics variables were calculated using the compartmental flux method (Ellis *et al*., 1994). Rumen fluid samples were mostly collected over 12 h after morning feeding to obtain fermentation parameters. The main volatile fatty acids (VFAs) were used to determine ratios between these (*e.g*. acetate: propionate) and production of both CH_4 and CO_2 per mol of volatile fatty acids (CH_4VFA and CO_2VFA, respectively) based on

stoichiometry principles according to Wolin (1960). Furthermore, CH_4VFA in addition to OM apparently digested in the rumen (OMADR) was used as the basis to predict total CH_4 production (g/day), which was further adjusted by hydrogen sinks such as microbial cells (Czerkawski, 1986) and biohydrogenation of fatty acids using the equation in the Karoline model (Huhtanen *et al*., 2015c). The predicted total CH_4 emissions were compared with two empirical equations, those presented by Yan *et al*. (2000), and Ramin and Huhtanen (2013), using data obtained from studies conducted in respiration chambers.

Variance components analysis was used for the most relevant variables associated with enteric CH_4 production to calculate the random effects of: experiment (Exp), Cow(Exp), Diet(Exp), Period(Exp) and residual variation. In addition, repeatability values were determined as in **Paper I**. Single regression models were developed based on their biological value in CH_4 production. The models included two random statements: a random intercept and slope of X1 with SUBJECT = Diet (Exp), and a random intercept with SUBJECT = Period (Exp), using the TYPE = VC as covariance structure for both random statements. The maximum likelihood method was used in the PROC MIXED model syntax (version 9.3; SAS Institute Inc., Cary. NC). The purpose was to remove both period and diet effects.

3.3 Paper III

A study was conducted at Röbäcksdalen experimental farm, Swedish University of Agricultural Sciences, Umeå, Sweden (63º45'N; 20º17'E), to investigate the effects of replacing late-harvested grass silage (LS) and barley by early-harvested grass silage (ES) on performance and CH_4 emissions in dairy cows. Sixteen Nordic Red cows with mean BW of 635 ± 76.0 kg, at 79 ± 14.4 days in milk (DIM) and producing 34 ± 6.9 kg milk/day at the beginning of the experiment were used in a replicate 4 x 4 Latin square design. Each 28-day period comprised 14 days of diet adaptation followed by 14 days of data collection. Cows were offered the diets *ad libitum* four times per day as TMR, with free access to water, and were milked twice daily.

Two grass silages were prepared from the same primary growth of a third-year ley dominated by timothy grass (*Phleum pratense*) with some red clover (*Trifolium pratense*) harvested two weeks apart. A mixture of LS and rolled barley was gradually replaced by ES (0, 33, 67, and 100% of the forage component of the diet), in order to obtain four diets defined as: Late-cut (L), late-early (LE), early-late (EL) and early-cut (E) silage. The proportion of forage increased from 42 to 64 % on DM basis with increasing proportion of

ES in the TMR diet without considering extra concentrate supplementation from the GreenFeed system attached to concentrate feeders. Heat-treated solvent-extracted rapeseed was used as the protein supplement. The diets were formulated to meet the metabolisable energy (ME) and metabolisable protein (MP) requirements for 35 kg energy-corrected milk (ECM) per day.

Apparent diet digestibility was assessed by collecting grab samples of faeces from the rectum of eight cows (two squares). Ash-free indigestible NDF (iNDF) concentration was used as an internal marker to calculate diet digestibility (Huhtanen *et al*. 1994). Milk yield was recorded daily, and samples were taken for milk composition analysis at four consecutive milkings. Gas emissions (CH_4 and CO_2) were measured using the GreenFeed system (C-Lock Inc., Rapid City, SD) as described by Huhtanen *et al*. (2015b) and Hammond *et al*. (2016). The GreenFeed system was programmed to allow each animal to visit the two units at minimum 5-h intervals and they were given eight 50 g servings of commercial concentrate at 40-s intervals during each visit.

Chemical composition and feeding values of the diets were calculated from the proportion of ingredients and their respective values. Energy-corrected milk was calculated according to Sjaunja *et al*. (1990). Feed efficiency was calculated as ECM yield (kg/d)/DMI (kg/d) and milk N efficiency (MNE) as milk N [CP (g/day)/6.38]/N intake (kg/day). Methane and CO_2 production were calculated as mean daily production during the last 14 days of each period. The experimental data were analysed by ANOVA for a replicate 4 × 4 Latin square design using the MIXED procedure of SAS (Version 9.3, SAS Inst., Inc., Cary, NC) and orthogonal polynomial contrasts were used to evaluate linear and quadratic effects of treatments.

3.4 Paper IV

The aim of **Paper IV** was to study the effects of the diets used in **Paper III** on rumen fermentation, microbial N synthesis, diet digestion and digestion kinetics using rumen-cannulated cows in a tie-stall system. This study was conducted in parallel with the production study (**Paper III**). Four multiparous rumen-cannulated Nordic Red cows averaging 676 ± 79 kg of BW, at 90 ± 19.1 days in milk and yielding 30.9 ± 6.27 kg of milk at the start of the experiment were used in a balanced 4 x 4 Latin square design. The experimental periods lasted for 21 days and were divided into 14 days of adaptation and seven days of data collection. The cows were fed manually with the experimental diets as TMR *ad libitum* and milked twice daily. To mimic extra concentrate supply from the GreenFeed system, 1 kg DM/day of the same

feed was added to give similar diet composition in both studies. Orts were recorded once daily and feeding rate was adjusted to provide 10% extra of the previous calculated intake except during the 4 days of sampling from the omasum, when feeding rate was restricted to 95% of previous *ad libitum* intake.

Total tract digestibility was assessed as described in **Paper III.** Two rumen evacuations were conducted, at 4 h after (d 12) and 1 h before (d 14) the morning feeding, to give a representative estimate of rumen digesta pool size and digestion kinetics. After the last rumen evacuation, *in situ* bags containing 2 g DM of early- or late-harvested grass silage were placed in the rumen for 24 h to evaluate the effects of diet composition on rumen fibrolytic activity. The omasal sampling technique (Huhtanen *et al*., 1997), as modified by Ahvenjärvi *et al*. (2000), was used for collection of digesta samples from the omasum on day 17 to day 20, with 4 h intervals between the three sampling occasions each day.

Omasal flow and ruminal digestibility of nutrients were calculated using the reconstitution system based on a triple marker technique (Cr-EDTA, Yb-acetate and iNDF; France and Siddons, 1986). Microbial protein synthesis was determined using ^{15}N as microbial marker (Broderick and Merchen, 1992). Samples of rumen fluid (n = 8 time points) were collected at 1.5 h intervals on day 21 to measure pH and VFAs concentrations. Production of CH_4 per mol of VFA (CH_4VFA) was calculated based on VFA stoichiometry equations (Wolin, 1960).

Calculation of omasal flow of nutrients was based on the triple-marker method (France and Siddons, 1986) and daily marker doses recovered from faeces according to Armentano and Russell (1985). True OM digestibility was corrected for VFA flow according to Huhtanen *et al*., (2010). Flows of OM and non-ammonia nitrogen (NAN) were corrected for microbial OM and microbial NAN, respectively. Digestion kinetic variables were calculated by the compartmental flux method (Ellis *et al*., 1994). The experimental data were analysed by ANOVA for a 4 × 4 Latin square design using the MIXED procedure of SAS (Version 9.3, SAS Inst., Inc., Cary, NC) and orthogonal polynomial contrasts were considered to assess the effect of the diets.

4 Results

4.1 Paper I

The study conducted in laboratory conditions (robot) demonstrated that for the sniffer method, the muzzle distance from the sampling point (0-30 cm) was a key factor determining gas concentrations. Muzzle distance had no effect on the recovery of CO_2 with the flux method, regardless of the cow head position, head movement or breath rate, and the variability was much smaller compared with the sniffer method throughout all recovery tests. Wind (6 m/s) was the only factor that clearly decreased the CO_2 recovery of the flux method, to about 85%.

In on-farm conditions (experiment 2), repeatability of muzzle position across the cows was 0.74 and 0.82 when analysed using daily (n = 40) and period (n = 4) data for each cow, respectively. When the flux-method data for all cows were not filtered according to muzzle position, fractional time with muzzle inside a manifold (*i.e.* from 0 to 1) and CH_4 flux showed a positive relationship ($R^2 = 0.31$; $P<0.001$). Weak relationships were found between the CH_4 concentration (ppm) determined by the sniffer method and by the flux method in both experiments. Between-cow coefficient of variation (CV) in CH_4 flux decreased from 21.2 to 17.6 % when using filtered data. The CH_4/CO_2 ratio determined by the sniffer method was negatively ($P<0.001$) related to muzzle position in experiment 2.

Total CH_4 flux was similar in both experiments, but CO_2 flux was numerically greater in experiment 2. Both CH_4 and CO_2 concentrations measured using the sniffer method were markedly lower in experiment 2 compared with experiment 1, indicating that the geometric structure of the head-box (GreenFeed compared with automatic milking system) influences the dilution of exhaled gases. The between-cow CV of CH_4 emissions was greater with the sniffer method compared with the flux method in both experiments.

However, between-cow CV values of the CH_4/CO_2 ratio for the flux and sniffer methods were rather similar (6.4 and 6.6 %, respectively, in experiment 1 and 8.8 and 7.5%, respectively, in experiment 2). Repeatability of gas measurements (CH_4 and CO_2) was generally high (>0.70), and the values were similar between the experiments and methods.

The relationship between the sniffer method CH_4 concentration and CH_4 flux was significant (P=0.02) in experiment 2, but this was not replicated in experiment 1 (P=0.11). The intercept (*i.e*. observed CH_4 flux at zero CH_4 concentration) was highly significant (P<0.001) for the sniffer method in both studies, suggesting larger random error compared with the flux method. The relationship between CH_4/CO_2 ratio and CH_4 flux determined by the sniffer and flux methods was statistically significant (P<0.01) in both studies, but R^2 values were higher in experiment 1 than in experiment 2. In experiment 1, the CH_4/CO_2 ratio was positively related to CH_4 emissions per kilogram of DMI when measured by the flux method, but not when measured by the sniffer method.

4.2 Paper II

The dataset was representative of feeding conditions for dairy cows in Northern Europe. The forage: concentrate proportion was 59:41 on DM basis and dietary concentrations of CP and NDF were 160 ± 21.3 and 394 ± 54.8 g/kg DM respectively. Dry matter intake was on average 18.9±3.35 kg/day.
The variability and repeatability in molar proportions of VFA were generally small. The variance component Diet(Exp) was on average two-fold larger than the observed for Cow(Exp), except for butyrate. Low variability in the main VFAs was reflected in calculated stoichiometric CH_4VFA. Between-cow variation and repeatability of CH_4VFA were very low (0.010 and 0.10, respectively), suggesting that rumen fermentation does not markedly contribute to between animal variation in CH_4 emissions. Total VFA concentration was more repeatable (0.48) than molar proportions of individual VFA.

Organic matter digestibility (OMD) was within the expected range for good quality grass silages (740 ± 39.9 g/kg). Between-cow variability in OMD and NDF digestibility (NDFD) was highly significant (P<0.001), but rather small (13 and 23 g/kg, respectively). For digestibility variables, the variance component Diet(Exp) was the largest source of variation. Digestibility variables had medium repeatability (Rep = 0.37 and 0.26 for OMD and NDFD, respectively). The contribution of Cow(Exp) variance component to the observed variation for both the OM and NDF pools per kg of BW was higher

than the observed for both fibre digestion and passage rate, and the same trend was observed for repeatability values (rumen pools >0.70).

Differences in rumen ammonia N (RAN) concentration were mainly related to differences in protein concentration and sources across the diets. Between-cow variation in RAN was of greater magnitude (CV = 0.149) than the variation in other N metabolism variables. However, repeatability for RAN was similar to the efficiency of microbial N synthesis per kg of OM truly digested in the rumen (OMTDR; Rep = 0.35 and 0.34, respectively). In general, between-cow variability and repeatability of variables related to ruminal N metabolism were greater than those related to rumen VFA pattern and diet digestibility.

Although *in vivo* CH_4 estimates based on total predicted CH_4 and those obtained by empirical equations were rather similar to each other (on average 392 g/day), the variation in total CH_4 production was around two-fold (CV = 0.28) higher than observed for the empirical models evaluated. Random variation in OM digested in the rumen across diets and studies could have contributed to this. Methane yield was less variable than predictions based on total CH_4 emissions. The estimated CV values for total CH_4 and CH_4 yield based on stoichiometric calculations were similar to those observed in respiration chambers.

Stoichiometric CH_4VFA increased (P<0.01) with increased OMD. For each unit (g/kg) increase in OMD, CH_4VFA decreased by 0.06 mmol/mol VFA. The variation in OMD was closely related to the variation in NDFD and it was reflected in the positive relationship (P<0.01) with digestion rate of potentially digestible NDF (pdNDF). Organic matter digestibility increased as RAN increased (P<0.01), but it was negatively (P<0.01) associated with microbial N flow and the efficiency of microbial N synthesis. Rumen ammonia N decreased (P<0.01) with increased passage rate of iNDF and molar proportion of propionate. In addition, RAN concentrations were positively associated with molar proportions of branched-chain VFA (BCVFA) in the rumen (P≤0.01) (models not shown). Overall, the effects of digestion and fermentation variables were additive.

4.3 Paper III

Differences between diets in which late-harvested grass silage (LS) and barley were replaced by 0% early-harvested grass silage (ES) (L diet) and by 100% early-harvested grass silage (E diet) in terms of dietary concentrations of digestible organic matter (DOM) and metabolisable energy (ME) were as expected, a consequence of different harvesting times. However, they were

slightly lower than expected for both silages, probably due to exceptionally warm weather conditions during early summer. In both cases, silage fermentation quality was good, as indicated by low pH and ammonia-N concentrations.

The main differences in diet composition between treatments were related to decreases in starch and increases in pdNDF supply due to graded addition of ES in the diet. Dry matter intake decreased linearly ($P<0.01$) with increasing proportion of early-harvested grass silage in the diet from 22.6 to 19.3 kg/day, whereas digestibility of nutrients increased linearly ($P<0.01$). The greatest numerical differences in digestibility between the L diet (0% ES) and the E diet (100% ES) were observed for NDF and CP, which accounted for 122 and 100 g/kg, respectively. The higher digestibility observed E diet was consistent with reduced faecal output of nutrients.

Decreased concentrate supplementation in the E diet did not have effect on milk or ECM yield, except for milk protein which decreased linearly ($P<0.01$). Milk fat concentration increased and protein concentration decreased with increased proportion of early-harvested silage in the diet. The concentration of milk urea N (MUN) increased linearly ($P<0.01$) from 9.7 to 11.9 mg/dL for diet L and diet E respectively, but milk N efficiency was not influenced by the diet. Total CH_4 and CO_2 production and gas emissions per kg of ECM were not influenced ($P>0.01$) by the addition of early-harvested silage in the diet, but CH_4 yield increased linearly ($P<0.01$). This reflected differences in DMI and OMD, and probably the composition of digested OM, among treatments. Greater faecal output (g/kg DMI) of potentially digestible nutrients (NDS + pdNDF) for diet L compared with diet E could counterbalance the reduced enteric CH_4 yield by increasing the potential for CH_4 emissions from manure, since more substrate is available for fermentation.

4.4 Paper IV

Overall, intake and milk production responses and total tract digestibility in rumen-cannulated cows were consistent with observed trends described in **Paper III**. Differences in rumen fermentation pattern between the diets were only detected for the molar proportions of isovalerate and valerate, which decreased linearly ($P\leq0.03$) when the proportion of early-harvested silage increased in the diet. Stoichiometric CH_4VFA molar concentration was not influenced by the dietary composition ($P>0.10$), which is consistent with observations for the major volatile fatty acids, indicating that rumen fermentation did not contribute to higher CH_4 yield with increased proportion of ES in **Paper III**. Both apparent and true ruminal OM digestibility increased

linearly ($P \leq 0.04$) with graded addition of ES silage in the diet and it was consistent with linear increases ($P < 0.01$) for both ruminal and total digestibility of NDF and pdNDF. Organic matter and NDF flows into the omasum were reduced ($P < 0.01$) in response to decreased intake of these nutrients in diets that included early-harvested silage.

There were no differences between treatments in terms of N intake or the flow of different N fractions into the omasum ($P > 0.05$), but the flow of feed N into the omasum tended to decrease ($P = 0.08$) as the inclusion rate of ES in the diet increased. The ruminal N degradability and the total tract N digestibility increased linearly ($P \leq 0.04$) with increased proportions of ES in the diet. Decreased ^{15}N enrichment ($P = 0.03$) of rumen bacteria was observed in diets containing early-harvested silage. Increased inclusion rate of ES in the diet resulted in linear decreases ($P < 0.05$) in both NDF and pdNDF pool sizes and this was reflected in faster ruminal turnover time of NDF, which decreased linearly ($P < 0.01$) from 24.9 with the L diet (0% early-harvested silage) to 18.7 h with the E diet (100% early-harvested silage). No differences were observed between treatments in passage rate of iNDF and pdNDF, but intake and digestion rates of pdNDF linearly increased ($P < 0.01$) as the proportion of ES in the diet increased. Diet did not have any influence on ruminal *in situ* degradation of silage DM or NDF. Improved OMD, numerically lower efficiency of microbial and probably minor changes in the site of digestion could explain the increased CH_4 yield with increasing proportion of early-harvested silage in the diet.

5 Discussion

5.1 Measurement technique

5.1.1 Between-cow variability related to methods

The literature reports considerable between-animal variation in CH_4 production values across different measurement techniques. The influence of between-cow variation compromises the repeatability of CH_4 measurements and highlights the need for revising the particularities of each technique, in order to minimise confounding effects from undesirable sources of variation. Technical limitations of the methods used in measuring CH_4 production may explain why it has been difficult to obtain consistent rankings in CH_4 yields when animals are measured on multiple occasions (Vlaming *et al*., 2008).

In the past, respiratory chambers have provided the most accurate and reliable between-animal coefficient of variation (CV) for CH_4 production by farm animals compared with other techniques, due to the characteristics of the equipment itself, and also due to the possibility of applying stronger controls on the experimental animals and thus reducing variation in the CH_4 measurements obtained. Blaxter and Clapperton (1965), using standard respiration chambers, reported 7-8% between-animal variation in CH_4 production, whereas studies conducted by Grainger *et al*. (2007) and Muñoz *et al*. (2012) using the SF_6 tracer technique reported greater between-cow CV (16.4 and 19.3 %, respectively). Compared with respiration chambers and SF_6 methods, studies using sniffer methods have reported even larger between-cow variation, *e.g*. Garnsworthy *et al*. (2012a) observed considerable variation between cows in their CH_4 emission rate index (mg CH_4/min) during milking (CV = 33%). A study by Bell *et al*. (2014) using a similar sniffer system on 21 commercial farms (n = 1964 cows) showed that the extent of between-cow CV for the CH_4 production index varied from 22 to 67 % within farms. In addition, there were six-fold differences between the farms reported in the study by Bell

et al. (2014), which might indicate difficulties in harmonisation of the sniffer method between farms. Although dietary and animal factors could contribute to variation in CH_4 measurements, such differences between dairy farms are not possible according to our current knowledge of factors influencing CH_4 production Overall, the between-cow variability observed for the sniffer methods within farms was much higher (17%) than reported in the dataset by Yan *et al*. (2010) obtained from respiration chamber studies (n = 579 cows), despite the large variability in individual animal and diet factors (BW: 379-733 kg; DMI: 7.5-25.0 kg/d; forage proportion: 18-100% of diet DM, NDF: 265-604 g/kg DM). Therefore, it is likely that high CV values for a group of animals in the same house, fed the same diet, reflect random error in measurements rather than true between-animal variation.

In addition to the between-cow variation in CH_4 production, it is also important to consider the variation in the CH_4/CO_2 ratio, which is far from being a constant value. In a dataset derived from studies conducted with dairy cows in respiration chambers fed a wide range of diets (n = 157 observations; 30 diets), the CV of the CH_4/CO_2 ratio was 0.095 (Hellwing *et al*., 2013). However, much greater variation in CH_4/CO_2 ratio (~0.15-0.20) has been reported with the sniffer method (Lassen *et al*., 2012; Lassen and Løvendahl, 2013; Haque *et al*., 2014).

Variation in methane production with the GreenFeed system

The extent of between-cow variation and repeatability for CH_4 production (g/day) in studies conducted with dairy cows at Röbäcksdalen Research Centre using the GreenFeed system is presented in Table 3.

The data in the Table 3 were taken from studies using either a Latin square, cyclic change-over or switch-back design. The diets used in these experiments were based on grass and can be considered representative of typical dairy cow diets in the Nordic countries, with mean forage to concentrate ratio of 40-45% on a DM basis. The concentrate supplements consisted principally of cereal grains, some fibrous by-products from the food industry and protein supplements, typically rapeseed meal. In all studies, the cows were fed a total mixed ration *ad libitum*. Mean and residual standard deviation (SD) values for CH_4 production were obtained from least squares (LS) means for cows using the general linear model procedure, while repeatability was assessed by the covariance test using the mixed procedure of SAS (SAS Institute, 2008). The use of a mixed model allowed the effects of diet and period to be removed, and therefore only between-animal differences were considered. Total CH_4 production was on average 435 g/day, in line with GreenFeed-measured data for dairy cows reported in the literature (Dorich *et al*., 2015; Gidlund *et al*.,

2015; Hristov *et al*., 2015a). Assuming a constant value of 18.5 MJ gross energy (GE) in DM in the forage, an estimated 6.5% of GE was lost in CH_4. This is in line with values reported by Yan *et al*. (2000; 2010) for cows in respiration chambers fed similar grass silage-based diets. In this thesis, the extent of between-cow variation (0.107) was higher than the residual variation, which in turn reflected the high repeatability of the GreenFeed technique in farm conditions (0.69) despite the contrasting dietary conditions across experiments. Average repeatability across experiments (Table 1) was consistent with repeatabilities presented in a previous report (>0.70; Huhtanen *et al*., 2013).

Table 3. *Total methane (CH_4) production (g/day) in dairy cows and its variation across different experiments conducted at Röbäcksdalen Research Centre (Umeå, Sweden) from 2012 to 2016 using the GreenFeed system*

Study	Exp. Design	Diet	N	Per.	Mean, g/d	SD	CV	Res SD	Res CV[1]	Rep[2]
1	Latin square	Forage	20	4	405	36.4	0.090	21.8	0.054	0.717
2	Latin square	Forage	30	5	421	34.2	0.081	29.3	0.070	0.529
3	Latin square	Straw	16	4	419	48.8	0.116	37.3	0.084	0.597
4	Switch-back	Concentrate	16	3	451	53.0	0.118	22.1	0.049	0.844
5	Cyclic-change over	For. x Conc.	16	4	443	48.8	0.110	37.3	0.084	0.597
6	Latin square	Oats	16	4	454	54.7	0.121	21.7	0.048	0.860
7	Cyclic change-over	Protein	29	3	455	44.4	0.098	24.3	0.054	0.755
8	Cyclic change-over	Protein	24	3	395	36.5	0.092	31.1	0.079	0.512
9	Cyclic change-over	Protein	25	2	453	48.5	0.107	30.2	0.066	0.654
10	Switch-back	Glycerol	22	3	452	60.1	0.133	27.6	0.061	0.814

[1]Residual coefficient of variation.
[2]Proportion of significant correlation coefficient (P<0.05).

The average between-cow CV for CH_4 production across different experiments using the GreenFeed system at Röbäcksdalen Research Centre (Umeå, Sweden) was greater (0.107) than observed in carefully conducted respiration chamber studies (0.072) (Blaxter and Clapperton, 1965), but even lower to values observed with respiration chambers in studies conducted more recently (0.178) (Grainger *et al*., 2007). In a recent review, Hammond *et al.* (2016) concluded that published GreenFeed estimates of daily CH_4 production (mean values) are in agreement with those measured in respiration chambers. In previous studies, those authors made direct comparisons between GreenFeed and respiration chamber techniques with growing (Hammond *et al*., 2015) and dairy cattle (Hammond *et al.*, 2016b). Since the principle of the techniques is different, comparison between the techniques using the same animals at the same time is not possible. In the dairy cow study, two separate experiments

evaluating the same diets were conducted simultaneously, to compare the measurement technique for CH_4 production: experiment 1 used a randomised block design for GreenFeed (40 cows) and experiment 2 used a Latin square design for respiration chambers (four cows) (Hammond *et al.*, 2016b). Both techniques were able to detect similar dietary treatment effects, despite differences in intake between studies, but the magnitude of the differences was considerably different, *e.g.* 24% lower in experiment 1 using GreenFeed and 8% lower in experiment 2 using respiration chambers for maize silage compared with grass silage as the only source of forage in the diet (Hammond *et al.*, 2016b). In the growing cattle study (Hammond *et al*., 2015), the GreenFeed system provided an average estimate of CH_4 production that was not different from respiration chamber measurements using the same experimental animals. However, GreenFeed and respiration chamber techniques showed poor agreement. According to those authors, this is partly attributable to the relatively small number of visits recorded by the GreenFeed method. Arthur *et al*. (2017) recommend a minimum of 30 flux records, with each record obtained from a minimum GreenFeed visit duration of 3 minutes. As a spot-sampling technique, GreenFeed relies on the number of animal visits during the day, whereas respiration chamber measurements are based on integrated measurements at specific time intervals according to the system set-up.

Thus, for the GreenFeed system, the greater the number of visits recorded, the better the accuracy of the measurements. Results from **Paper III** (study 5 in Table 3), showed that the diurnal pattern in CH_4 production recorded by the GreenFeed system did not differ across diets and there was no clear diurnal pattern (Figure 9). Conversely, in the study by Hammond *et al*. (2015) there were some diurnal variations in number of visits.

An indirect comparison was performed by Huhtanen *et al*. (2016), by comparing CH_4 measured with the GreenFeed system in cattle with model-predicted (six models) CH_4 production. Mean CH_4 production estimated by GreenFeed was close to values predicted by models developed from respiration chamber data (386 and 384 g/day, respectively). However, it was much higher than that predicted by the model proposed by Ellis *et al*. (2007), which was developed from data determined by different techniques (386 and 294 g/day, respectively). Root mean square prediction error (RMSPE) ranged from 6.0 to 8.9 % of observed mean for models developed from the respiration chamber data. Huhtanen *et al*. (2016) concluded that CH_4 production estimated by the GreenFeed system is consistent with values predicted by models derived from large datasets from respiration chamber studies.

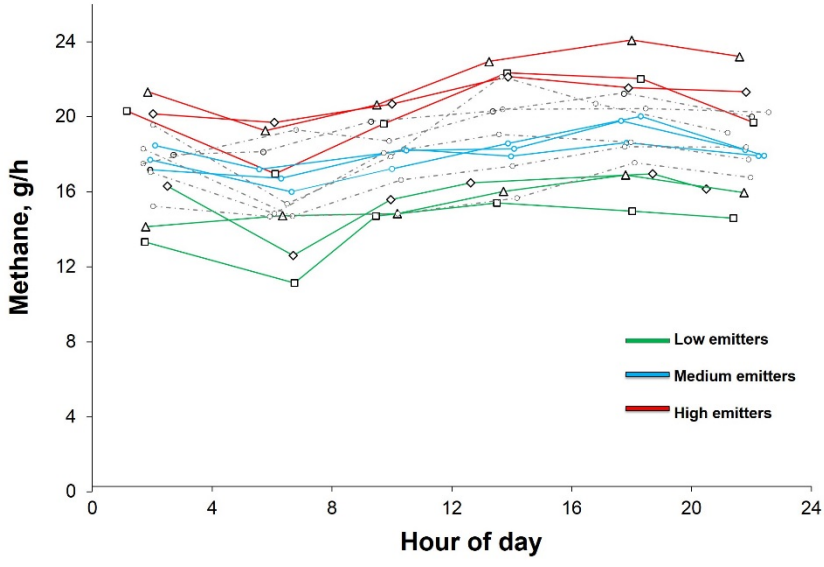

Figure 9. Mean diurnal pattern of methane (CH_4) production observed in dairy cows (n=15) using the GreenFeed system (**Paper III)**. One cow was excluded owing to insufficient data.

Residual CV in general linear model analysis was on average 0.065 (range 0.048-0.084). Considering that 20 cows can be measured in one GreenFeed unit in normal conditions, the probability of detecting biologically meaningful differences in change-over studies is rather high. For example, in quadruplicated 4 × 4 Latin square studies with a 2 × 2 factorial arrangement, the probability of detecting differences (P<0.05) of 10, 7.5 and 5% between main factors (n = 32) was >99, 93 and 61 %, respectively, for the highest observed residual CV (0.084) obtained here. The corresponding probability using the mean residual CV (0.065) was >99, 99 and 83 %, respectively.

GreenFeed compared with other methods to measure methane production

The GreenFeed technique has recently been compared with the SF_6 tracer technique. Dorich *et al*. (2015) performed a direct comparison between the two techniques by measuring CH_4 production in dairy cows with the same diet (52:48 % on DM basis) fed either *ad libitum* or restricted feed to 90% of the baseline DMI (cross-over design). The results showed that the SF_6 tracer method produces larger CV than obtained by the GreenFeed system, despite the average values being virtually the same (468 and 467 g/day for GreenFeed and SF_6, respectively). When outliers were removed from the SF_6 data, mean value decreased to 405 g/day but, although variation was substantially reduced, it still remained considerably higher than for GreenFeed data (CV = 0.41 and

0.22 for SF_6 and GreenFeed, respectively). A moderately strong relationship between CH_4 production and DMI was observed for the GreenFeed system (R^2 = 0.42) and a weak relationship for the SF_6 technique (R^2 = 0.17). Dorich *et al.* (2015) attributed the higher variability in the SF_6 measurements to the high concentration of background gases, as a result of poor house ventilation in indoor conditions. This is in agreement with findings by Hristov *et al.* (2016) that correlation and concordance between the two methods are relatively low. In addition, the difference between the methods was not consistent over time, most likely influenced by house ventilation and background methane and SF_6 concentrations. One major requirement for any tracer gas technique is that the background concentrations in the environment should be low relative to the concentration of the tracer in the samples collected (Berndt *et al*., 2014).

Paper I compared the GreenFeed system (flux method) with a modification of the original system set-up, in order to mimic the mechanism of the sniffer technique based on analysis of gas concentrations (Garnsworthy *et al*., 2012a). Details of the experimental conditions applied in **Paper I** can be found in the material and methods section of this thesis. Between-cow coefficient of variation in CH_4 was smaller for the GreenFeed system (range 0.11-0.18) than for the sniffer technique (range 0.18-0.28). Although the repeatability of the measurements by both methods was high for CH_4 production ($\geq$ 0.72) and CH_4/CO_2 ratio ($\geq$ 0.59), the relationship between the CH_4 concentration (ppm) determined by the sniffer method and CH_4 production (g/d) determined by the GreenFeed method was rather poor, as indicated by the low coefficient of determination of the linear regression (R^2 = 0.09). In contrast, Garnsworthy *et al.* (2012a) reported a good relationship between methane emission index measured by the sniffer method and respiration chambers.

In **Paper I**, CH_4 values from the GreenFeed system were strongly related either to DMI (experiment 1) or BW (experiment 2), whereas for the sniffer method no significant relationships were observed for these variables. Similarly, Garnsworthy *et al.* (2012a,b) and Bell *et al.* (2014) reported that increased DMI was poorly associated with increases in CH_4 emission rate. Since DMI is the main driver determining CH_4 production in ruminants, as determined in large datasets derived from respiration chamber studies (Yan *et al*., 2000; Ramin and Huhtanen, 2013), sniffer values lack biological value in terms of animal physiology mechanisms related to CH_4 production. In addition, in both the study by Garnsworthy *et al.* (2012a) and **Paper I,** the intercepts of regressions predicting fluxes from CH_4 concentrations were highly positive. Theoretically this is not possible (positive flux at zero concentration) and it most likely reflects random variation in the gas concentration measurements. High repeatability in both CH_4 concentrations and CH_4/CO_2 ratio for the sniffer

method were at least partly associated with greater variability of the data and not necessarily the accuracy of the technique. Results from both laboratory and on-farm studies indicated that, for the sniffer method, the muzzle distance from the sampling point is a critical factor in determining the concentrations. Indeed, the repeatability of muzzle position was as high as 0.82 for experiment 2 in **Paper I**. This may seem surprising, but different characteristics of animal behaviour can be highly repeatable (*e.g.* Napolitano *et al*., 2005). In experiment 1 in **Paper I**, repeatability of number of visits was also highly variable (0.50-0.68). In addition to the muzzle position, differences in manifold geometry and number of cow visits between concentrate feeders and automatic milk stations seemed to contribute to larger variation in CH_4 and CO_2 concentrations in the sniffer method in experiment 2, which were similar to values reported by de Haas *et al*. (2013). From an image of a cow breathing (Figure 10), it is clear that exhaled air goes in two directions at a near 90-degree angle and that small changes in head position can influence measured CH_4 concentrations by the sniffer method. Therefore, the combined effects of the smaller head-box for concentrate feeders attached to the GreenFeed unit compared with the automatic milking system and the muzzle position may add non-accounted variation to the predictions.

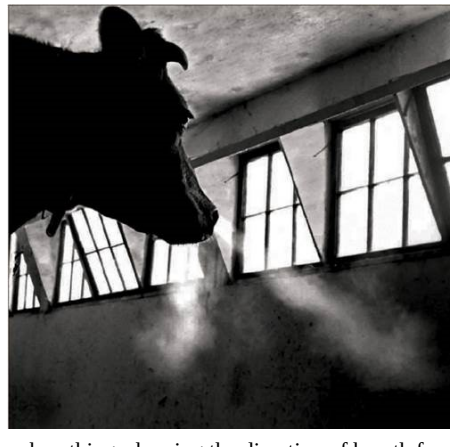

Figure 10. Image of a cow breathing, showing the direction of breath from each nostril. http://1.bp.blogspot.com/_6Zl8x3ZGFMY/TQTIr9xDjXI/AAAAAAAAAG48/05AQZdFZFHI/s1600/cow_breath.jpg (Accessed 8 May, 2014).

Overall, **Paper I** showed that CH_4 measured by the sniffer method is a poor indicator for ranking animals for selection purposes, whereas GreenFeed showed more realistic results in terms of CV and goods agreement with respiration chamber data, both in direct and indirect comparisons.

5.1.2 CH_4/CO_2 ratio

Carbon dioxide comes from fermentation of the feed in the gastrointestinal tract and also from tissue mobilisation, whereas CH_4 can only be produced from enteric fermentation in the rumen and to a limited extent in the hindgut. However, CO_2 and CH_4 production are positively associated because both are highly correlated with DMI (Pinares-Patiño *et al*., 2007). Therefore it can be expected that the CH_4/CO_2 ratio is not constant. In a dataset derived from respiration chamber studies conducted with dairy cows (157 observations, 30 diets), the CH_4/CO_2 ratio varied between 0.053 and 0.105 (Hellwing *et al*., 2013). However, in **Paper III,** which compared the effects of graded replacement of late-harvested and early-harvested grass silage and barley, the CH_4/CO_2 ratio (g/kg) was rather constant (33.2-34.2), despite the large differences in dietary carbohydrate composition. Because both ECM yield and BW were similar between the diets, calculated CO_2 production was also similar. In **Paper III**, moderate relationships were observed between the CH_4/CO_2 ratio ($R^2 = 0.56$) or CH_4 production ($R^2 = 0.57$) predicted according to Madsen *et al*. (2010) and observed CH_4 production, but predicted values were on average 11% lower. However, it should be noted that the CH_4/CO_2 ratio was measured by the GreenFeed mode that resulted in much better relationship between gas ratio and CH_4 flux than the gas ratio measured in the sniffer mode. In **Paper I**, the CH_4/CO_2 ratio (ppm/ppm) was 0.107 and 0.088 for the GreenFeed method and 0.094 and 0.100 for the sniffer method in experiment 1 and 2, respectively. In that study, the better relationship between CH_4/CO_2 ratio and CH_4 flux than the corresponding relationship between CH_4 concentration and flux suggests that CH_4/CO_2 ratio could be more useful in ranking cows as emitters than CH_4 concentration, as is the case for the sniffer method.

The problem with CH_4/CO_2 is that it can be influenced by the CH_4 and the CO_2 concentrations, both of which can vary because of different biological mechanisms. High CH_4/CO_2 ratio can result from increased CH_4 production as a consequence of increased DMI and/or high CH_4 yield and from improved feed efficiency due to reduced CO_2 production per unit intake as the result of allocation to both milk and body tissues. Conversely, in addition to low CH_4 emissions, low CH_4/CO_2 ratio can also result from mobilisation of body tissues, which produces CO_2 but not CH_4.

Monte Carlo simulation

One problem with using CH_4/CO_2 and estimated CO_2 production for predicting total CH_4 production is that it is not known whether the gas ratio changes due to increased CH_4, decreased CO_2 or both. To evaluate this, a Monte Carlo

simulation study was conducted to evaluate the effects of efficiency of ME utilisation on predictions of total CH_4 production. The following default values were used: DMI 20 kg/d (CV = 2.4 kg), CH_4 production was adjusted for differences in DMI (-0.35 g per kg/DMI deviation from 20 kg/day; Ramin and Huhtanen, 2013); a value of 0.10 was used for CV in CH_4 production and 11.5 MJ/kg DM for dietary ME concentration; a constant value of 70 MJ/d was assumed for maintenance heat production; yield of ECM was calculated as 0.62 × (ME intake – ME for maintenance)/3.14 (MJ/kg ECM); and total estimated heat production (MJ/d) was calculated as 70 + ECM yield (kg/d) × 1.92 MJ/kg (3.14 / 0.62 – 3.14). A herd of 100 cows was simulated 1000 times assuming CV values of 0.06, 0.08 and 0.10 for k_l (efficiency of ME utilisation for lactation above maintenance; default = 0.62). The correlation between DMI and dietary ME concentration and between CH_4 production and the efficiency of ME utilisation was assumed to be 0. Irrespective of CV for k_l, the efficiency of ME utilisation for lactation was consistently negatively related to residual CH_4 production (observed – predicted), *i.e.* CH_4 production estimated from CH_4/CO_2, and predicted CO_2 production was overestimated with improvements in feed efficiency (FE; Table 4).

The greater the variation in k_l, the stronger the negative relationship between FE and residual CH_4 production was. With no or small (*e.g.* CV = 0.04) variability in k_l, the CH_4/CO_2 ratio was not associated with FE (results not shown), but with a CV value of 0.06 for the correlation between CH_4/CO_2 and FE, the probability of a positive correction coefficient was 0.907 and the probability of a significantly positive ($r > 0.165$) correlation was 0.369 (Table 4). With higher CV values in FE (0.08 and 0.10), the probability of a significant relationship between CH_4/CO_2 and FE was high. Examples of the simulation results representing average correlations (CV = 0.08) are shown in Figure 11. The CH_4/CO_2 ratio was positively related to predicted CH_4 prediction (Figure 11). The relationships were broadly similar to *in vivo* data (**Paper I**) when the measurements were based on the flux method ($R^2 = 0.55$ and 0.23 in experiment 1 and 2, respectively), indicating that default values and variations used in the simulation were relevant. However, the relationships between gas ratio and CH_4 flux were weaker ($R^2 = 0.27$ and 0.09) when the CH_4/CO_2 ratio was measured with the sniffer method (**Paper I**). The results of this simulation indicate that care should be exercised when applying predictions of CH_4 production based on determined CH_4/CO_2 ratio and predicted CO_2 production in breeding programmes, since it may favour cows with low FE.

Table 4. *The effects of the variability in the efficiency of ME utilisation to milk production on the relationships between feed efficiency (FE) and residual of CH_4 production (default observed – predicted from gas ratio and CO2 production) and between gas ratio /CH_4/CO_2) and feed efficiency (kg ECM/kg DMI)*

CV of k_l[1]	Relationship	Mean r	Min r	Max r	$r^2 > 0$	*P*-value[3] <0.05
0.06	FE vs. CH_4 Res	-0.751	-0.866	-0.549	1.000	1.000
	CH_4/CO_2 vs. FE	0.129	-0.265	0.498	0.907	0.369
0.08	FE vs. CH_4 Res	-0.828	-0.905	-0.699	1.000	1.000
	CH_4/CO_2 vs. FE	0.285	-0.042	0.600	0.996	0.898
0.10	FE vs. CH_4 Res	-0.875	-0.939	-0.753	1.000	1.000
	CH_4/CO_2 vs. FE	0.410	0.077	0.697	1.000	0.997

[1]k_l = Efficiency of ME used for lactation.
[2]Proportion of correlation coefficient < 0 (FE vs. CH_4 Residual) or >0 (Ratio vs. FE).
[3]Proportion of significant correlation coefficient (*P*<0.05) in 1000 simulations.

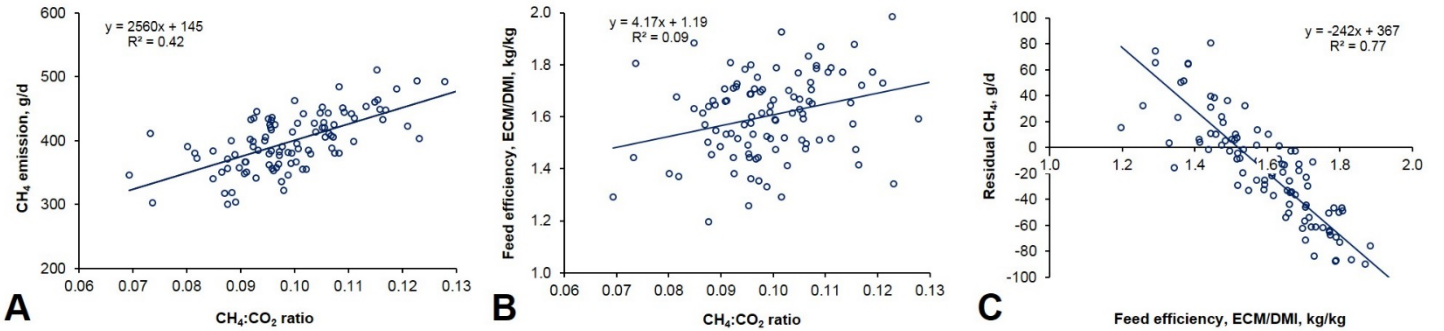

Figure 11. An example of simulation results for a 100 cow herd when CV of efficiency of ME utilisation was assumed to be 0.08. A) Methane (CH_4) emissions as a function of CH_4/CO_2 ratio, B) Feed efficiency as a function of CH_4/CO_2 ratio and C) Residual of CH_4 emissions as a function of feed efficiency.

5.2 Animal-related factors

Evidence from several studies indicates that CH_4 production in cattle is partly under genetic control, and therefore it could be possible to decrease CH_4 production through genetic selection for low-emitting animals (de Haas *et al*., 2011; Pickering *et al*., 2015; Negussie *et al*., 2017). Indeed, studies conducted in sheep (Pinares-Patiño *et al*., 2013) and Dutch dairy cows (de Haas *et al*. 2011) have shown medium heritability values for total CH_4 production (0.29 and 0.35, respectively). In the long run, the success of strategies to mitigate CH_4 production in cattle will rely on how the mitigation target is defined and the implications of the chosen variable in practice. Animal breeders have defined four different CH_4 phenotypes for genetic selection purposes that have been widely used during the past decade (de Haas *et al*., 2017; Negussie *et al*., 2017).

From a general perspective, total CH_4 production (CH_4, L/day, g/day or MJ/day) is the clean trait that animal breeders want to improve in terms of CH_4 mitigation strategies in ruminants (de Haas *et al*., 2017). However, as mentioned before, total CH_4 production is strongly positively correlated with DMI or gross energy intake (Yan *et al*., 2000; Ramin and Huhtanen, 2013; Hristov et *al*., 2013). One of the limitations of considering CH_4 production as an isolated mitigation target is that it is a poor indicator of the efficiency of utilising dietary energy, and thus lacks economic value. In addition, reliable measurement of CH_4 production based on DMI by individual animals still represents a major challenge in large-scale practical farming.

Efforts aimed at reducing CH_4 production from ruminants should also consider reducing CH_4 production per unit of edible product. For meat-type animals, CH_4 intensity (CH_4/unit of edible product, g/kg) is usually measured in terms of kg of BW or carcass gain. In dairy cattle, by default, it is quantified in terms of g CH_4 per kg of milk, or preferentially per kg energy-corrected milk (ECM). Methane intensity is mostly influenced by milk production level in dairy cows and BW gain (gr/day) in growing animals. In addition, BW influences CH_4 intensity via CH_4 produced from maintenance feed. At individual animal level, this means that the total energy requirement per kg of milk produced is reduced by dilution of the energy requirement for maintenance and hence the cows are more efficient in feed conversion. In a global perspective, CH_4 intensity should be considered a target to mitigate production considering that the demand for ruminant products (beef, milk) is likely to increase in the future. This is the case especially in tropical countries, where there is great potential for substantial reductions in CH_4 production in

the near future by improving management and nutrition of the animals (FAO, 2016).

Methane yield (CH_4/DMI, g/kg or proportion of GEI) describes the arithmetic ratio between daily CH_4 production (output) related to the DMI or GEI (input) per animal. Therefore, this criterion is important in understanding the mechanism of digestive physiology and rumen microbiology involved in enteric CH_4 production. While CH_4 yield may better explain the biological mechanisms involved in CH_4 production among CH_4 phenotypes, the use of ratio traits has been criticised by animal breeders, as the genetic parameters may not truly represent the trait under consideration, because there is always extra variability of the denominator trait (Pickering *et al*., 2015). As an alternative to overcome this issue, calculation of residual CH_4 (observed minus predicted CH_4 production) has been suggested, based on its advantages in terms of statistical properties (de Haas *et al*., 2017). In a large dataset (n = 1000) of dairy cows across Europe fed the same diet within-farm (RuminOmics EU project), both high and low CH_4 emitters were ranked according to residual between observed and predicted CH_4 production taking into account the effects of DMI, BW and period (takes into account possible variation in diet composition within herd). However, the use of residual CH_4 on-farm conditions still remains unpractical because DMI cannot be determined for individual animals.

Methane production expressed in terms of CH_4 yield is probably the most appropriate CH_4 trait in order to understand biological mechanisms involved in between-animal variation in CH_4 production in experimental conditions. However, difficulties in measuring DMI limit its use on-farm conditions. As indicated before, total CH_4 production is mainly driven by DMI and CH_4 intensity by production level. Therefore, for the purposes of the present discussion, animal factors influencing between-cow variation in CH_4 production are addressed in terms of CH_4 yield. This trait allows integration in a more comprehensive manner of physiological mechanisms such as: rumen fermentation, passage rate, digestibility and ruminal N metabolism.

5.2.1 Effects of rumen fermentation pattern

Methane can only be produced from available substrate for enteric fermentation; in other words, its rate of production relies on the type of feed ingested by the animal. The amounts of specific VFAs produced in the rumen (*i.e*. acetate, propionate and butyrate) change depending on the diet. These VFAs are the major determinant of the amount of H_2 produced, and consequently CH_4 produced, in the rumen (Wolin, 1960; Czerkawski, 1986; Van Soest, 1994). Hydrogen production is a thermodynamically unfavourable

reaction, but methanogens scavenge H_2 and relieve this inhibition (Russell and Rychlik, 2001). Equations based on stoichiometric principles (Czerkawski, 1986; Van Soest, 1994) demonstrate that acetate and butyrate production promotes CH_4 production, whereas high propionate production acts as an H_2 sink and consequently reduces CH_4 production (Johnson and Johnson, 1995; Moss *et al*., 2000). In such a scenario, it is expected that increased proportion of concentrates in the diet reduces CH_4 production in cattle by promoting propionate fermentation in the rumen. In feed-lot type diets fed to growing beef cattle (>90% of concentrate in the diet on DM basis) substantial reductions in CH_4 production have been reported (Johnson and Johnson, 1995). However, within the common ranges used in dairy cow diets the effect of concentrate proportion on CH_4 energy losses is marginal until 59% and only tends to decrease at 70% of concentrate supplementation (Ferris *et al*., 1999). Methane yield is 3% of GE intake of a grain ration and 6% of a roughage ration (Johnson *et al*., 1995). Jonker *et al*. (2016) performed a study in sheep in order to evaluate the effects of graded substitution of lucerne silage with maize silage or maize grain in a diet fed at a fixed DMI level (2% of BW) on rumen fermentation characteristics in both *in vivo* and *in vitro* conditions. A quadratic effect was observed for both supplements on CH_4 yield, to a maximum at 50%, and thereafter it decreased more rapidly for the maize grain supplement. Ruminal fermentation pattern was significantly related to CH_4 yield with the ratio of (acetate + butyrate) / (propionate + valerate) and the propionate concentration alone being the best single predictor of CH_4 yield (Jonker *et al*., 2016).

In the meta-analysis approach by Ramin and Huhtanen (2013), mixed regression equations were developed for predicting CH_4 yield (CH_4-E/GE; kJ/MJ) in ruminants. Dry matter intake as a proportion of BW, OMD at maintenance level and dietary fat concentration were the major factors predicting CH_4-E/GE. When rumen fermentation pattern was determined, the effect of CH_4VFA on CH_4 yield was significant and it was the best predictor among VFA measurements. Regression coefficient of CH_4VFA on CH_4 yield was close to that expected from stoichiometric relationships.

In order to demonstrate the relationship between CH_4VFA and CH_4 yield, primary data were taken from a study conducted by Kittelmann *et al*. (2014) on 118 low- and high-CH_4 emitting sheep selected from a group of 340 animals (Figure 12). The sheep were fed the same diet (2.2 times maintenance). Daily CH_4 production from individual animals was measured in respiration chambers (2-3 days) and rumen fluid samples were taken by stomach tube before feeding. To calculate stoichiometric CH_4VFA from acetate to propionate ratio (AP) from Kittelmann *et al*. (2014) dataset, the molar proportion of butyrate

was estimated using the relationship between AP and butyrate from the dataset in **Paper II**. In that study, rumen fermentation pattern and CH_4 yield in respiration chambers (2-3 days) from individual animals were measured on two occasions. There was a positive (P<0.001) relationship between CH_4VFA and CH_4 yield (Figure 12). Two approaches were taken into account to calculate stoichiometric CH_4VFA (x-axis) as follows: 'observed' refers to relationship based on reported VFA values and 'predicted' is based on expected CH_4 production assuming only changes in the molar proportions of major VFAs, but constant amount of fermentable substrate, without considering other animal or dietary factors. For each unit increase in CH_4VFA, CH_4 yield increased by 0.053 and 0.037 g/kg DMI for 'observed' and 'predicted', respectively.

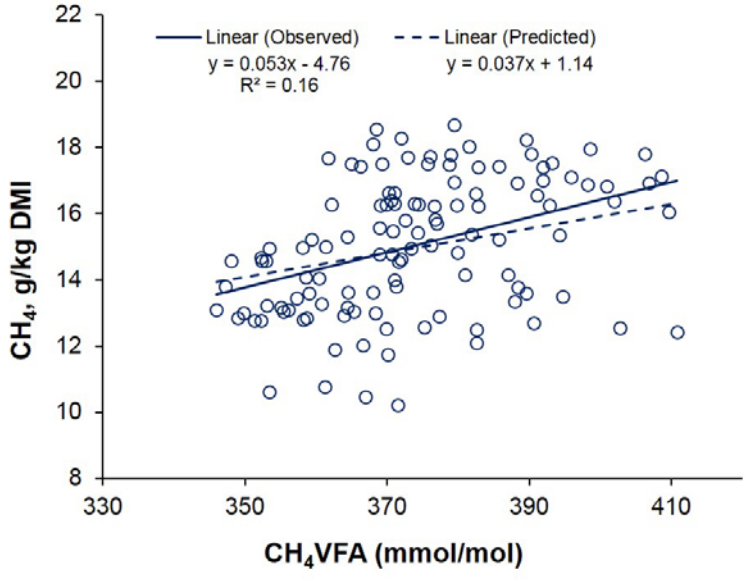

Figure 12. Relationship between stoichiometric CH_4VFA (Wolin, 1960) and observed CH_4 yield in sheep (n = 118 animals) fed a standard lucerne pellet diet (19% CP, 43% NDF and 10 MJ ME/kg DM) at 2.2 times the maintenance ME requirement (CSIRO, 2007). Calculated from Kittelmann *et al*., (2014; supplementary data). Reproduced with the author's permission.

Differences between observed and predicted CH_4VFA responses in observed CH_4 yield can be related to improved diet digestibility, as the positive relationship between these variables suggests (**Paper II**). Based on the relatively low coefficient of determination (R^2 = 0.16), although significant, this analysis demonstrated that rumen fermentation pattern explains a relatively small proportion of the variation in CH_4 yield in sheep. Use of the Wolin

equation (Wolin, 1960) to calculate CH_4VFA can be criticised mainly because it assumes that all fermented substrates are expressed in terms of hexose equivalents ($C_6H_{12}O_6$), and due to the fact that it does not take into account microbial cells as a H_2 sink (Czerkawski, 1986). However, this approach, based on stoichiometric principles, is still valid, since a major part of the substrate available for fermentation comes from dietary carbohydrates, which in turn are converted mostly to glucose units, and to the fact that acetate production is a major factor for CH_4 production in rumen fermentation conditions.

5.2.2 Variability and repeatability of volatile fatty acids in the rumen

In **Paper II**, low variation in rumen fermentation variables was observed when accounting for differences between diets across changeover studies in dairy cows. The CV for molar proportions of individual main VFAs ranged from 0.022 to 0.061. Among these, propionate and butyrate made similar contributions to the total variation in the Diet(Exp) variance component (CV = 0.055 and 0.061, respectively). The between-cow variation observed in CH_4VFA derived from fermentation pattern (**Paper II**) was very low (CV = 0.010). The CV in CH_4VFA calculated from Kittelmann *et al*., (2014) data was higher (0.033), but still rather low to account for the variation in observed CH_4 yield. However, differences in rumen fluid sample collection method could have influenced the variation. In most cases in **Paper II**, the samples were collected during a 12-h sampling period at 1.5 h intervals through a rumen cannula in dairy cows, whereas in Kittelmann *et al*. (2014) rumen fluid was taken once using a stomach tube in sheep. In that sheep study, the 118 animals used for rumen fluid collection were intentionally selected as high and low CH_4 emitters from a larger group of animals (340), which could have contributed to the higher CV values obtained compared with the dataset in **Paper II.** Results from the variance components analysis performed in **Paper II** basically confirmed findings obtained from analysis of data reported by Kittelmann *et al*. (2014) and models proposed by Ramin and Huhtanen *et al*. (2013). In **Paper II**, variance component analysis for major VFAs in terms of concentrations (mmol/L) was not performed, but they are included in Table 5.

Overall, comparisons between individual VFAs (Table 5) demonstrated that between-cow CV is a more important source of variation for VFA concentrations than for molar proportions of VFA. As a consequence, VFA concentrations display higher repeatability values. Among individual VFAs, butyrate concentration was more repeatable (Rep = 0.55) than acetate or propionate (Rep = 0.46 and 0.33, respectively). This implies that animal physiology factors such as passage rate or VFA absorption through the rumen wall may have a major impact on VFA concentrations, whereas molar

proportions of individual VFA reflect changes in fermentation pattern mainly as a consequence of the type of diet ingested by the cow. The data in **Paper II** suggested that that repeatability of VFA fermentation pattern decreased as a function of time, which may indicate changes in microbial population over time.

Table 5. *Variance components and repeatability estimates for major volatile fatty acids (VFAs) in rumen fermentation of dairy cows fed typical grass-silage based diets in the Nordic countries. For further details, see* ***Paper II***

		Variance component[1]				
		Diet		Cow		
VFA	units	SD	CV	SD	CV	Rep[2]
Acetate	mmol/L[3]	2.4	0.032	3.4	0.046	0.46
	mmol/mol[4]	14.9	0.022	7.4	0.011	0.28
Propionate	mmol/L	1.2	0.058	1.3	0.062	0.33
	mmol/mol	10.4	0.055	4.6	0.025	0.06
Butyrate	mmol/L	1.0	0.071	1.4	0.098	0.55
	mmol/mol	7.9	0.061	6.6	0.051	0.23

[1]Diet(Exp) = diet within experiment; Cow(Exp) = cow within experiment respectively.
[2]Rep = Repeatability calculated as Rep = δ^2 Cow / (δ^2 Cow + δ^2 Residual).
[3]Volatile fatty acids concentrations.
[4]Volatile fatty acids molar proportions.

Variation in VFA concentrations can be related to differences in bicarbonate secretion, saliva production, short chain fatty acid absorption and fluid passage rate out of the rumen. These factors are more likely related to animal physiology than rumen microbiome and may be partly genetically controlled. On the other hand, variations in VFA pattern are mainly related to diet composition and to a smaller extent variations in rumen microbiome. Variation in the physical structure and size of the rumen, as well as the intensity of contractions and rate of passage of digesta are all expected to have an influence on the rumen microbial community (Roehe *et al*., 2016).

Since microbial populations in the rumen act directly on the available substrate by modulating the rates of VFA production, it could be expected that the possible effects of the rumen microbiome on enteric CH_4 production would be also reflected in variation in the VFA fermentation pattern. However, the relatively small CV calculated in both studies does not support a major contribution of the rumen microbiome to between-animal CV in CH_4 yield. Different metabolic pathways in rumen fermentation pattern, *e.g*. acetogenesis, would weaken the relationship between CH_4VFA and CH_4 yield. Although

induction of rumen acetogenesis was proposed by Van Nevel and Demeyer (1995) as an interesting alternative to reduce CH_4 production in ruminants, all attempts to establish it have failed so far (Fievez *et al*., 1999). Because quantitative relationships between CH_4VFA and CH_4 yield were close to the theoretical potential both when analysed from treatment mean data (Ramin and Huhtanen, 2013) and from individual animal data from Kittelmann *et al*. (2014), any other major fermentation pathways are unlikely. Emissions of free H_2 can be an alternative fate of ruminal H_2 production, but with normal diets its contribution is likely to be minimal. Conversely, it could be high with halogenated hydrocarbons, the increase in hydrogen production is generally of a similar order of magnitude to the decrease in CH_4 production as discussed by Hristov *et al*. (2015a).

It seems that between-animal variability in CH_4 production and rumen fermentation pattern is greater for high concentrate diets than the estimated for forage diets or mixed diets. In a study by Roehe *et al*. (2016) with Aberdeen Angus and Limousine steers, average CV in total CH_4 was 0.166 and 0.283 and in CH_4 yield 0.180 and 0.263 for low and high concentrate diets, respectively. Similar differences between forage and concentrate diets were observed in a study by Herd *et al*. (2016) (0.125 and 0.217 in total CH_4 and 0.100 and 0.207 in CH_4 yield, respectively). Consistently, analysis of primary data from the study of Jaakkola and Huhtanen (1993) indicated increased between-animal variability in CH_4VFA with increased proportion of concentrate in the diet (CV: 0.016, 0.020 and 0.096 with 25, 50 and 75% concentrates on DM basis, respectively). In **Paper II**, it was not possible to estimate variance components separately for the low and high concentrate diets (average 41% on DM basis), but animal + residual variance was greater for high concentrate diets, indicating greater between-cow variation with high than low concentrate diets in rumen fermentation pattern.

Individual animal data from a study conducted with primiparous cows by Zhu *et al*. (2014) showed that the estimated repeatability was rather low for the main VFAs: acetate (0.03), propionate (0.18), and butyrate (0.03). The acetate: propionate ratio had slightly higher repeatability compared to individual VFAs. Moreover, a study by Robinson *et al*., (2010) showed repeatabilities in the magnitude of 0.20 for VFA profiles in 708 sheep. In the same study, phenotypic correlations between individual VFAs and short-term CH_4 production (1 h) were relatively low, ranging from 0.15-0.20. An experiment by Pinares-Patiño and Clark (2010) in lactating cows under grazing conditions showed very small animal variation in rumen fluid osmolarity (CV < 0.05), which may indicate little variation in rumen fermentation pattern.

It is important to note that in the studies by both Zhu *et al*. (2014) and Robinson *et al*. (2010), the rumen fluid samples were collected by stomach tubing, which could alter VFA concentrations due to saliva contamination (more diluted samples) compared with the rumen fluid samples used in the study by Pinares-Patiño and Clark (2010) and in **Paper II**, in which grab samples were collected directly through the rumen cannula. Another consideration is the effect of time of rumen fluid collection, which in addition to diet composition also influences the VFA production rate (Bergman, 1990). In **Paper II**, rumen fluid samples were taken on several occasions. Nevertheless, despite the differences in animals and experimental conditions, all the data found in different studies display a similar general trend, which indicates small variation in rumen fermentation pattern.

Overall, observed CV and repeatability in rumen fermentation pattern are much smaller than those in CH_4 production, suggesting that variations in the rumen microbiome are not likely be the major factor influencing between-animal variation in CH_4 production. Much smaller repeatability of CH_4VFA than CH_4 yield (0.20 compared with 0.59) calculated from the primary data published by Kittelmann *et al*. (2014) is in line with this suggestion. Repeatability in CH_4VFA is more 'time-dependent' than estimated repeatability for VFA concentrations.

The effects of microbiome could derive indirectly from differences in the physical structure and size of the rumen, while the intensity of contractions and rate of passage of digesta can also be expected to have an influence on the rumen microbial community, as suggested by Roehe *et al.* (2016). Therefore, there is a limited room to select low CH_4 emitters by taking into account only rumen fermentation pattern due to the rather small repeatability values, which in turn may compromise the accuracy of animal selection.

5.2.3 Passage rate and associated factors

In a study by Pinares-Patiño *et al*. (2003) on sheep, fractional passage rate (reciprocal of mean retention time; MRT) of particulate matter was strongly and negatively (R^2 = 0.57) related to CH_4 yield. More recently, Goopy *et al*. (2014) selected 10 low and 10 high emitters from 170 ewes and found that, over the measurement period, the difference between low and high groups in CH_4 yield was 2.7 g/kg DM intake. High emitters had 5.5 h longer particulate mean retention time (MRT). Particulate and fluid MRT explained 56% and 69% of the variation in CH_4 yield, respectively (Goopy *et al*. 2014). When expressed per hour difference in MRT, CH_4 yield (g/kg DM intake) declined by 0.48 and 0.49 g/h in the study by Pinares-Patiño *et al*. (2011) and Goopy *et*

al. (2014), respectively. A simulation study by Huhtanen *et al*. (2016) also showed a positive relationship between MRT and CH_4 production.

Increased retention time of rumen digesta is related to higher reticulorumen volume and consequently higher CH_4 production per unit intake can be expected. Higher OM rumen pool size was observed for high CH_4 yield emitters (7.4 L) compared with low CH_4 yield emitters (5.9 L) in a study by Goopy *et al*. (2014) conducted on ewes fed 1.2-fold the maintenance requirement. In an earlier study by Pinares-Patiño *et al*. (2003) in sheep at a fixed level of intake, a high and positive correlation between CH_4 production and rumen fill was observed (0.84). Robinson *et al*. (2010) suggested two main mechanisms which may explain reduced VFA concentrations at larger rumen volumes: i) with increased volume of rumen water VFA concentrations are diluted, and ii) changes in the absorptive surface area could reduce VFA absorption through the rumen wall.

It seems that the effects of MRT on CH_4 yield are similar when variation in MRT is related to increased feeding level of group of animals or between individual animals fed the same level of intake. According to Yan *et al*. (2000), proportion of CH_4 energy decreased 0.78 %-units per multiple of maintenance, whereas Ramin and Huhtanen (2013) reported 0.7 kJ/MJ per 1 g/kg BW increase in DMI. Both estimates are close to a 10% reduction in the maintenance requirement. It is also possible that physiological mechanisms are involved in the relationship between MRT and CH_4 yield when MRT is affected by individual animal variation or by feeding level. According to the Karoline model (Huhtanen *et al*., 2015c), reduced CH_4 with increased intake is associated with reduced digestibility, repartitioning of fermentation products between gases and VFA compared with microbial cells and uptake of H_2 by microbes.

Link between passage rate and digestibility

The differences in CH_4 yield between low- and high-emitting sheep have been associated with digesta retention time (Pinares-Patiño *et al*., 2003, 2011; Goopy *et al*., 2014), with diet digestibility being significantly (Pinares-Patiño *et al*., 2011) or numerically (Goopy *et al*., 2014) lower in low-emitting sheep. In the study by Goopy *et al*. (2014), for each kg increase in rumen particulate-phase MRT, CH_4 yield increased by 11.5 g/kg DMI in combined individual animal data for low and high CH_4 emitters (R^2= 0.56). The modelling approach by Huhtanen *et al.* (2016) predicted similar relationships between MRT and OMD. Positive correlations between CH_4 production and cellulose digestibility have been reported in sheep (Pinares-Patiño *et al*., 2003), and positive

correlations between NDF digestibility and CH_4 production in dairy cows (Pinares-Patiño and Clark, 2010).

The simple equation proposed by Waldo (1970) for calculating digestibility from digestion and passage rates [Digestibility = digestion rate / (digestion rate + passage rate)] indicates that with increased passage (reduced MRT), digestibility decreases when digestion rate is constant. The effect of passage rate on digestibility calculated by a biologically more correct two compartment model considering selective retention of particles in the rumen also predicts reduced digestibility with shorter retention time in the rumen (Allen and Mertens, 1988).

Schiemann *et al*. (1971) presented individual data for eight cows fed either at maintenance or production level. There was a strong positive relationship between diet digestibility and CH_4 yield in both cases (Figure 13). In that respiration chamber study with dairy cows (Schiemann *et al*., 1971) and in the modelling study by Huhtanen *et al*. (2016), the slope between digestibility and CH_4 yield was about three-fold the average CH_4 yield, suggesting that incremental digestion produced more CH_4 per unit of digested OM than the diet on average. It is possible that between-cow differences in OMD result mainly from digestion of the slowly digestible NDF fraction, which can produce more acetate and CH_4. The positive relationships found for OMD and NDF in relation to molar proportion of acetate, and the negative relationship found for propionate (**Paper II**), support this suggestion.

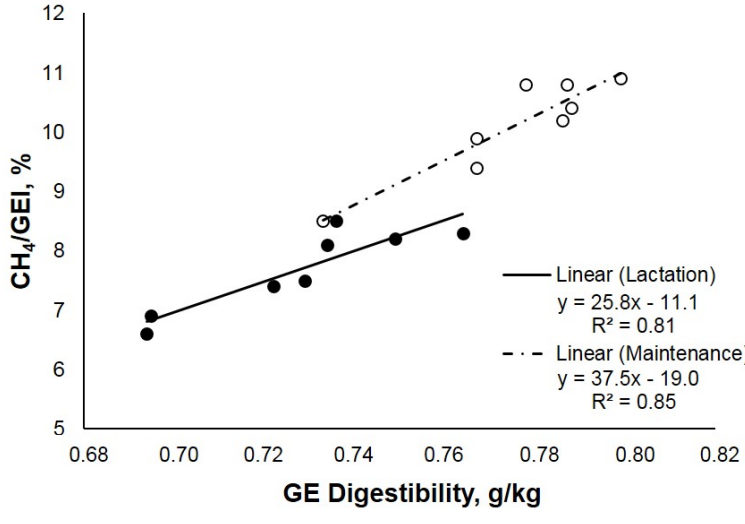

Figure 13. Relationship between gross energy (GE) digestibility and CH_4 yield at individual animal level based on data from Schiemann *et al*. (1971).

In **Paper II**, improved OMD was positively associated with the molar proportion of acetate and CH_4VFA and negatively with the molar proportion of propionate. Thus, the effects of OMD and CH_4VFA are additive. Because most of the variation in OMD was due to NDFD (cow variance for NDFD was three-fold that for digestibility of ND solubles) and CH_4VFA ratio was positively related to dietary NDF concentration, incremental digestibility increased CH_4VFA.

Between-cow variation in OMD was small (SD = 10 g/kg; CV = 0.013). Mehtiö *et al*. (2016) reported a value of 12.3 g/kg for between-cow variation in OMD determined using acid-insoluble ash as an internal marker. Similarly, small between-animal variation (CV = 0.012) was observed in a meta-analysis of individual cow data from 21 studies using acid insoluble ash as a marker (Huhtanen *et al*., 2015a). These values are consistent with the 0.016 in OMD predicted by the Karoline model (Huhtanen *et al*., 2016) using the same between-animal CV (0.085) as the dataset in **Paper II**. Based on Ørskov *et al*. (1988) and the results described above, between-animal variation in digestibility is strongly influenced by MRT.

In general, between-cow variation in digestibility can explain only a small part of the observed variation in CH_4 production. Similarly, analysis of the data from respiration chamber studies (Yan *et al*., 2000, 2010; Ramin and Huhtanen, 2013) indicates that CH_4 yield decreases by about 10% per multiple of maintenance increase in feeding level. This effect is much greater than observed decreases of approximately 2-3% in OMD per multiple of maintenance (Yan *et al*., 2002; Huhtanen *et al*., 2009).

Link between passage rate and efficiency of microbial protein synthesis

As discussed before, between-animal variation in VFA pattern and digestibility cannot explain observed effects of between-animal variation and feeding level on CH_4 yield. One possible mechanism can be improved efficiency of microbial synthesis in the rumen associated with increased passage rate. Because bacteria pass with digesta, their growth rate increases with increasing digesta passage rate in the rumen. Increasing passage rate by nutritional manipulation could be one strategy to decrease the relative impact of maintenance energy and improve growth efficiency (Hackman and Firkins, 2015). The relationship between passage rate and microbial efficiency can be demonstrated from the positive relationship between feed intake and microbial efficiency. It is well-known that increased feed intake would increase ruminal passage rate (*e.g*. NRC, 2001) and reduce microbial retention time, and thus increase microbial cell yield per unit of energy fermentation by diluting maintenance expenditure (Russell *et al*., 1992). Several studies have shown

that the efficiency of microbial N synthesis is positively related to feed intake (Chen *et al*., 1992; Volden, 1999; Broderick *et al*., 2010). The relationship between passage rate and the efficiency of microbial N synthesis could be expected to be similar when the differences in passage rate derive from differences in feeding level or from between-animal differences.

With improved efficiency of microbial synthesis, more fermented carbon is partitioned to microbial cells instead of VFA and fermentation gases. In addition, microbial cells are more reduced than fermented carbohydrates (Czerkawski, 1986; Van Soest, 1994) and act as a H_2 sink. According to Czerkawski (1986), at microbial hydrogen uptake of 8.1 g/kg cells:

Production of hydrogen, mol $2A + P + 4B + 3V + L$

Utilization of hydrogen, mol $2P + 2B + 4V + L + 4CH_4$ + 8.1 (kg cell DM),

where A. P, B, V, L and CH_4 are the amounts of acetic, propionic, butyric, valeric and lactic acid and CH_4 (mol) produced, respectively.

Methane production would clearly have been overestimated in **Paper II** if microbial uptake of H_2 had not been included in stoichiometric predictions. Applying the equations of Czerkawski (1986), CH_4 yield per mol of VFA was 0.25-0.26, which is considerably lower than the 0.312 calculated by Wolin (1960) equations for VFA ratio in the example. In *in vitro* studies, the recovery rate of metabolic H_2 varies between 78 and 96 % (Demeyer, 1991). Considering a mean H_2 recovery of 90%, CH_4 production should be 10% lower than the stoichiometric fermentation equation suggests (Moss *et al*., 2000). In **Paper II,** microbial N efficiency was negatively relatively related to OMD, indicating that the effects of these variables on CH_4 yield were additive. The positive relationship between rumen ammonia N concentration and OMD is consistent with this. Variation in rumen ammonia N concentration in animals fed the same diet reflects differences in the balance between microbial synthesis and protein degradation.

Between-cow CV in parameters related to passage kinetics (0.077-0.090) and rumen digesta pools (0.130) was much greater than in parameters related to rumen fermentation pattern or digestibility. Other studies have also indicated similar or higher between-animal variation in passage rate or retention. In the study by Pinares-Patiño and Clark (2010), the CV of mean retention time determined using particle marker was 0.209, whereas CV of rumen evacuation derived from lignin passage rate was 0.14. Between-cow CV of the passage rate of chromium-mordanted straw was 0.10-0.11 in a study by Ørskov *et al*. (1988). Variables related to ruminal N metabolism also showed high between-

animal variation (ammonia N concentration 0.149, microbial N flow 0.078, efficiency of microbial N synthesis 0.078). In studies by Pinares-Patiño *et al*. (2003) and Pinares-Patiño and Clark (2010), between-animal CV in microbial N flow was 0.209 and 0.179, respectively, and between-animal CV in microbial N efficiency was 0.166 and 0.180, respectively. In both studies, microbial N was estimated from urinary purine derivative excretion.

In **Paper II**, passage rate of iNDF was moderately repeatable (0.38). Earlier studies with sheep (Faichney, 1993) and cattle (Ørskov *et al*., 1988) found that the ranking of animals on the basis of rumen fractional passage rate was consistent among diets and feeding levels. Smuts *et al*. (1995) reported that rumen digesta retention time is a repeatable physiological trait (Rep = 0.45). Other physiological traits that can reflect variation in passage rate, such as OMD (rep = 0.365), microbial N flow (rep = 0.509), microbial N efficiency (rep = 0.354) had moderate repeatability. It can be concluded that both variability and repeatability are greater for physiological animal-related factors such as passage rate and rumen pool size than for rumen fermentation pattern, which is more related to microbial ecology in the rumen.

In the modelling approach by Huhtanen *et al*. (2016), the coefficient of variation of predicted CH_4 yield was 0.052 for cows and 0.045 for sheep (DMI = 20 and 1 kg/day respectively)when the simulations were made using the variation in MRT of iNDF. Variation in model predictions was smaller than observed CV in animal studies. This may be because i) fixed intake was used in the modelling approach and ii) possible variation in rumen fermentation pattern was not taken into account. In addition, iii) random measurement errors increase observed between-animal CV of CH_4 production, especially when measurements are based on single observations from one animal. Differences in predicted microbial efficiency were the main contributor to variation in CH_4 production. A proportion of between-animal variation can be related to digestive processes. As a consequence, selecting animals for low CH_4 production could lead to selection of low digester animals. Finally, a summary of the effects of animal-related factors on selecting low and high CH_4 emitters is presented in Figure 14. The contribution of isolated factors and mechanisms was discussed above.

5.3 Dietary factors

Mitigation of greenhouse gas emissions in cattle production systems should focus on reducing CH_4 production per unit of edible product. Methane intensity, measured as the ratio CH_4/ECM, is by default the most important trait in dairy production, which requires further research. Forage quality and

the level of concentrate supplementation are the most important practical tools available on dairy farms to optimise production. Because these factors represent a high proportion of total diet, they can also have a critical impact on CH_4 production.

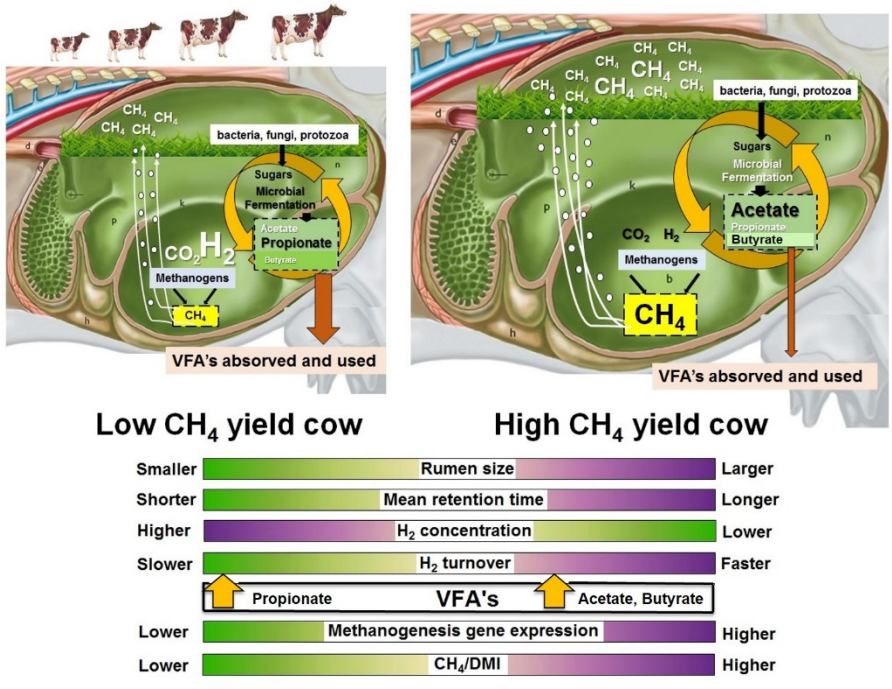

Figure 14. Summary of animal-related factors influencing CH_4 yield as a criteria to identify both low and high CH_4 emitters. (Modified from slide of Dr Sidney Leahy presented at METHAGENE Training School on Rumen Microbial Ecosystem. University of Porto, Porto, Portugal. September 11 – 14, 2016, (https://twitter.com/METHAGENE?ref_src=twsrc%5Etfw). Reticulorumen figures were taken from www.scanvetpress.com (accessed 1 October, 2016; Copyright © (2016).

Dietary carbohydrate composition affects digestion site, fermentation pattern and digestibility of different nutrients. In practice, it is determined by the forage to concentrate ratio, forage type, forage maturity and concentrate source. The results in this thesis (**Papers III** and **IV**) demonstrated that it was possible to reduce the amount of concentrate supplementation by early harvesting to improve forage quality, without compromising the performance or increasing CH_4 production or N excretion per kg ECM, and even improving feed efficiency.

Although the reported effects of forage maturity at harvest (digestibility) are variable (Thomas, 1987; Kuoppala *et al*., 2008), improved digestibility increases DMI (Huhtanen *et al*., 2007), and consequently ME intake at a fixed

level of concentrate. Therefore it is likely to increase nutrient intake, with improved forage digestibility and increased production and decreases in CH_4 production per kg of ECM. **Paper IV** showed reduced rumen NDF pool size with increased inclusion of early-cut silage in the diet. This is an indication that rumen fill was not a limiting factor in intake with higher inclusion of better quality forage in the diet. Rumen NDF pool size decreases with increasing digestibility (Bosch *et al*., 1992; Rinne *et al*., 2002; **Paper IV**), suggesting that this strategy can work even at higher production levels.

Increased concentrate supplementation is often considered as an effective strategy to reduce CH_4 production. With high grain diets in a feedlot situation, CH_4 losses may drop to approximately 3% of gross energy (Johnson *et al*., 1993), which is much lower than the 6-7% of gross energy reported for typical grass silage-based diets for dairy cows (*e.g.* Yan *et al*., 2000). However, within typical ranges of concentrate supplementation for dairy cows, the effects are relatively small (Sauvant and Giger-Reverdin, 2009; Ramin and Huhtanen, 2013). For example, in the study by Ferris *et al*. (1999), CH_4-E/GE only tended to decrease when the proportion of concentrate gradually increased from 37 to 70%. Although the effects of concentrate level on CH_4 yield are not very large for dairy cow diets, increased feed intake and production will most likely decrease CH_4 production per unit of product.

When using high concentrate diets for dairy cows, it is important to be aware that some concentrate ingredients such as cereal grains and soybean can be used directly as human food or more efficiently in monogastric animals with minimal CH_4 production. With high concentrate diets, the special advantage of ruminants in human food production – microbial digestion of fibre in the rumen – is also partly or completely neglected. The results in **Papers III** and **IV** indicated that with increased concentrate proportion in the diet and reduced forage quality, more potentially digestible nutrients were excreted in faeces. It is possible that the greater faecal output of fermentable substrate with increased concentrate at least partly compensates for the lower CH_4 yield from rumen fermentation. In addition, the carbon footprint of feed production should be taken into account when comparing different nutritional mitigations strategies. According to Mogensen *et al*. (2014), carbon footprint is 1065 and 671 g CO_2-eq/kg DM for barley grain and grass silage, respectively, in Danish conditions. This difference corresponds to 14 g CH_4 (1 g CH_4 = 28 g CO_2) when replacing 1 kg DM of grass silage with barley. It is also important to consider the contribution from soil carbon storage or loss potential from different land uses and manure systems when identifying appropriate strategies for reducing greenhouse gas emissions from dairy production.

6 Conclusions

- The GreenFeed system (flux method) was shown to be a useful tool for measuring CH_4 emissions from large numbers of animals in on-farm conditions. Repeatability was high, while between-animal variation and measured emissions were within expected biological ranges.
- Methane emissions measured by the sniffer method were poorly correlated to CH_4 flux measured by the GreenFeed system. Head position had a strong influence on measured CH_4 values. A sniffer method based on CH_4/CO_2 ratio was better correlated to CH_4 flux than CH_4 concentration.
- Repeatability and between-cow variation in stoichiometric CH_4 production per mole of volatile fatty acids (VFA) were small and can only make a minor contribution to observed between-cow variation in CH_4 emissions. Variation and repeatability were greater for ruminal VFA concentrations than molar proportions.
- Between-cow variability in digestibility was small, but repeatability was moderate.
- Greater between-cow variability and repeatability was observed in digesta passage rate and rumen pool size variables.
- Between-cow variation in digesta passage rate-associated variables can explain more of the between-cow variation in CH_4 emissions than rumen fermentation patterns associated with differences in rumen microbial population.
- Decreased CH_4 emissions with increased digesta passage rate are related to reduced diet digestibility, improved efficiency of microbial protein synthesis, which repartitions fermented carbon from VFAs and gases to microbial cells, and uptake of hydrogen by microbial cells. Selection for low CH_4 emissions can decrease the efficiency of cell wall digestion.
- By improving forage digestibility, the amount of concentrate supplementation could be reduced and milk production level could be

maintained, without increasing CH_4 emissions or nitrogen excretion per unit of product.

- The depression in digestibility from maintenance level to production level was greater for diets based on medium-quality silage and a high level of concentrate than for diets based on high-quality silage and a moderate level of concentrate supplementation. The difference was mainly due to lower digestibility of potentially digestible neutral detergent fibre (pdNDF).
- Higher CH_4 yield with increased proportion of early-harvested silage was not related to rumen fermentation pattern. The differences were mainly related to higher total digestibility of organic matter and especially to higher apparent organic matter digestibility in the rumen.
- Feed efficiency in terms of ECM yield per kg dry matter intake improved with increased inclusion of early-harvested silage in the diet. No difference in the efficiency of nitrogen utilisation was observed.
- Ruminal and total tract NDF digestibility improved with increased inclusion of early-harvested silage in the diet, reflecting differences in intrinsic characteristics of fibre and negative effects of higher starch content in diets with increased proportion of late-harvested silage.

7 Future perspectives

Based on the results obtained in this thesis, future studies focusing on the study of between-animal variation in CH_4 emissions should consider:

- Investigate the potential for ranking cows as high and low CH_4 emitters and for establishing links with their productive performance (*i.e*. dry matter intake, milk yield), their ability to digest fibre, rumen microbial ecology and fermentation characteristics *etc*.
- Quantify the real effect of animal variation on methane emissions, measure the repeatability of specific animal characteristics as a tool for animal breeders, identify biomarkers from low CH_4 emitters (*i.e*. fatty acids in rumen bacteria) and then suggest protocols for future research and develop useful CH_4 mitigation strategies for dairy cows.
- Examine why rumen fermentation pattern is not enough to explain individual differences in CH_4 emissions.
- Compare digestibility and microbial community, *e.g*. when selecting for low emitters then also select for low digesters.
- In selection of low methane emitters, determine the relationships between digestibility and CH_4 emissions.
- Study whether increasing feed conversion is a more effective way to reduce CH_4 emissions than selecting for low CH_4 emitters.

8 Popular scientific abstract

Climate change is kind of every day's issue that contemporary society has to deal with. The growing human population represents a constant threat to the ecosystems since it demands increased amount of food and natural resources to supply its demands. Greenhouse gas (GHG) emissions to the atmosphere is just one example as a consequence of livestock production. Due to the particularities of digestive tract of ruminants, they are able to convert non-edible foods (*i.e.* forages) into highly valuable products for human consumption such as milk. However, ruminants also produce methane (CH_4) which contributes significantly to global warming. Several attempts to account for CH_4 emissions around the world and across different production systems have been made but many of them fail in get realistic numbers, especially at large farm scale.

Accurate and reliable methods for measuring CH_4 emissions in dairy cows at individual-animal basis are needed in order to develop successful CH_4 mitigation targets in cattle production. The first study of the present thesis demonstrated GreenFeed is a reliable tool for ranking animals as low CH_4 emitters in farm conditions. Gas concentrations from sniffer method were poorly correlated to dry matter intake (DMI) and it lacks of biological value. In addition to be repeatable, GreenFeed proved to be an accurate method to measure CH_4 emissions despite of being based on spot sampling.

Interest on selecting low emitters as a long term strategy to mitigate methane CH_4 emissions from ruminants has increased significantly mainly due to promising heritability values. Traditional selection for total CH_4 emissions may have an impact on selecting efficient animals since the selection of low CH_4 emitters could lead to select for low fibre digesters which is not very convenient for farmers' income. A meta-analysis of experiments conducted in the Nordic countries was performed to investigate the effects of animal-related factors on the variation in in vivo CH_4 yield emissions. Results from study 2,

showed that among of the studied animal-related factors, passage rate is the key variable in modulating CH_4 yield emissions since their contribution to the observed between-cow variation was much higher than digestibility, microbial N synthesis or rumen fermentation patterns. Since passage rate is positive and strongly correlated to DMI, it may be a strong evidence to support selecting individual animals for feed efficiency rather than selection for low CH_4 emissions.

In the Nordic countries, diets for dairy cows are based on grass silage forages. By harvesting in an early stage the forage, it is expected that forage quality improved compared to late cut harvest. Combined results from two experiments (studies 3 and 4), conducted at the same time in either 16 intact cows (production trial) or 4 rumen-cannulated cows (flow study), demonstrated that by the graded addition of early-cut silage in the diet is possible to reduce concentrate supplementation in practical diets without compromising milk production or total CH_4 emissions. Differences in forage digestibility partly explained the differences between treatments. Although, medium quality forage (late-cut silage) and increased amount of concentrate was able to reduce CH_4 yield, higher faecal output of potential digestible nutrients was observed in these treatments compared with early-cut silage diets. This compromises give a final recommendation on a life-cycle assessment approach since potential nutrients are wasted to the environment and its CH_4 production was not measured in those studies. Future GHG mitigation strategies have to consider all nutrient outputs of the system to better understanding the real impact of ruminants on the environment.

9 Populärvetenskaplig sammanfattning

Exakta och tillförlitliga metoder för att mäta produktionen av CH_4 från mjölkkor på individnivå behövs för att utveckla realistiska mål för begränsningar av utsläppen av växthusgaser från nötkreaturssektorn. Den första studien i denna avhandling visar att GreenFeed är ett pålitligt verktyg för att rangordna djuren som hög- eller lågemitterande kor och utan att behöva flytta djuret från sin naturliga miljö under mätningarna. GreenFeed har utvecklats för att i realtid mäta flöden av CO_2 och CH_4 från en större grupp djur i en besättning genom upprepade regelmässiga individbaserade mätningar under flera dagar. Att bara mäta gaskoncentrationer med den så kallade Sniffer metoden visade sig vara dåligt korrelerat till kornas konsumtion. Konsumtionen är den viktigaste faktorn som bestämmer den totala produktionen av CH_4 hos en mjölkko. Sniffer metoden bedöms därför vara missvisande och sakna biologisk relevans för att mäta kornas metanproduktion.

På grund av den individuella variationen borde det kunna vara möjligt att välja ut kor som ger lägre metanproduktion som en långsiktig strategi för att minska klimatpåverkan från idisslare. Att ensidigt selektera för en målsättning kan dock påverka andra funktionella egenskaper, och just metanproduktionen har visat sig ha ett nära samband med kornas förmåga att smälta växtfibrer. Den här doktorgradsavhandlingen undersökte också hur andra funktionella egenskaper relaterade till mjölkkornas matsmältning inverkade på metanproduktionen. I en metaanalys där flera försök med mjölkkor, som genomförts i de nordiska länderna, användes för att undersöka vilka djurrelaterade faktorer som har störst inverka på metanproduktionen visade sig passagen av fodret ut ur vämmen vara mycket viktigare än fodrets smältbarhet, den mikrobiella proteinsyntesen eller jäsningsmönstret av flyktiga fettsyror i vämmen. Eftersom fodrets passagehastighet är positivt korrelerat till foderkonsumtionen innebär det att genom att hellre avla för bättre foderutnyttjande än låg metanproduktion per se har man större förutsättningar

för att minska metanproduktion per kg mjölk än om man gör ett direkt urval av kor med låg produktion av CH_4. Detta eftersom det senare kan leda till nedsatt smältbarhet och fodereffektivitet.

I de nordiska länderna baserar sig utfodringen av mjölkkor i hög grad på gräsensilage. Genom att skörda fodret tidigt i säsongen uppnår man ett högkvalitativt vallfoder. Detta innebär att man kan spara kraftfoder i produktionen jämfört med att utfodra ett senare skördat vallfoder i foderstaten. I två olika försök i den här doktorgradsavhandlingen undersöktes effekten av att gradvis ersätta ett sent skördat gräsensilage och kraftfoder med ett energirikt och tidigt skördat gräsensilage på mjölk- och metanproduktionen samt mer detaljerat med avseende på fodrets omsättning i korna. Produktionsförsöket visade att det är möjligt att ersätta kraftfoder med ett tidigt skördat gräsensilage i foderstaten till mjölkkor och upprätthålla produktionen utan att öka metanproduktionen hos korna. Även om det senare skördade ensilaget och mera kraftfoder minskade metanproduktionen relaterat till konsumtionen utsöndrades mer näringsämnen i kornas träck än när tidigt skördat gräsensilage utfodrades. Slutgiltigt poängterar detta vikten av en helhetlig betraktning av nötkreaturssektorns klimatpåverkan för att minska metanproduktionen från idisslarna i framtiden.

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Keywords:
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