Predicting Methane Production in Dairy Cows

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(Karoline)

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Abstract

Methane is a potent greenhouse gas, to which enteric fermentation from ruminants contributes significantly. Reliable and accurate predictions of methane (CH_4) production from dairy cows would be of interest to develop mitigation strategies and for national inventories. Thus, the overall aim of this thesis was to predict CH_4 production in dairy cows by modelling approaches.

Predicted *in vivo* CH_4 production decreased with increased sample size in the gas *in vitro* system. Molar proportion of acetate decreased at the expense of propionate. Digestibility also decreased with increased sample size. Predicted CH_4 production based on stoichiometric equations of volatile fatty acids was in good agreement with observed values of CH_4 production from the gas *in vitro* system.

Dry matter intake per kilogram of body weight, organic matter digestibility and dietary concentrations of neutral detergent fibre, non-fibre carbohydrates and ether extract were the variables of the best fit model predicting CH_4 energy as a proportion of gross energy (prediction error 4.65% of the observed mean). The non-linear models developed proved to be more applicable over a wider range of intake for predicting total CH_4 production than linear models. Adjusting the exponents for dietary concentration of fat, proportion of non-fibre carbohydrates in total carbohydrates and organic matter digestibility improved the model.

The sub-model predicting CH_4 production in the Karoline model was revised. Modifications were made to equations predicting digesta passage kinetics, microbial cell synthesis, digestion in the hind-gut and utilisation of hydrogen. The sensitivity analysis suggested that accurate values for digestion kinetic variables are required for accurate and acceptable predictions of CH_4 production with mechanistic models. The Karoline model was evaluated against published data (n=184 diets) reporting CH_4 production from *in vivo* trials. There was a good relationship between observed and predicted values of CH_4 production, with a small root mean square error of prediction (10.1% and 6.1% of the observed mean for fixed and mixed models, respectively). The mean bias was small (<2%) but statistically significant, and there was no slope bias. Most of the error was due to random bias (96.4%), whereas the contributions of mean and slope bias were small (3.4 and 0.2%, respectively).

Keywords: Dairy cow, diet composition, empirical models, *in vitro* gas production, methane production, mechanistic modelling, methane kinetics, rumen model, volatile fatty acids.

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Dedication

To the beloved memory of my mother, who is always in my heart…

To my family for their encouragement

To **Narges** and **Ilya**, with gratitude for their love and for tolerating all the extra hours I always had to spent on my research work

Contents

List of Publications

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

I **M. Ramin and P. Huhtanen.** (2012). Development of an *in vitro* method for determination of methane production kinetics using a fully automated *in vitro* gas system - A modelling approach. *Animal Feed Science and Technology* 174, 190-200.

II **M. Ramin and P. Huhtanen.** (2013). Development of equations for predicting methane emissions from ruminants. *Journal of Dairy Science* 96, 2476-2493.

III **M. Ramin and P. Huhtanen.** (2013). Development of non-linear models for predicting enteric methane production. *Acta Agriculturae Scandinavica Section A-Animal Science* 62 (4), 254-258.

IV **P. Huhtanen, M. Ramin and P. Udén.** (2013). Nordic dairy cow model Karoline in predicting methane emissions: 1. Model description and sensitivity analysis (submitted).

V **M. Ramin and P. Huhtanen.** (2013). Nordic dairy cow model Karoline in predicting methane emissions: 2. Model evaluation (submitted).

The contribution of Mohammad Ramin to the papers included in this thesis was as follows:

I Planned the experiment jointly with the co-author and performed the experiment, analysed the data and wrote the manuscript.

II Planned the research jointly with the co-author, collected the data set in collaboration with the co-author, analysed the data and wrote the manuscript.

III Planned the research in collaboration with the co-author and participated in writing the manuscript.

IV Planned the experiment jointly with the co-authors and participated in performing the analysis and writing the manuscript.

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

Abbreviations

CH_4-E/GE	CH_4 energy as a proportion of gross energy
CV	Coefficient of variation
DM	Dry matter
DMI	Dry matter intake
DMIBW	Dry matter intake per kg body weight
EE	Ether extract
EMPS	Efficiency of microbial protein synthesis
GHG	Greenhouse gas
iNDF	Indigestible neutral detergent fibre
IPCC	Intergovernmental Panel on Climate Change
NDF	Neutral detergent fibre
NFC	Non-fibre carbohydrates
OM	Organic matter
OMD	Organic matter digestibility
pdNDF	Potential digestible NDF
RMSE	Root mean square error
RMSPE	Root mean square prediction error
RUSITEC	Rumen simulation technique
VFA	Volatile fatty acids

1 Introduction

1.1 Methane and composition of gases in the atmosphere

Methane is a chemical compound with a high combustion energy, 55.5 MJ/kg (Crutzen, 1995). It is a potent greenhouse gas (GHG) contributing 20% to total anthropogenic emissions (Lassey, 2007), and it is responsible for one-third of all global warming over the last 250 years (Thorpe, 2009). The methane concentration in the atmosphere has increased 2.5-fold over three centuries. The agriculture sector contributes to a large extent to GHG emissions (Lassey, 2007). Methane (CH_4) gas has a shorter turn-over time (about 10 years) in the atmosphere than carbon dioxide (CO_2), and can trap heat about 20 times more effectively than CO_2. Methane is therefore one of the gases that needs to be considered in climate mitigation approaches, as it is responsible for the destruction of the ozone layer and increased global temperature (Immig, 1996).

Unpolluted air mainly contains 78% nitrogen, 21% oxygen and 1% other gases. The other gases are: argon (0.9%), neon (less than 0.01%), helium (less than 0.01%), krypton (less than 0.01%) xenon (less than 0.01%) and radon (less than 0.01%). The concentration of CO_2 has been about 0.03% in the past, but it is increasing and approaching 0.04% as a result of human activities. Water vapour contributes the most to the greenhouse effect, followed by CO_2 and CH_4 gases (Table 1). Carbon dioxide together with CH_4 is considered a GHG and there has been significant interest in their atmospheric composition and relative contribution to the greenhouse effect (Moss *et al.*, 2000).

Table 1. *Contribution of different compounds to the greenhouse effect*

Compound	Formula	Contribution (%)
Water vapour and clouds	H_2O	36 – 72
Carbon dioxide	CO_2	9 – 26
Methane	CH_4	4 – 9
Ozone	O_3	3 – 7

Source: Kiehl & Trenberth (1997).

1.2 Contributors to methane production

1.2.1 Sources of methane production

Methane originates from different biological sources such as natural wetlands, landfills, rice paddies, livestock, termites, solid wastes and burning biomass, as shown in Figure 1a (Immig, 1996). It also originates from coal mining and leakages from natural gas production. Rice paddies are an important source of increased atmospheric CH_4 production, with annual emissions of about 115 Teragrams (Tg) per year (Dannenberg & Conrad, 1999; Thorpe, 2009). However, seasonal variations in the contribution of rice paddies to atmospheric CH_4 production may be caused by different factors such as organic amendments, water management and fertilisation (Dannenberg & Conrad, 1999). The global anthropogenic CH_4 production from different sources is given in Figure 1b. Moss *et al.* (2000) estimated that 689 Tg CH_4 are produced annually by different sectors. Total CH_4 production is 84 Tg/year, which is greater than the CH_4 sink capacity (reaction in the atmosphere to produce CO_2 and microbial uptake in soil), increasing the CH_4 concentration in the atmosphere (Figure 1c).

The agricultural sector contributes a total of 10-12% of global anthropogenic GHG emissions (McAllister *et al*., 2011). The livestock sector is one of the largest CH_4 producers, with total emissions from the livestock sector (enteric fermentation) estimated to be 70-100 Tg/year (Hegarty, 1999b; Thorpe, 2009).

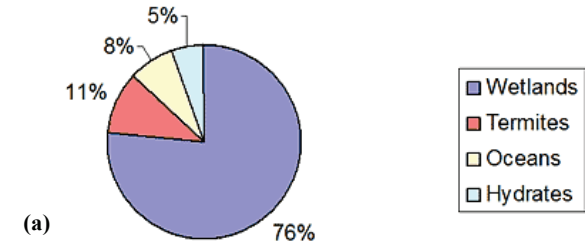
There is considerable variation in the contribution of the livestock sector to total anthropogenic GHG emissions as reported in the literature. Part of this variation relates to different methods of calculation, *e.g*. how land use change is taken into account. Based on a life cycle assessment, the livestock sector contributes about 18% of the total global anthropogenic GHG emissions (Steinfeld *et al*., 2006). Meale *et al*. (2013) reported that agriculture is responsible for 10-12% of global anthropogenic GHG emissions. The differences between these numbers can be due to accounting for land use changes. Moss *et al*. (2000) reported that livestock CH_4 production is about

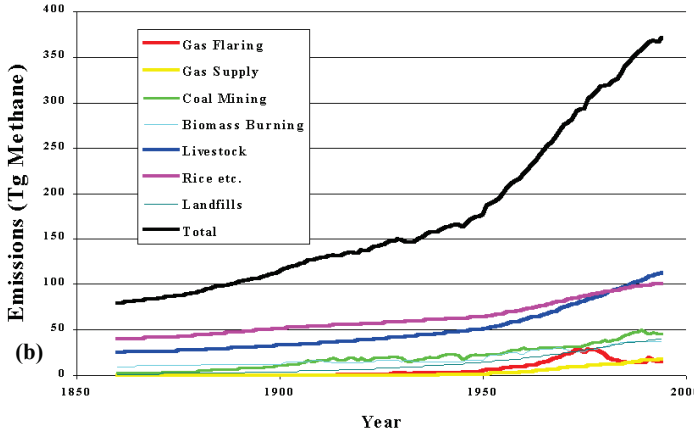
51% of total agricultural CH_4 production and that agriculture contributes about 21-25, 60 and 65-80% of the total anthropogenic emissions of CO_2, CH_4 and N_2O, respectively. According to those authors, the contribution of livestock (fermentation + manure) and rice paddies is rather similar (110 and 100 Tg/year, respectively). They also claim that globally, CH_4 emissions account for 40-45% of GHG emissions from ruminant livestock, in which around 90% of the emissions arise from enteric fermentation. In a recent study by Meale *et al*. (2013), it was reported that CH_4 production from ruminant livestock accounts for 37% of total anthropogenic CH_4 emissions. Earlier, Crutzen *et al.* (1986) reported that ruminants contribute 15% of total CH_4 emissions.

1.2.2 Global warming

Global warming due to the increase in atmospheric gases such as CH_4 and CO_2 is an important issue (Klieve & Hegarty, 1999). The greenhouse effect is due to absorption of solar infrared radiation by gases and the Earth's surface, which are then heated and re-emit infrared radiation of a lower frequency with higher absorptive power. It has been predicted that by the year 2030, the world will be 1-2 °C warmer than today.

The Intergovernmental Panel on Climate Change (IPCC) is asking developed countries to evaluate the amounts of gases produced in their country and to develop research and techniques to reduce these emissions within an assigned period (Moss *et al*., 2000). It is predicted that the human population will reach 9 billion by 2050, and the demand for livestock products is predicted to double. This need will lead to an increase in total GHG from the livestock sector (McAllister *et al*., 2011). It is assumed that the increase in livestock population will mainly occur in the developing countries. Because agriculture is a major choice, opportunities to mitigate emissions will be limited (McAllister *et al*., 2011).





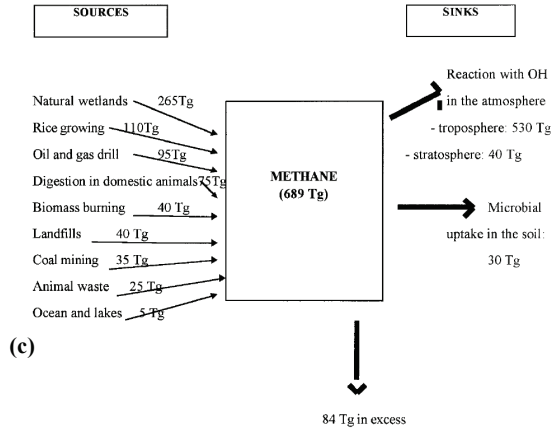


Figure 1. (a) Natural sources of atmospheric CH_4 production (source: Courtesy United States Environmental Protection Agency), (b) global anthropogenic CH_4 emissions (source: Stern & Kaufmann, 1998) and (c) sources and sinks for methane production on Earth and in the atmosphere (source: Moss *et al.,* 2000).

As stated above, there are different sectors contributing to the total GHG. The most recent National Inventory Report in Sweden published by the Swedish Environmental Protection Agency (Naturvårdsverket, 2013) showed that the contribution of agriculture in Sweden to total GHG production is about 13% (Figure 2).

In Sweden, total anthropogenic CH_4 production has fallen by 28% since 1990, mainly due to measures taken in the waste sector (Figure 3). Here, methane was converted to CO_2-equivalents by assuming that 1 tonne of CH_4 has the same effect as 21 tonnes of CO_2. However, the contribution of the agriculture sector to total emissions of CH_4 seems to have remained constant since 1990 (Figure 3). In 2011, CH_4 production from enteric fermentation contributed one-third of the emissions from agriculture (33% or 2.6 million ton CO_2-eq). Methane production from enteric fermentation decreased by about 12% over the period 1990-2011, mainly due to reduced livestock farming activities (decreased population of cattle) (Naturvårdsverket, 2013).

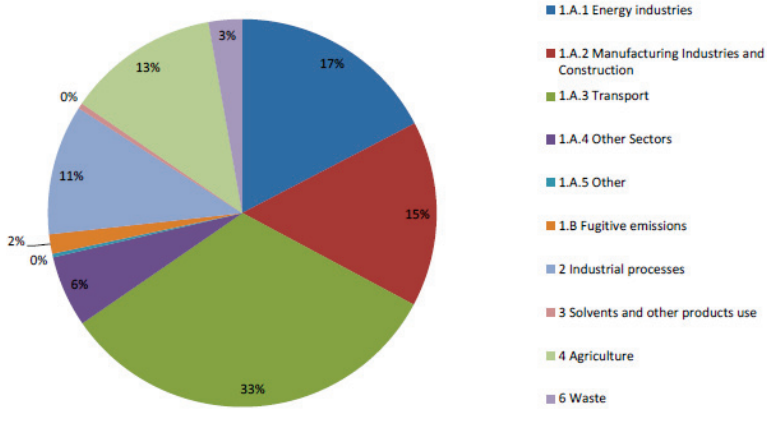


Figure 2. Greenhouse gas emissions broken down by sectors in Sweden. 0% means a share lower than 0.5% (source: Naturvårdsverket, 2013).

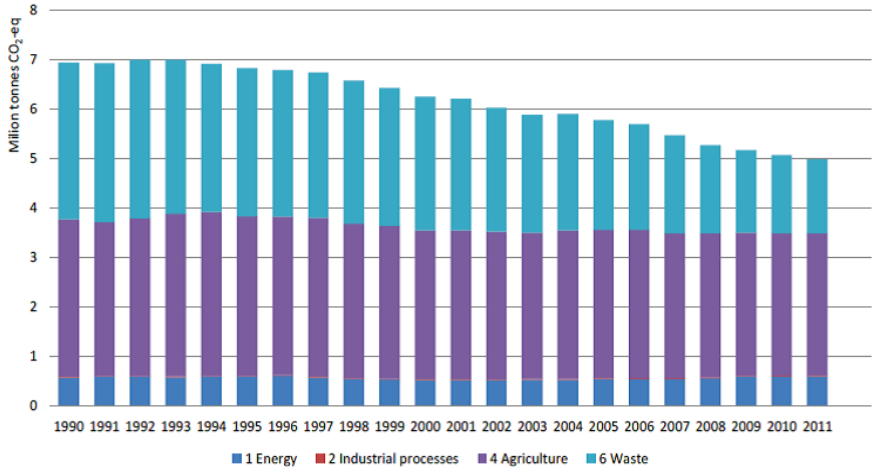


Figure 3. Total emissions of CH_4 from different sectors in Sweden, calculated as CO_2-equivalents, where 1 tonne of CH_4 has the same effect on climate as 21 tonnes of CO_2 (source: Naturvårdsverket, 2013).

Methane production from the agriculture sector in the European countries has been estimated to be mostly the result of enteric fermentation (two-thirds, 80 million tons per year) and livestock manure (one-third) (Moss *et al*., 2000).

For example, the contribution of enteric fermentation is 50.2% of total CH_4 emissions in Sweden, 49.6% in Finland, 49.6% in Denmark, 44.0% in Iceland and 24.5% in Norway (Thorpe, 2009). Recently, Lesschen *et al*. (2011) reported the distribution of main livestock types within the 27 member states of the European Union (EU-27) based on data from 2003. As shown in Figure 4, cattle production (dairy and beef) showed the highest intensity (livestock units per ha arable land) in the Netherlands and Belgium and in some regions of Germany, France, Austria and Ireland. However, it is worth noting that proportion of arable land in the total land area is much greater in *e.g.* the Netherlands than in Northern Europe, where animal intensity is moderate.

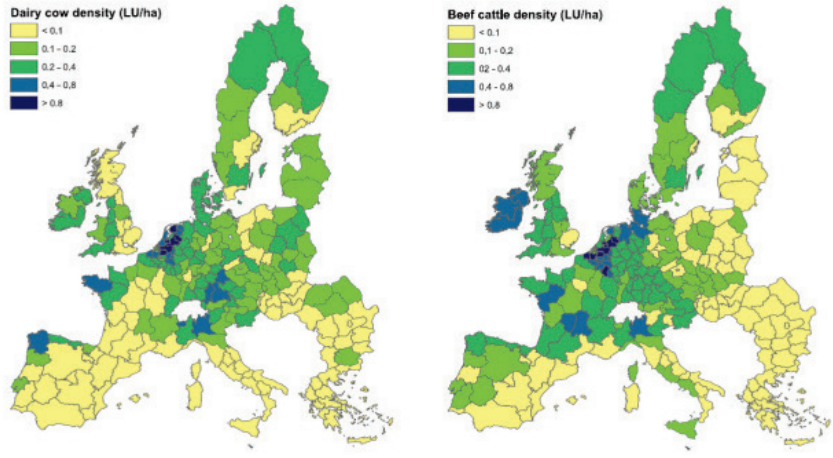


Figure 4. Distribution of the main livestock types in the EU-27. Animal density is expressed in livestock units (LU) per ha utilised agriculture area, in which the relative weight of a mature dairy cow is set at 1 and that of a mature beef animal at 0.5 (source: Lesschen *et al*., 2011).

Lesschen *et al*. (2011) also reported the contribution of different animal species (dairy cows, beef cattle, pigs, broiler chickens and laying hens) to GHG emissions. The dairy cow and beef cattle sectors had the highest GHG emissions in the EU-27. The annual emissions from the dairy cow sector were 195 Tg CO_2-eq., marginally greater than the emissions from the beef cattle sector (Figure 5).

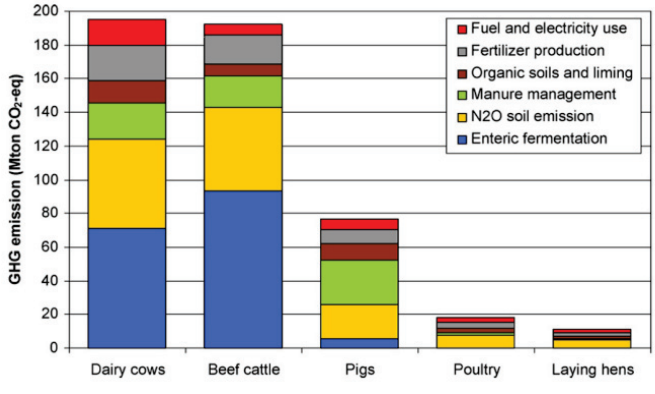


Figure 5. Total greenhouse gas emissions from the various emission sources associated with livestock production in the EU-27 (source: Lesschen *et al*., 2011).

The main source of GHG emissions from both the dairy cow and beef cattle sectors was enteric fermentation (Figure 5), but the contribution of enteric

fermentation was disproportionately greater for the beef cattle sector. The contribution of manure was greatest for the pig sector.

1.3 Methane production in ruminants

In 1879, an efficient respiration chamber apparatus for cattle was invented in Germany by Gustav Kühn (Breirem, 1952). In this well-equipped laboratory, Oskar Kellner was given the opportunity to work on feed evaluation. Towards the end of the 19th century, Kellner developed a net energy system for feedstuffs and found that fermentation in the rumen disturbed calculation of the net energy value for feeds. The fermentation losses were most pronounced when animals were fed with roughage (Breirem, 1952).

Methane is produced from the fermentation of feeds in the rumen. Methanogens are a unique group of microorganisms belonging to the domain Archaea, which produce CH_4 in order to gain energy for growth. Methanogens are strictly anaerobic, which means they can only grow in an oxygen-free environment. Almost two-thirds of the rumen archaea belong to the *Methanobrevibacter* spp. (Morgavi *et al*., 2010). Methanogens are found in both the liquid and solid phase of the rumen contents, as well as on the rumen epithelium (Morgavi *et al*., 2010). *Methanobacterium ruminantium* is one of the main methanogens detected in the rumen and its population is greater than 1×10^6 mL^{-1} in the rumen of a dairy cow (Miller *et al*., 1986). The bacterium is Gram-positive and active at pH values above 5.5 (Crutzen, 1995). Recently, a new group of methylotrophic methanogens that use methylamines has been detected in the bovine rumen (Poulsen *et al*., 2013). Methane production in cattle begins approximately 4 weeks after birth, when solid particles are retained in the reticulo-rumen. It starts to increase as the animal matures (Johnson & Johnson, 1995), due to increased intake but also to an increased contribution by rumen fermentation to feed digestion. Czerkawski (1986) postulated that CH_4 production from 12 cows daily can provide an average household with its domestic gas.

Feed entering the rumen is primarily digested by rumen microorganisms such as bacteria, fungi and protozoa, which are known as primary fermenters. They digest feed components to simple monomers, which are then utilised by both primary and secondary fermenters to produce final end-products such as volatile fatty acids (VFA) hydrogen (H_2) and CO_2 (McAllister *et al*., 1996). Methane is then produced in the final stage by methanogens, using H_2 (80%) or formate ($HCOO^-$) (18%) together with CO_2 as their main substrates (Figure 6).

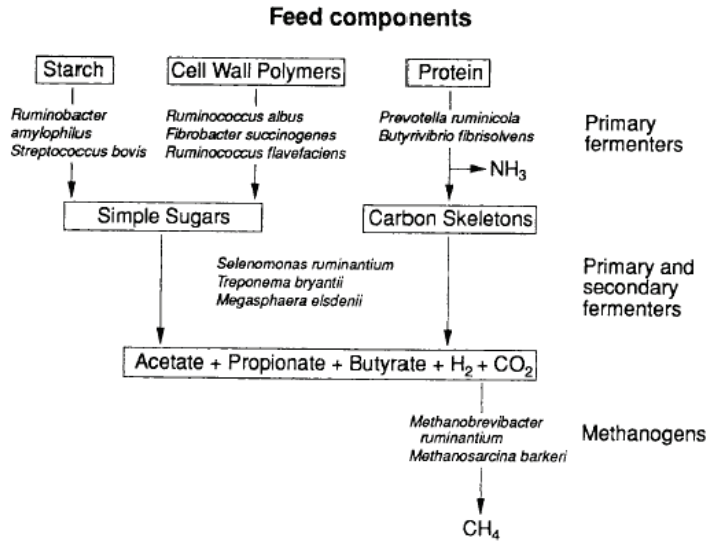


Figure 6. Microbial fermentation in the rumen (source: McAllister *et al*., 1996).

More than 95% of the CH_4 produced during enteric fermentation in the rumen is lost via the mouth to the atmosphere, whereas rectal emissions account for only 2-3% (Murray *et al*., 1976). It has been estimated that an adult cow can produce about 300-600 L CH_4 per day (Jouany, 1994). However, the variation in CH_4 production is large depending on several factors, with dry matter intake (DMI), digestibility and composition of the diet being the most important (Johnson & Johnson, 1995).

Depending on different factors such as DMI, feed quality, digestibility, type, size and weight of the animal and its production, CH_4 production as a proportion of gross energy (GE) varies from 2-12% of gross energy intake (GEI) (Johnson & Johnson, 1995; Sauer *et al*., 1998). An example of distribution and losses of feed energy from ruminants by considering a 6% GE loss via CH_4 eructation is illustrated in Figure 7. Metabolisable energy (ME) calculated as digestible energy (DE) – CH_4 energy – urinary energy (UE) is not completely available for maintenance and production, as part (5 MJ in this example) is lost as fermentation heat.

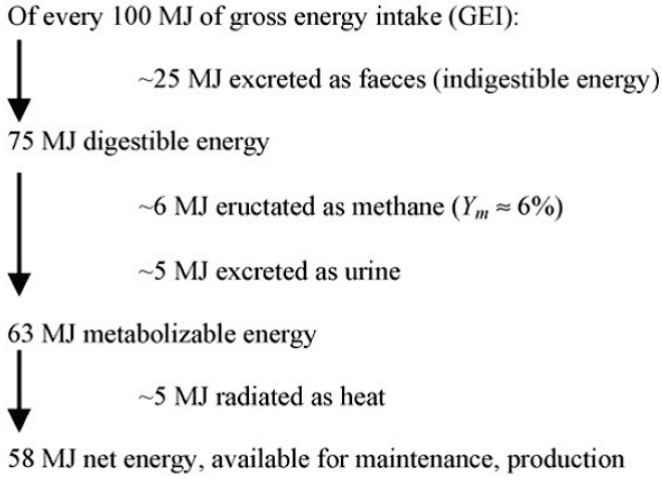


Figure 7. Energy losses during ruminant digestion of a high-quality forage diet (source: Lassey, 2007).

1.4 Hydrogen production and sinks in the rumen

It has been reported that immediately after feeding, the rate of CH_4 production is higher due to the higher partial pressure of H_2 and the high rate of fermentation (Baker, 2002). Approximately 100 L H_2 gas are produced in the rumen of sheep (Hegarty & Gerdes, 1999). Hydrogen production plays an important role in the rumen, and its partial pressure is an important factor for methanogenesis (Hegarty & Gerdes, 1999). Feeding increases the partial pressure of H_2 in the rumen, consequently increasing the headspace H_2 (Hegarty & Gerdes, 1999).

Hydrogen gas is a central metabolite in rumen fermentation. It originates from the reduction of protons by the hydrogenases associated with ferredoxin enzyme systems in rumen microbes (Hegarty, 1999b). Accumulation of H_2 in the rumen inhibits re-oxidation of NADH. Lactate accumulates and the pH decreases. A consequence of the accumulation of H_2 is a depression in fibre digestibility (Joblin, 1999). From the scientific point of view, in the absence of methanogens NADH is regenerated through other H_2-consuming processes, such as the formation of lactate, ethanol or succinate. Lactate and succinate can then be converted to propionate (Schönhusen *et al*., 2003).

Methanogens convert H_2 to CH_4 to keep the H_2 pressure (accumulation) low in the rumen. For continuous monomer fermentation, NADH has to be re-oxidised to NAD^+ (Figure 8). Most rumen microbes use the Embden-Meyerhof-Parnas pathway to oxidise sugar units (glucose) to pyruvate. Hydrogen is then produced during the enzymatic oxidation of NADH formed

during glycolysis to NAD^+. The metabolism of NADH H^+ occurs in anaerobic conditions and the electron sink products are given in Figure 9.

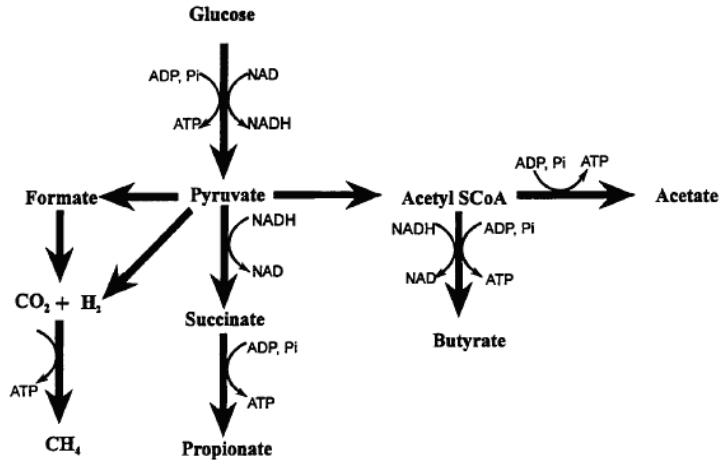


Figure 8. End-products of carbohydrate fermentation in the rumen (source: Hungate, 1966).

The symbiotic relationship between fermenting species and H_2 using methanogenesis is termed interspecies H_2 transfer (Crutzen, 1995). Acetate production promotes CH_4 production, while production of propionate depresses CH_4 production, as H_2 is used for the formation of propionate (Moss *et al*., 2000) (see Figure 9).

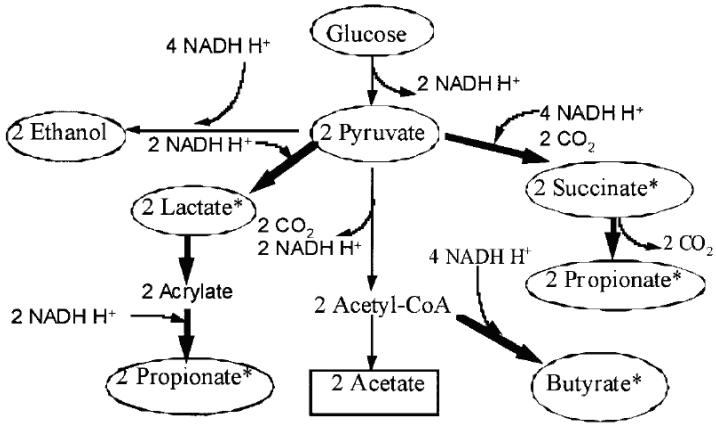


Figure 9. Metabolism of NADH H^+ and the electron sink products* in anaerobic conditions (source: Moss *et al*., 2000).

There are several H_2 sinks in the rumen, the main one being the production of CH_4. Other sinks are microbial cell synthesis (approximately 10%), biohydrogenation of fatty acids (1-2%), acetogenesis and production of propionic acid (Czerkawski, 1986). The recovery rate of metabolic H_2 varies between 78 and 96% (Demeyer, 1991). Assuming a mean H_2 recovery of 90%, CH_4 production should be 10% lower than the stoichiometric fermentation suggests (Moss *et al*., 2000). Nitrate and sulphates are also H_2 sinks in the rumen (Van Soest, 1994).

1.5 Factors influencing methane production

Enteric CH_4 production is the most important source of GHG emissions to be used as a target for mitigation within the ruminant production cycle (Meale *et al*., 2013). In this section of the thesis factors influencing CH_4 production are discussed. However, more details of animal and dietary factors affecting CH_4 production are given in Papers II and V and section 5.2. The objective of this section is to give some examples of possible strategies for mitigating CH_4 production, not to make a comprehensive review of feed additives and other methods used to mitigate CH_4 production.

1.5.1 Feed characteristics and methane production

There are some feed characteristics which influence CH_4 production, as the rumen-fermented organic matter has a close relationship with CH_4 production. Diets containing highly digestible fibre tend to lead to an increase in digestibility and consequently promote CH_4 production. Factors such as forage maturity and its physical form also influence CH_4 production (Moss *et al*., 2000). For example, CH_4 production is lower in animals fed milled and pelleted forages compared with chopped forages (Hironaka *et al*., 1996).

One strategy that has recently been investigated is the type of diet fed to ruminants in the 2 months after weaning. Lambs were fed on a hay only diet at weaning or hay plus concentrate to examine the effect of concentrate on the population of methanogens in the rumen. Methane production after 8 weeks tended to be lower in lambs fed on hay plus concentrate compared with the hay only diet. The difference resulted from a higher acetate/propionate ratio in lambs on the hay only diet (McAllister & Newbold, 2008). However, this did not lead to a permanent change in the potential methanogen population in the rumen after 4 months, when both groups of lambs were fed the same diet (grass and concentrate) (McAllister & Newbold, 2008).

1.5.2 Digesta passage rate and ambient temperature

It has been suggested that a cold environment may influence CH_4 production, mainly because the rumen passage rate increases in cold climates, thereby decreasing CH_4 production (McAllister *et al*., 1996). Lower temperature decreases the ratio of acetate/propionate in sheep, resulting in a shift to propionate production instead of CH_4 production (McAllister *et al*., 1996). Kennedy & Milligan (1978) observed that the exposure of sheep to cold resulted in a decrease in rumen turnover time, which increased the efficiency of microbial synthesis and decreased the digestibility of organic matter (OM). Shibata & Terada (2010) assigned a regression equation between DMI and CH_4 production at 18°C and 30-32°C. Methane production per DMI increased at the higher temperature and was reported to be even 10% higher at a temperature above 26°C than at 18°C in cows kept at the maintenance level of intake.

It has also been reported by Shibata & Terada (2010) that in natural tropical environments, the content of components in the cell wall of plants such as acid detergent fibre (ADF) and lignin tend to increase. These increases result in lower digestibility of feed and higher energy losses, causing an increase in CH_4 production per unit product through the decrease in the efficiency of animal production (Shibata & Terada, 2010).

1.5.3 Diet composition

Lovett *et al*. (2003) indicated that feeding low forage:concentrate ratio diets to finishing beef animals is an effective way to reduce CH_4 production per unit product while improving animal productivity. They claimed that adding coconut oil could further reduce CH_4 production. Inclusion of concentrate is a feeding strategy in which reductions can be achieved by inclusion of concentrate, especially when the concentrate proportion is above 90% of diet dry matter (DM) (Johnson & Johnson, 1995).

Inclusion of concentrate is suggested to be a solution for mitigation purposes, as it shifts the fermentation towards propionate production. However, Sveinbjörnsson *et al*. (2006) found that the effect of starch on propionate production was small. Findings in the literature regarding this matter are contradictory. McGinn *et al*. (2006) found the opposite effect, *i.e*. that increased concentrate in the diet increased CH_4 production per unit DMI. Inclusion of concentrate generates a higher amount of fermentable organic matter per unit feed than with roughage alone, resulting in increased CH_4 production. The effect mainly depends on the inclusion level, the effect on fibre digestibility and the type of grain used (Hristov *et al*., 2013a). Feeding level can also influence the effects of concentrate proportion on CH_4 energy as a proportion of gross energy (CH_4-E/GE). In the study by Moss *et al*. (1995),

CH_4-E/GE increased more with increased concentrate in sheep fed a low rather than high level of intake.

Forage quality is another important factor influencing CH_4 production. Forages are very variable in nature in terms of digestibility, time of harvest and concentration of neutral detergent fibre (NDF). These factors should be taken into account when considering mitigation of CH_4 production (Hristov *et al*., 2013a).

Inclusion of high sugar grasses is one feeding management approach that can influence CH_4 production (Kim *et al*., 2011). Inclusion of high sugar grasses can increase the efficiency of microbial growth in the rumen, by directing feed N into microbial protein and diverting H_2 to microbial cells rather than CH_4 production (Hristov *et al*., 2013a).

1.5.4 Dietary fat supplements

Supplementation of the ruminant diet with fat (oleic, linoleic and linolenic) is an effective method of suppressing CH_4 production (Beauchemin *et al*., 2009; Grainger & Beauchemin, 2011). However, supplementation with fat is not always effective, as reported by Johnson *et al*. (2002). Using high levels of fat to reduce CH_4 production can decrease DMI and productivity (Hristov *et al*., 2013a). In most cases the diet costs also increase, since feed energy is usually more expensive in fat than in cereal grains. However, according to a meta-analysis of a large data set from production trials, the optimal concentration of concentrate fat is 30-40 g/kg diet DM (Huhtanen & Nousiainen, 2012). Consequently, small amounts of supplementary fat can both increase productivity and reduce CH_4 production.

Recently, the effect of distillers' grains on CH_4 production has been investigated. Hales *et al*. (2012) fed wet distillers' grains with solubles to Jersey steers and observed a linear increase in CH_4 production per unit DMI due to increased NDF intake, despite increased fat intake. However, using high levels of distillers' grains will increase dietary crude protein concentration and consequently urinary N. This will increase ammonia (NH_3) and nitrous oxide (N_2O) emissions from stored manure (McAllister *et al*., 2011; Hristov *et al*., 2013a). Furthermore, Grainger & Beauchemin (2011) suggested that the inclusion level of fat does not suppress digestibility or depress milk fat synthesis. Other potential oils such as castor, lauric, myristic and cashew nutshell extract oils for mitigation purposes can also be mentioned.

1.5.5 Methane inhibitors

A large number of chemicals have been studied for their efficacy in inhibiting CH_4 production. Some chemicals, including chloroform, amichloral and 2-

bromoethanesulphonic acid (BES), can be used directly to suppress methanogenesis, but they have an adverse effect on the animal (liver damage and even death) (Dong *et al*., 1999). However, the effect of BES is momentary and CH_4 production rapidly returns to its original level of emission within some days (McAllister & Newbold, 2008). Statins are chemical compounds used in humans to reduce cholesterol synthesis, but they can also reduce CH_4 production by inhibiting the synthesis of mevalonate (a key factor for isoprenoid). Ramin *et al*. (2010) reported a reduction in total gas production after fermenting palm kernel cake with fungi. The reduction could be due to production of some special compounds such as statins during the fermentation, thereby inhibiting the emission of gases.

Wolin & Miller (2006) reported that adding HMG-CoA (mevastatin) *in vitro* to a co-culture comprising CH_4-producing bacteria and other eubacterial species, mostly cellulolytic, inhibited the growth of *Methanobrevibacter* without causing any growth inhibition on other bacterial species. Chemical therapy is not an option for mitigation purposes in the European Union due to the restrictions on use of chemically synthesised additives in livestock.

1.5.6 Ionophores

Ionophores are lipid-soluble molecules. Some ionophores are used as antibiotics or as growth-enhancing feed additives for growing cattle. Adding ionophores to the diet of ruminants has been shown to reduce CH_4 production (Moss *et al*., 2000). It promotes the production of propionate at the expense of acetate and reduces feed intake (Johnson & Johnson, 1995; Baker, 1999; Hegarty, 1999b). Wolin & Miller (2006) reported that CH_4-producing Gram-positive bacteria are not resistant to ionosphere antibiotics (monensin) but the Gram-negative bacteria are. Monensin causes high intracellular NADH/NAD^+, which reoxidises NADH while limiting the H_2-yielding acetate pathway (Hegarty, 1999b).

Ionophoric antibodies (*e.g*. monensin and related compounds) are prohibited within the European Union countries. Research has focused on the use of other compounds that can be substituted for ionophores but have the same inhibitory effect. Plant extracts have shown inhibitory effects on CH_4 production. Using a rumen simulation technique, McAllister & Newbold (2008) reported that a commercial allicin product originating from garlic was effective in inhibiting CH_4 production at a dose of 20 µg/mL without any adverse effect on daily VFA production. They concluded that the inhibitory effect was mainly a direct effect on methanogen DNA. Hops acids are effective additives that have been used to inhibit CH_4 production. Narvaez *et al*. (2011) showed that by increasing the concentration of hops acid from 50 to 400

µg/mL in an *in vitro* gas production system, CH_4 production quadratically decreased per unit DM digested. Tannins have also been reported to reduce emissions of CH_4 from ruminants, either by reducing the methanogen population in the rumen directly or by reducing the protozoa population, which tends to increase the molar proportion of propionate in the rumen (Bhatta *et al*., 2009). Tavendale *et al*. (2005) reported a decrease in CH_4 production when legume forages containing condensed tannins were incubated *in vitro*. A reduction in H_2 production or direct inhibitory effects on methanogens were the suggested mechanisms by which tannins reduced CH_4 production.

1.5.7 Defaunation

Protozoa are microorganisms living in the rumen that contribute up to 50% of fibrolytic activity within the rumen (Coleman, 1986). Defaunation is an effective mitigation strategy, *i.e*. elimination of protozoa from the rumen results in a decrease in CH_4 production (Moss *et al*., 2000; McAllister & Newbold, 2008). Defaunation can be done by lowering the pH in the rumen (feeding grain) or giving oils such as highly unsaturated C18 fatty acids (linseed oil) to the animal (Hegarty, 1999a). Defaunation is a process that disrupts the cross-feeding between ruminal protozoa and *Archaea* in the rumen. Defaunation increases the proportion of propionate in the rumen, improves the efficiency of microbial cell synthesis and decreases diet digestibility (Eugène *et al*., 2004), all of which can contribute to reduced CH_4 production. In a study by Schönhusen *et al*. (2003), reduced CH_4 production in defaunated calves was associated with lower OM and carbohydrate digestibility.

1.5.8 Acetogenesis

Potential microbes for CO_2-acetate fermentation exist in the rumen, but CO_2-CH_4 fermentation is always predominant in the rumen (Crutzen, 1995; Fievez *et al*., 1999). Methanogens keep the rumen H_2 pressure low, which is an inhibitory factor for CO_2-acetate fermentation microbes in the rumen. Elimination of methanogens from the rumen and their replacement with acetogens has been reported in the literature (Crutzen, 1995). Adding acetogenic bacteria daily as a feed additive to the diet of ruminants can inhibit CH_4 production, as they compete with methanogens by using H_2 and producing acetic acid. On the other hand, it has been reported that in the rumen conditions, acetogens are unable to compete with methanogenic *Archaea*. Acetogens are more active in the gut of termites and the human colon than in the rumen (Immig, 1996; Klieve & Hegarty, 1999). Fievez *et al*. (1999) reported that acetogens are more numerous in the hind-gut than in the rumen of the dairy cow. Methane production was much lower with faecal inoculum

compared with rumen fluid in an *in vitro* study by Ramin *et al*. (2013a), also indicating less CH_4 production from hind-gut fermentation compared with rumen fermentation. Thermodynamically, the conversion of CO_2 and H_2 to CH_4 is much more favourable than the conversion to acetate via acetogens ($\Delta G°$=-135.6 KJ and $\Delta G°$=-104.6 KJ, respectively) (Joblin, 1999). If it takes place in the rumen, the reaction is:

$$8H_2+4CO_2 \rightarrow 2CH_3COOH+4H_20$$

1.5.9 Vaccines and other factors

Vaccination is a novel strategy to reduce CH_4 production in ruminants. It is based on a continuous supply of antibodies to *Archaea* through the saliva in the animal. However, attempts to use vaccines *in vivo* against methanogens have not been successful (Wright *et al*., 2004). One reason could be the growth of other methanogenic strains in the rumen to replace those methanogens against which the antibodies are generated (McAllister & Newbold, 2008). However, the vaccination strategy to reduce CH_4 production is an attractive method, as it can be applied to all types of ruminants (Clark, 2013).

Biological strategies to control methanogens are one approach influencing CH_4 production. *Archaeal* viruses and bacteriocins, *e.g*. nisin produced by *Lactococcus lactis*, are safe, natural feed additives that can be used to control rumen methanogens. *Archaeal* viruses (*e.g*. bacteriocins) are biological treatments that raise the H_2 pressure sufficiently in the rumen to initiate acetogenesis (Klieve & Hegarty, 1999).

Exogenous enzymes and direct-fed microbials (*e.g*. yeast-based products) have also been examined as CH_4 inhibitors, but data concerning the effect of both these groups as CH_4 inhibitor agents are limited. Because of the inconsistent effects of these groups on CH_4 production, they cannot be recommended as an effective mitigation practice (Hristov *et al*., 2013a).

Supplementation of diets with electron receptors such as fumarate, nitrates and sulphates is a CH_4-mitigating strategy that has recently received attention. The reduction of nitrate and sulphate is energetically more favourable than CH_4 production (Van Zijderveld *et al*., 2010). Nitrates may be a promising enteric CH_4 mitigation agent, especially in low-protein diets, but care should be taken to avoid any toxicity to the animals (Hristov *et al*., 2013a). In principle, nitrates can replace urea as a source of rumen-degradable N, but they are more expensive than urea and the health risks are greater.

One problem with many additives for reducing CH_4 production could be an excess of H_2 in the rumen if alternative H_2 sinks cannot completely replace CH_4 production. This phenomenon has been observed especially with CH_4

inhibitors such as trichloroethyladipate (Czerkawski & Breckenridge, 1977) (Figure 10).

Figure 10. Cartoon showing the side effects of dietary additives to inhibit CH_4 production Reprinted from: An Introduction to Rumen Studies by J.W. Czerkawski, page 172. Copyright © (1986).

Generally, some additives and other factors have the potential to mitigate CH_4 production from ruminants, as discussed earlier. However, there are also some disadvantages, *e.g*. they are costly and not economical to apply at farm level, and many have only short-term effects on CH_4 production. Therefore, multi-factorial mitigation approaches, such as inhibition of methanogens, involvement of other H_2 alternative sinks and *e.g*. inclusion of dietary fat, are needed to result in a reasonable reduction in CH_4 production.

The most cost-effective way to reduce CH_4 production is to improve feed efficiency. In low intensity systems, CH_4 production per unit product can be markedly reduced by improving feed quality and feeding intensity, thereby diluting the maintenance costs. However, in intensive systems the potential to reduce CH_4 production by increasing production level is rather limited. Selecting animals for improved feed efficiency can be the best strategy to reduce CH_4 production per unit product in these systems. Increasing the longevity of dairy cows also has high potential to improve lifetime feed efficiency and reduce CH_4 production per kg milk.

1.6 Measurements of methane production

1.6.1 Equipment and devices

Different methods have been developed to measure CH_4 production in ruminants. The respiration chamber technique (Figure 11) is the most accurate method of measuring CH_4 production by ruminants (Johnson & Johnson, 1995). The chamber method can thus be considered a reference method for evaluating the accuracy of other experimental techniques, as well as the performance of empirical and mechanistic models in predicting CH_4 production. The principle of the respiration chamber is to collect all exhaled breath from the animal and to measure gas, *e.g*. CH_4 concentration. The animal is placed in a chamber for about 2-4 days with ventilation for intake and exhaust air. To keep the air moving within the chamber, fresh air flow is also applied to the recycling fan (McGinn *et al*., 2006). Methane flux (g/h) is calculated as air flow (L/h) × CH_4 concentration (g/L). Concentrations of CH_4 and other gases are corrected for background concentrations. The chambers described by Hellwing *et al*. (2012) are covered with transparent polycarbonate walls so that animals have visual contact with other animals in the house, to ensure that animal welfare and DMI are not influenced. One disadvantage of the chamber technique could be underestimation of fluxes due to the installation of ventilators inside the chamber, enhancing air exchange through the entrance (Greatorex, 2000). The construction costs and labour requirement are the main disadvantages with this technique. It has also been argued that the animals are not in their natural environment, which can influence their behaviour and feed intake, but Hellwing *et al*. (2012) found no reduction in DMI when cows were confined in chambers.

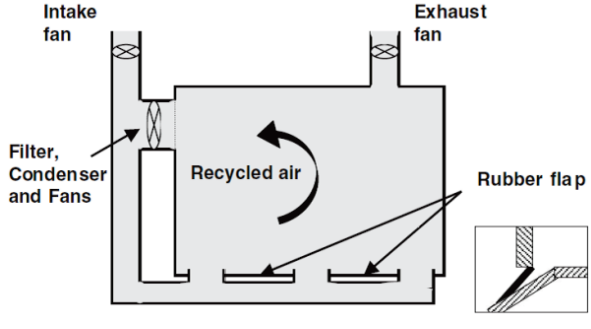


Figure 11. Respiration chamber technique (source: McGinn *et al*., 2006).

Sulphur hexafluoride (SF_6) determination is an alternative method for measuring CH_4 production *in vivo*. This technique was first developed by

Johnson *et al*. (1994) and is known as the tracer technique. In this technique, a known source of a gas (permeation tube) is placed inside the rumen before the experiment starts and the breath of the animal is sampled. The concentration of the gases is determined using gas chromatography, and the ratio of CH_4 to SF_6 is determined. It has been reported that 95% of CH_4 is emitted through the mouth and nose, suggesting that the contribution of rectal emissions to the error in measurements of CH_4 production is small (Ulyatt *et al*., 1999). The tracer technique is mainly designed for grazing cattle, but measurements can also be performed in regular farming situations (Ulyatt *et al*., 1999; McGinn *et al*., 2006). In animal house conditions, care should be exercised to take background concentrations into account. The SF_6 gas itself is a GHG and it is not recommended for use with cannulated animals, as leakage from the cannula could lead to underestimation of CH_4 production if the gas ratios in leakage air differed from those in outbreath air (Greatorex, 2000; Beauchemin *et al*., 2012).

The release rate of the SF_6 gas from the permeation tubes is an important factor, as a variation in measurements of CH_4 production might occur if the release rate changed. Grainger *et al*. (2007) compared the SF_6 method with the chamber method and concluded that CH_4 production measured by both methods was similar. However, they reported higher variability between days within cows with the SF_6 method. Variability among cows was also substantially higher than within cows and was higher for the SF_6 technique than for the respiration chamber (Grainger *et al*., 2007).

Recently, methods based on sampling the air released by eructation during milking have been developed. In the method developed by Garnsworthy *et al*. (2012), air is sampled continuously from the feed bins in the milking stations. The device can also be installed in the concentrate feeder. Methane concentration is measured using one infrared CH_4 analyser per unit. The relationship between CH_4 production index and CH_4 production measured by respiration chambers is good, but between-animal variability is much greater with the CH_4 production index than for CH_4 production measured in chambers. In farm conditions, CH_4 production index was highly variable between the cows (overall coefficient of variation, CV=0.63, in individual farms 0.25-0.69) and also the range between farms (2.4-fold) was greater than could be expected (Bell *et al*., 2013).

The technique developed by Madsen *et al*. (2010) is based on the principle of using CO_2 as a tracer gas. Methane and CO_2 concentrations are measured from air samples when the cows visit automatic milking systems or automatic concentrate feeders. Total CO_2 production is estimated from information on intake of metabolisable energy or heat-producing units. Methane production is

then calculated from CH_4:CO_2 ratio and CO_2 production. Estimation of CO_2 production is based on the assumption that there is no variation in the efficiency of feed utilisation between cows, which may not hold true.

A new method called GreenFeed (C-Lock Inc, Rapid City, South Dakota, USA) has recently been developed to measure real-time CO_2 and CH_4 mass fluxes from a herd/flock of animals. The number and length of the visits can be adjusted. One unit can be used for 25-30 animals. A small amount of concentrate feed is released from the feed bin, which attracts the animal. Once the animal is in, the breath together with the air flow enters the system, travels through the pipes and gets mixed within a fan. After passing through the fan, a sample of gas is taken and subsequently analysed for CH_4 and CO_2 concentrations (Figure 12). The system also includes a head position sensor and when the head of the cow is not in the right position, the data are filtered out.

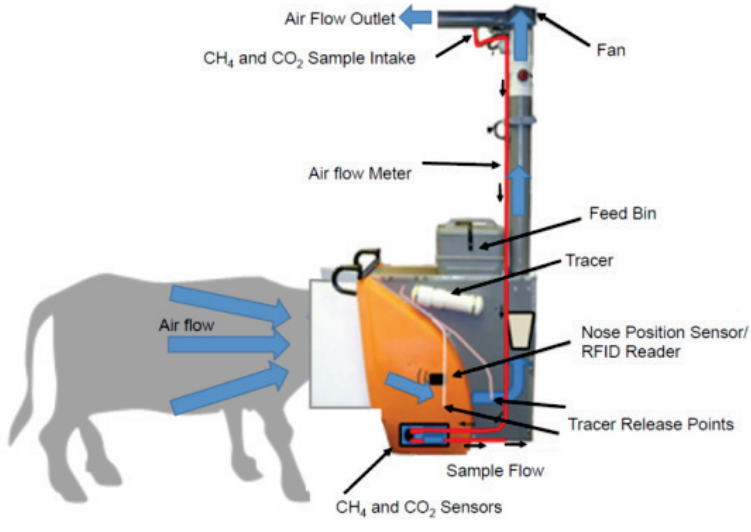


Figure 12. General layout of the GreenFeed system in the Stand-Alone Feeder (source: GreenFeed Stand-Alone Feeder instruction manual, C-lock; Zimmerman, 2011).

Both the SF_6 technique and methods based on sampling of outbreath air tend to give larger between-animal variability than respiration chambers. Huhtanen *et al*. (2013a) found that between-animal variability was similar or slightly greater with the GreenFeed measurements compared with chamber studies. Hammond *et al*. (2013) reported a non-significant difference between mean CH_4 production from heifers when CH_4 production was measured by the GreenFeed device and by respiration chamber (215 and 198 g/d, respectively).

Average DMI was the same for both techniques. In the same study, values obtained using the GreenFeed method were not comparable with those from the SF_6 method. The authors attributed this to a lower number of visits to GreenFeed during grazing measurements (Hammond *et al*., 2013). An advantage of the methods described by Madsen *et al*. (2010) and Garnsworthy *et al*. (2012) and the GreenFeed method is that a large number of animals in their natural environment can be measured with relatively low investment and labour costs.

The hand-held laser method, which uses a near-infrared diode laser to detect CH_4, has also been used for measuring CH_4 production (Chagunda *et al*., 2013). However, there was a greater variation for cows when CH_4 production was measured with the hand-held laser method compared with respiration chamber (CV=46.1% and 9.54%, respectively).

In vitro gas production methods have been developed to evaluate factors influencing digestibility and rumen fermentation, but the systems can also be used to measure CH_4 production from a given feed or diet. *In vitro* methods also allow screening of a large number of samples. Czerkawski & Breckenridge (1977) designed a continuous culture, rumen simulation technique (RUSITEC) in which the fermentation continues for several days or even weeks, so that CH_4 measurements can be applicable for a given amount of feed. Batch culture is another *in vitro* method for measuring fermentation parameters from feed samples (Van Nevel & Demeyer, 1981; Demeyer, 1991). Feeds can be incubated in bags and then incubated in buffered rumen fluid. In batch cultures, CH_4 measurements are made using end-point measurements, or occasionally two time-points. These methods do not take into account the dynamics of digestion and passage kinetics in the rumen. One advantage of the *in vitro* methods is that H_2 recovery can be calculated.

Methane can also be measured using *in vitro* gas production techniques. Menke & Steingass (1988) developed a syringe-based *in vitro* method which has been widely used for CH_4 measurements from feed samples. Cone *et al*. (1996) developed a fully automated *in vitro* gas production method in which the recordings of gas production are based on the number of openings of gas valves at an adjustable overpressure. This allows digestion kinetics to be investigated in a large number of samples without any possible influence of excess pressure on fermentation.

Recently, Pellikaan *et al*. (2011) developed the application of an *in vitro* gas system in order to measure CH_4 production over time. A sample of gas is drawn from the headspace of the fermentation unit and injected into a gas chromatograph. By knowing the concentration of CH_4 in the headspace and the volume of the bottle, CH_4 production can be measured. The *in vitro* methods

suffer from the disadvantage that they only simulate ruminal fermentation of feed, not the emissions and digestibility from the entire animal. Comparison of results obtained with *in vitro* techniques with more standard methods such as respiration chambers is desirable. Such work is being conducted in our laboratory.

2 Objectives

Several *in vitro* methods for measuring CH_4 production are described in the literature, but to date the *in vitro* data obtained have rarely been used to predict actual CH_4 production *in vivo*. Therefore, there is a need for such work. In addition, models for predicting CH_4 production would be useful for national inventories and for developing mitigation strategies. It would be practical and useful if reliable prediction models could be developed using animal and feed data that are easily available without extra costs. The overall aims of the studies described in this thesis were to determine the factors influencing CH_4 production in ruminants, simulate CH_4 production (empirical and mechanistic models) and validate the models predicting CH_4 production in dairy cows, with specific emphasis on diets fed at northern latitudes. Specific objectives of investigating CH_4 production in dairy cows were:

- To develop an application of an *in vitro* method to predict CH_4 production *in vivo* from kinetic parameters of CH_4 production.
- To develop empirical models predicting CH_4 production in ruminants.
- To revise and describe the sub-model of the dynamic, mechanistic dairy cow model Karoline in predicting CH_4 production.
- To evaluate the Karoline model in predicting CH_4 production.

3 Materials and methods

3.1 Paper I

In order to improve application of the automated gas *in vitro* system and to allow prediction of CH_4 production *in vivo*, four different levels (300, 600, 900 and 1200 mg) of milled timothy hay were used in Paper I. The *in vitro* method used was that described by Cone *et al*. (1996). Figure 13 shows the system used in the Paper I. Appropriate amounts of sample were placed in bottles, which were then filled with 60 mL buffered rumen fluid and placed in a water bath at 39°C. Readings of total gas production were made every 12 min and corrected to the standard air pressure (101.3 kPa).

A sample of gas (200 µL) was drawn from the headspace of each bottle at 2, 4, 8, 24, 32 and 48 h and injected into a gas chromatograph (Varian, USA) to determine the concentration of CH_4 gas. Methane concentration in each bottle measured at different time-points was then plotted against time to develop a model for estimating CH_4 concentration at each time-point. A logarithmic model resulted in the best fit between incubation time and CH_4 concentration. A mechanistic model based on fermentation stoichiometry and fermentation kinetics was developed to estimate relative CH_4 concentration in outflow and headspace gas. Methane and total gas production, separately estimated at each 12-min interval, were then subjected to the two-pool Gompertz model to estimate kinetic parameters. The kinetic parameters for CH_4 data were introduced into a dynamic, mechanistic model (Huhtanen *et al*., 2008) to predict CH_4 production *in vivo*. The predictions were made for maintenance level of intake using 50 h rumen residence time (20 + 30 h in the two rumen compartments) corresponding to the maintenance level of intake.

In order to evaluate the *in vitro* gas production system, CH_4 production was also predicted from VFA production using equations on rumen VFA fermentation stoichiometry as described by Wolin (1960).

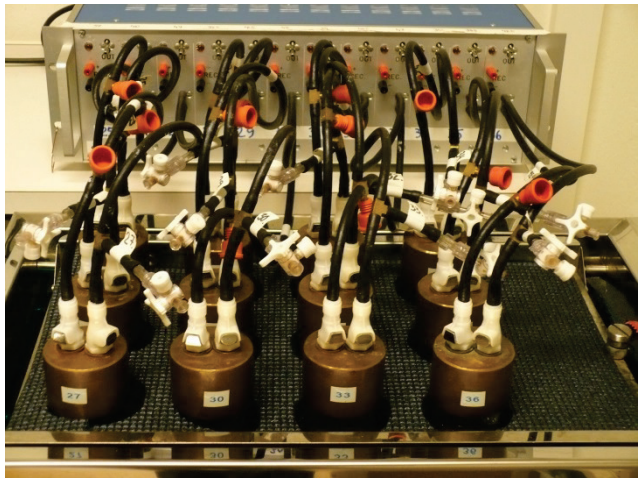


Figure 13. The fully automated gas *in vitro* system used for recording total gas and CH_4 production at SLU, Umeå (photo: Mohammad Ramin).

3.2 Paper II

In Paper II, a data set of CH_4 measurements was collected from 52 papers published 1960-2011, including a total of 298 observations (treatment means). Only the data from studies conducted using respiration chambers were included. The minimum prerequisite for inclusion in the data set was that DMI, diet digestibility, dietary ingredient composition, and some parameter of forage digestibility or fibre were reported. Because the objective of Paper II was to develop prediction models for dairy cow diets, diets containing more than 75% concentrate on a DM basis were excluded from the analysis. Sheep data were included in the data set to develop the model predicting CH_4-E/GE, as the relationship between sheep and dairy cattle fed the same diets was high in a study of Schiemann *et al*. (1972) (R^2=0.85, n=21). A mixed model regression analysis with random study effect using the SAS procedure was used to evaluate the relationship between independent and dependent variables. The multiple regression models were developed by running the iterations in the mixed model procedure beginning from combinations of intake, digestibility and fat variables. The first three variables were selected because it is known that CH_4 production is related to feeding level, digestibility and dietary fat concentration. The best fit model was selected based on the smallest root mean square error (adjusted for random study effect) and Akaike's information criterion. Thereafter, additional feed variables were included in the models. These variables were included in the final model if they improved it according to the two criteria and if the effect was significant (P<0.05). The models

developed were then evaluated within the same data set using cross-validation by dividing the data into six subsets, five of which were used for model development and one for model evaluation. The split was made experiment-wise, so that all data from one study were in the same subset. Each subset was omitted in turn and the CH_4 production model was developed based on the remaining five subsets. The resulting model parameters were used to compute predicted CH_4 production for the observation in the excluded subset. The procedure was repeated for all subsets. The same approach was used after dividing the data set into two subsets.

3.3 Paper III

Because the models developed in Paper II for predicting total CH_4 production can result in biased estimates beyond the range of DMI in the data from which the models were developed, a non-linear mixed modelling approach was applied in Paper III. Two different non-linear models, power and exponential (Mitscherlich) functions, were used for model development. The data set used in Paper II (only those reported for cattle data; n=207 treatment means) was used for developing these non-linear models, both of which are based on DMI. Because any increase in CH_4 production with increasing DMI would depend on dietary composition and diet digestibility, both models were extended to include these factors. The factors adjusted the exponent of both models. The PROC NLMIXED in SAS was used to estimate parameter values, using study as the random effect and diet variables as the fixed effects to adjust the exponent (power) of both functions.

3.4 Paper IV

The aim of Paper IV was to describe and develop the sub-model of the Nordic dairy cow model Karoline in predicting CH_4 production. The Karoline model is a dynamic and mechanistic model that describes digestion and metabolism in dairy cows. In the model, dietary carbohydrates are divided into the following fractions: NDF, which in turn is divided into forage potential digestible NDF (pdNDF) and concentrate pdNDF and corresponding indigestible fractions of forage indigestible neutral detergent fibre (iNDF) and concentrate iNDF, starch, lactic acid, acetic acid, propionic acid and butyric acid and the rest fraction. Dietary crude protein (CP) is described as ammonia N, amino acids, peptides, soluble true protein, insoluble protein and potentially indigestible protein. Dietary fat concentration is described as ether extract (EE), which is converted to fatty acids using separate empirical models for forage and

concentrate EE. The rest fraction is calculated as the difference between organic matter and the sum of other carbohydrates, CP and EE. It is a heterogeneous fraction including different components such as water-soluble carbohydrates (WSC), pectins, plant organic acids and alcohols produced in silage fermentation. Feed fractions have specific feed digestion rates and WSC, lactic acid and soluble non-protein fractions have a common general rate.

In the sub-model of CH_4 production, the following descriptions and changes were included: Carbon from the fermentation of carbohydrates and deamination of amino acids was distributed according to stoichiometric equations. The amount of truly fermented substrate was adjusted for the microbial uptake of carbon. This process is regulated by the ATP supply for microbial cell synthesis. Methane production in the hind-gut was adjusted to 60% of the value predicted on the basis of stoichiometric principles. This adjustment was made to account for the observed lower CH_4 production in the hind-gut, probably as a result of acetogenesis.

Because microbial cells are more reduced than dietary carbohydrates, stoichiometric fermentation balance will overestimate CH_4 production. To account for the more reduced status of microbial cells compared with dietary carbohydrates, CH_4 production was adjusted to an uptake of 8.1 H_2/kg cells. The reduction in CH_4 (moles) was calculated as H_2 (moles)/4. As dietary fatty acids are extensively bio-hydrogenated in the rumen, thereby acting as a H_2 sink, the contribution of bio-hydrogenation to CH_4 production was taken into account.

A sensitivity analysis was conducted in order to evaluate the robustness of the model and the importance of the accuracy parameters required in the Karoline model for predicting CH_4 production. The effects of intake and of some diet and digestion parameters on CH_4 production were evaluated with a mixed dairy cow diet. The diet consisted of 60% grass silage and 40% concentrate on a DM basis. The model simulations were made for feed intake by increasing DMI from 10 to 26 kg/d at 2-kg intervals for a 600 kg dairy cow. The effects of some diet and digestion variables on predictions of CH_4 production were evaluated using a constant DMI of 20 kg/d (forage:concentrate ratio 60:40 on a DM basis) for a 600 kg dairy cow. The effects of iNDF, protein, digestion rates of pdNFD in forage and concentrate feeds on CH_4 production were estimated at 5-6 levels. The effects of increased concentrate feeding were evaluated by increasing concentrate DMI from 4 to 16 kg/d. At the same time, forage DMI was decreased from 14 to 8 kg/d (substitution rate 0.5). The effects of the extent and type of silage fermentation acids on CH_4 production were also evaluated.

3.5 Paper V

In Paper V, the Karoline model was evaluated for its ability to predict observed *in vivo* CH_4 production in experiments conducted using respiration chambers. A data set consisting of 184 treatment means was used in the evaluation. Most of the data were from the same data set as used in Paper II. Studies were selected using the same criteria as in Paper II, plus that the parameters required in the Karoline model could be estimated with reasonable accuracy. Some studies used in Paper II were excluded because it was not possible to derive required parameter values. Tabulated values from the Cornell Net Carbohydrate and Protein System (Tylutki *et al*., 2008) and MTT (2013) were used for missing values of feed composition that were not reported. In most cases forage iNDF was not reported and it had to be estimated from organic matter digestibility (OMD) using empirical relationships. Potentially digestible neutral detergent fibre was calculated as NDF – iNDF and pdNDF digestibility (pdNDFD) was calculated as digested NDF/pdNDF. For the concentrate ingredients, iNDF values were based on 12-d ruminal *in situ* incubations (MTT data) or, when data were not available, iNDF was calculated as 2.4 × lignin. If forage ammonia N was not reported, values of 0, 100 and 50 g/kg N were assigned for hay, grass and legume silages and maize silage, respectively. Indigestible protein (IDP) in forages was estimated from iNDF using empirical relationships. For soluble true protein (SPN), values of 50 and 100 g/kg N were used for silage and hay, respectively. Insoluble protein (ISP) values and their corresponding degradation rate were taken from the Cornell Net Carbohydrate Protein System (Tylutki *et al*., 2008). Amino N + Peptide N (ratio 75:25) was calculated as: 1000 – Ammonia N – SPN – ISP – IDP. The results obtained after Karoline simulations were then subjected to the fixed and mixed model regression analysis of the SAS programme in order to further evaluate the CH_4 predictions. Residual analysis was performed by regressing the residual (observed CH_4 production – predicted CH_4 production) against centred predicted values. Some dietary factors such as DMI, EE, NDF, starch, proportion of concentrate and OMD known to affect CH_4 production were regressed against residuals (Observed – Predicted) of CH_4 production in order to detect possible sources of failure in model performance.

4 Results

4.1 Paper I

In Paper I, when the sample size was increased from 300 to 1200 mg, predicted *in vivo* CH_4 production at maintenance level of intake decreased linearly (P_{LIN}<0.01), from 36.9 to 28.2 mL/g DM. The first-order rate of CH_4 production was not influenced by the sample size (P=0.12). The effects of sample size on total gas production followed the same pattern as CH_4 production, decreasing linearly (P_{LIN}<0.01) from 209 to 177 mL/g DM. The rate of gas production remained unchanged (P=0.18). The molar proportion of acetic acid decreased, whereas that of propionate increased with increasing sample size. Digestibility of NDF decreased from 0.479 to 0.369, and true OMD from 0.681 to 0.614, with increased sample size. There was a good relationship (R^2=0.97) between predicted (stoichiometry) and observed (determined with the *in vitro* gas system) CH_4 production, indicating the accuracy of the system. Mean or slope biases were not detected. The model predicted the ratio between headspace and outflow gas to be 0.55 and it was not markedly influenced by fermentation pattern or digestion rate, but it increased with fermentation time.

4.2 Paper II

A set of models was developed in order to predict CH_4 production from variables that are known or could be predicted with a reasonable accuracy at the time of feeding. The selected variables known to influence CH_4 production most were DMI, digestibility and fat. Dry matter intake, expressed as a proportion of body weight (DMIBW), OMD estimated at the maintenance level of feeding (OMD_m) and dietary concentrations of NDF, non-fibre carbohydrates (NFC) and EE were the variables of the best-fit model predicting

CH_4 energy as a proportion of GE intake: CH_4-E/GE (kJ/MJ) = -0.6 (±12.76) - 0.70 (±0.072) × DMIBW (g/kg) + 0.076 (±0.0118) × OMD_m (g/kg) - 0.13 (±0.020) × EE (g/kg DM) + 0.046 (±0.0097) × NDF (g/kg DM) + 0.044 (±0.0094) × NFC (g/kg DM). Adjusted root mean square error (RMSE) for the best model was 3.26 kJ/MJ (4.65% of the observed mean). Total CH_4 production (L/d) in the cattle data was closely related to DM intake. However, further inclusion of other variables improved the model: CH_4 (L/d) = -64.0 (±35.0) + 26.0 (±1.02) × DMI (kg/d) - 0.61 (±0.132) × $DMI^2_{(centred)}$ + 0.25 (±0.051) × OMD_m (g/kg) - 66.4 (±8.22) × EE (kg DM/d) - 45.0 (±23.50) × NFC / (NDF + NFC), with adj. RMSE of 21.1 L/d (5.6% of the observed mean). The quadratic term was centred in order to avoid high correlation and variance inflation factor between the linear and quadratic effects of DMI. Cross-validation of the CH_4-E/GE model (Observed CH_4-E/GE = 0.96 (±0.103) × Predicted CH_4-E/GE + 2.3 (±7.05); R^2=0.85, 4.82% of the observed mean) indicated that the differences between the diets in terms of CH_4 production could be predicted accurately.

4.3 Paper III

Non-linear models were developed using a data set of only cattle data from studies in which CH_4 production was measured in respiration chambers (n=207). The simple power function model developed was CH_4 (L/d) = 51.5(±4.5) × DMI, kg/d $^{0.792(\pm0.034)}$ (adj. RMSE = 25.5 L/d), whereas the exponential model developed (Mitscherlich) was CH_4 (L/d) = 976(±95.3) × [(1 – $e^{(-0.0407(\pm0.00510) \times \text{DMI (kg/d)})}$], (adj. RMSE = 25.0 L/d). Adjusting the exponents for dietary concentration of EE, proportion of NFC in total carbohydrates and OMD_m improved the models. The effects of all these factors were significant (at least $P<0.05$) with both models. In both models, the effects of the changes in adjustment factors (*e.g.* EE) increased with increased DMI.

4.4 Paper IV

Modifications were made in the equations predicting digesta passage kinetics, microbial cell synthesis, digestion in the hind-gut, and utilisation of H_2. The Karoline model predicted similar decreases in OMD and NDF digestibility and improvements in the efficiency of microbial nitrogen synthesis with increasing DMI, as reported in the published meta-analysis based on large data sets. The proportion of ruminal digestion in total NDF digestibility (0.95) and faecal metabolic and endogenous output (98 g/kg DMI) also agreed with the literature data. The results of the sensitivity analysis indicated that by increasing DMI

from 10 to 26 kg/d, predicted OMD and NDF digestibility could be decreased and the efficiency of microbial protein synthesis (EMPS) increased. Total predicted CH_4 production increased quadratically from about 300 L/d to 700 L/d. However, when CH_4 production was expressed as a proportion of GE intake, it decreased from 80 to 57 kJ/MJ. Increased iNDF concentration in both forages and concentrates decreased OMD and CH_4 production, while increased rates of pdNDF increased OMD and total CH_4 production (L/d). Predicted CH_4 production increased with the level of concentrate supplementation, mainly as a result of increased total DMI. However, it decreased when expressed as CH_4-E/GE. Increasing the concentrate fat concentration in the diet decreased predicted CH_4 production. Increased fat supply also increased the molar proportion of propionate of total VFA. Total acids in silage were negatively related to total CH_4 production, as increasing total acids from 40 to 160 g/kg DM decreased total predicted CH_4 production from 597 to 580 L/d. However, the effects of digestion rate of soluble N components (free AA, peptides, soluble true protein) were small, as were the effects of distribution of AA and peptide N in soluble non-ammonia N. The results indicated that accurate estimates of digestion parameters are essential for good performance of mechanistic models, whereas the accuracy of protein variables is less important.

4.5 Paper V

One problem in evaluating mechanistic models predicting CH_4 production is inadequate input data. Much of the input data were based on tabulated values or had to be estimated from other variables. The Karoline model predicted the mean of OMD accurately, with no mean bias compared with published values (723 g/kg). Molar proportions of VFA were predicted with reasonable accuracy by the Karoline model compared with the published values, but the predicted values were less variable than the observed values. Karoline slightly under-estimated the mean of total CH_4 production compared with published values (413 and 421 L/d, respectively). When analysed with the fixed model regression, the correlation between predicted and observed CH_4 production was quite high (R^2=0.93, 10.1% of the observed mean). However, the R^2 increased to 0.98 (6.1% of the observed mean) when analysed with the mixed model regression (n=184). The relative error (adj. RMSE) was 5.1% when analysed with the mixed model regression, which corresponds to random variation around the regression line. When laboratory was assigned in the model as subject variable instead of study, the relative error increased to 8.8% (of the observed mean) and R^2 declined to 0.95. This increase is an indication

of high contribution of study within laboratory to overall variability. The Karoline model slightly underestimated CH_4 production (7.9 L/d; $P<0.05$), but the slope bias was not significant ($P=0.53$). The relationships between dietary input variables (DMI, OMD, CP, EE and NDF) and the residuals of CH_4 production (Observed CH_4 production – Predicted CH_4 production) were not significant. Furthermore, dietary starch concentration ($P=0.60$) and proportion of concentrate in the diet (P=0.72) were not significantly related to the residuals. However, there was a significant relationship between the residuals of CH_4 production and the digestible OM concentration and intake. Laboratory had a significant effect on the residuals of CH_4 production.

5 Discussion

The contribution of ruminants to anthropogenic CH_4 production is high. Enteric fermentation is the main contributor to CH_4 production, while CH_4 production from manure contributes to a smaller extent. Various experimental techniques and mathematical models for determining or estimating CH_4 production are described in the literature. Methods able to measure CH_4 production from ruminants vary from very simple (*e.g. in vitro*) to more sophisticated and reliable apparatuses (*e.g*. respiration chambers). However, the application of the automated *in vitro* gas production system could be upgraded to determine kinetic parameters of CH_4 production. These kinetic parameters could then be used in mechanistic models taking into account the dynamics of rumen passage kinetics to predict CH_4 production *in vivo* for animals fed at different intake levels.

In vitro methods could be an appropriate choice for screening purposes. Different feeds and additives could be tested to evaluate their possible effects on CH_4 production in *in vivo* conditions. The choice between different techniques measuring CH_4 production depends on investment and labour costs, number of animals to be measured, and the requirements on data accuracy. In addition, mathematical models (empirical and mechanistic) are an alternative approach to predict CH_4 production; for example, models are now applied in IPCC and national inventories. Reliable methods or models are also required to develop mitigation strategies.

It would be an advantage for practical models predicting CH_4 production to be based on simple input variables, without extra analytical costs. Most of the models developed to date are mainly based on regional databases and may not be suitable for animals fed different types of diets. For example, Shibata & Terada (2010) pointed out that due to differences in diet composition and production systems, the models developed using regional databases may not perform well in other conditions, *e.g*. the types of diets used in North America

differ from those fed to dairy cows in Europe (high grain and maize and grass silage-based diets, respectively). If the objective is to predict CH_4 production in certain conditions, the ideal would be to generate empirical models based on published data from studies in which similar rations were used. On the other hand, mechanistic models could be better able to handle different dietary conditions, provided that the biological mechanisms and their regulation are accurately described in the model and that the required input data are accurate.

Models can be classified into empirical models (*e.g.* Axelsson, 1949; Yan *et al.*, 2000; Jentsch *et al.*, 2007) and mechanistic models (*e.g.* Dijkstra *et al.*, 1992; Baldwin, 1995; Danfær *et al.*, 2006). Empirical models are based on nutrient intake and diet composition, whereas mechanistic models are based on mathematical description of rumen fermentation stoichiometry and digestion kinetics. One advantage of dynamic mechanistic models is that they can describe the system behaviour, and can therefore be useful in understanding the mechanisms influencing CH_4 production. Mechanistic models can be more applicable to different conditions provided that the input data are accurate. On the other hand, they require input variables that are seldom reported in published papers and need to be estimated in order to conduct the model simulations. Karoline is a mechanistic, dynamic model of a whole dairy cow developed in the Nordic countries (Danfær *et al.*, 2006). The Karoline model predicts nutrient digestion and metabolism, including CH_4 production. The transactions in the digestive tract are described by different equations based on first-order digestion rates or enzyme kinetics.

The studies included in this thesis focused on developing models predicting CH_4 production. For the *in vitro* approach (Paper I), the main target was to develop an application for the fully automated *in vitro* gas production system to predict CH_4 production *in vivo*. In Papers II and III, models predicting CH_4 production for typical dairy cow rations were developed. Paper IV describes the revisions made to the Karoline model in predicting CH_4 production. In Paper V, the Karoline model was evaluated using published data on CH_4 production from respiration chamber studies.

5.1 Estimation of CH_4 production by *in vitro* methods

Different *in vitro* methods have been used to measure CH_4 production. Van Nevel & Demeyer (1977) used batch cultures to measure CH_4 production, whereas Czerkawski & Breckenridge (1977) developed a continuous culture (RUSITEC) technique to investigate rumen metabolism, including measurements of CH_4 production. *In vitro* methods allow estimation of H_2 recovery, which provides useful information for the development of

mechanistic models. Batch systems are mostly used for screening purposes. End-point measurements are used for gas recordings. The *in vitro* gas production system was first developed to determine forage digestibility (Menke & Steingass, 1988). Automated systems (*e.g*. Cone *et al*., 1996) made it possible to determine gas production at different intervals during the incubation period. The problem with end-point measurements is that they do not take into account the dynamic nature of the rumen. Paper I showed that depending on the digestion rate, the length of the incubation period should be different with end-point incubations to correspond to a certain mean residence time in the dynamic rumen system. Recently, Pellikan *et al*. (2011) described a novel method for measuring CH_4 production at different time-points. Their method gives more detailed information on CH_4 production from different diets compared with end-point measurements. However, it still has the disadvantage that it does not predict *in vivo* CH_4 production.

In Paper I, the application of the *in vitro* gas production system was developed. To our knowledge, this is the first study to describe the kinetics of CH_4 production from an *in vitro* gas production system and to predict CH_4 production *in vivo* from ruminants. The same approach (kinetic parameters determined by the *in vitro* gas system and the same rumen model) has been found to predict *in vivo* NDF digestibility and digestion rate of pdNDF accurately and precisely (Huhtanen *et al.*, 2008). This confirms that the method can also be successfully used to predict CH_4 production. Frequent measurements of total gas and CH_4 production provide enough data points for estimating kinetic parameters with more sophisticated models such as the two-pool Gompertz model used in the present study (Paper I). Methane production can then be estimated at different feeding levels by regulating mean rumen retention time in the rumen model. With *in vivo* data, the model consistently predicted reduced CH_4 production per unit intake with increased feeding level. The first-order production rate of CH_4 was slower than that of total gas. This is consistent with the greater relative decreases in CH_4 production than diet digestibility with increased feeding level *in vivo*. The slower rate of CH_4 production compared with total gas production indicates that the proportion of CH_4 in total gas production increased with advancing incubation period. This can be related to fermentation of the least digestible components of feeds towards the end of the incubation period. The CH_4-E/GE in trials (data not shown) conducted using the *in vitro* method described in Paper I ranged from 6.5 to 8.8% (Table 2). These values compare well with *in vivo* data in animals fed similar diets (Blaxter & Clapperton, 1965; Yan *et al*., 2000). For calculating CH_4-E/GE, it was assumed that the dietary GE was 18.5 MJ/kg DM. Johnson & Johnson (1995) reported that depending on the type of feed

used, a loss of 2-15% CH_4-E/GE can occur. The typical range of CH_4-E/GE is somewhere between 6-7% with grass silage-based dairy cow diets (Yan *et al*., 2000). Some examples of calculated CH_4-E/GE from *in vitro* studies are given in Table 2. Calculated values of CH_4-E/GE were higher, 8-9% of CH_4-E/GE (Table 2), for the *in vitro* system used by Getachew *et al*. (2005). The values could be comparable with those reported by Ramin *et al*. (2012b), as Getachew *et al*. (2005) only used total mixed ration samples in their experiment. In both cases, a high CH_4-E/GE could be explained by the higher digestibility of feeds, leading to increased CH_4-E/GE. This thesis also showed that digestibility has a positive relationship with CH_4-E/GE (Papers II and IV). It should be noted that the predictions in the thesis were made for the maintenance level of intake.

To evaluate the *in vitro* gas production system (Paper I), CH_4 production was estimated by predicting CH_4 production based on VFA stoichiometric equations (Wolin, 1960). As shown in Figure 14, the good relationship between observed and predicted CH_4 production (R^2=0.97) showed the accuracy of the system in estimating CH_4 production. In line with this good relationship of predicted CH_4 from VFA stoichiometry (CH_4VFA) and actual measured CH_4 production (Paper I), the developed model (Eq. 26 in Paper II), also showed that the stoichiometric relationship between CH_4 and VFA improved the model more than single VFA or VFA ratios. The fermentation variables were included in the model with intake, digestibility and EE.

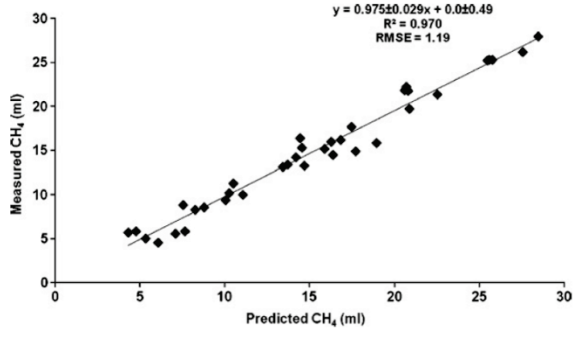


Figure 14. Relationship between CH_4 production predicted from VFA stoichiometry and measured using the *in vitro* gas system, as reported in Paper I.

The rumen simulation technique developed by Czerkawski & Breckenridge (1977) is a continuous culture *in vitro* method which has been widely used for determination of digestibility, fermentation pattern and gas production, including CH_4 production. Gas is usually collected in sampling bags,

increasing the risk of possible leakages and underestimation of CH_4 production. The values are clearly lower (by between 2-4%) for the RUSITEC technique (Machmüller *et al*., 1998; Bhatta *et al*., 2006a, 2006b; Giraldo *et al*., 2007; Garcia-Gonzalez *et al*., 2010) than in other studies in which the *in vitro* CH_4 production was measured by end-point batch cultures, or by the automated *in vitro* method developed in the present study (6.5-9%).

One reason for the lower values with the RUSITEC system could be the incubation of feeds in the bags. The exchange of fluid with samples could be low, resulting in biased estimates of CH_4 measurements (Ramin *et al*., 2013b). Ramin *et al*. (2013b) also reported an interaction between feed and method (incubated in bags or directly dispersed in the medium) for CH_4 measurements, indicating that even ranking of the feeds may not be correct by incubating feeds in bags. Possible reasons for the lower activity within bags are discussed in more detail by Krizsan *et al*. (2013). The second reason could be leakage of gases from the sampling bags, underestimating CH_4 production. A meta-analysis conducted by Hristov *et al*. (2012) also reported lower values for NDF digestibility in the RUSITEC continuous culture (34.2%, n=203) compared with non-RUTITEC continuous culture (45.5%, n=308), in which feeds are incubated freely dispersed. Longer incubation times in the RUSITEC system may also lead to the death of protozoa, resulting in lower production of butyrate and CH_4 (Bhatta *et al*., 2006b).

Table 2. *Methane production as a proportion of gross energy intake (GEI) calculated from different experiments in the literature*

Study	Type of feeds incubated	Method used	CH_4-%GEI
Ramin *et al*. (2013a)	Grass silage, hay, silage:barley	*In vitro*	6.5, 5.2, 7.8
Ramin *et al*. (2013b)	Alfalfa, grass silage, grass hay	*In vitro*	8.2, 8.0, 7.6
Ramin *et al*. (2012a)	Grass silage, whole crop silage	*In vitro*	6.5, 7.1
Ramin *et al*. (2012b)	Barley:grass silage	*In vitro*	8.8
Getachew *et al*. (2005)	TMR[1]	*In vitro*	8-9
Pellikaan *et al*. (2011)	Grass silage	*In vitro*	10.6
Narvaez *et al*. (2011)	Grass hay:alfalfa hay:barley silage	*In vitro*	7.4
Bhatta *et al*. (2006a)	Timothy hay: maize:soybean meal	RUSITEC[2]	2.1
Garcia-Gonzalez *et al*. (2010)	Hay:barley straw:barley grain	RUSITEC	2.5
Giraldo *et al*. (2007)	Grass hay:concentrate	RUSITEC	3.4
Machmüller *et al*. (1998)	Maize silage:barley:soybean meal	RUSITEC	1.7

[1]Total mixed ration including alfalfa hay, maize, cottonseed and soybean meal.
[2]Rumen simulation technique.

5.2 Factors influencing CH_4 production

Many dietary factors influence CH_4 production, with DM intake or feeding level being the main factor. In addition, diet digestibility and composition affect CH_4 production at a given intake level. In the following sections, the effects of these factors on CH_4 production and how these factors are taken into account in the models or the *in vitro* gas production procedure are discussed.

5.2.1 Dry matter intake

The significant effect of DMI on total CH_4 production in dairy cattle is well known in the literature, and feed intake is clearly the most important factor influencing CH_4 production in ruminants (Axelsson, 1949; Johnson & Johnson, 1995; Yan *et al*., 2000). Hristov *et al*. (2013b) showed a strong relationship between DMI and CH_4 production (R^2=0.86, n=377). Most of the models predicting CH_4 production published in the literature are based on some intake variable (DM, GE, digestible energy and metabolisable energy). Therefore, it is necessary to model the effect of DMI correctly on CH_4 production, and to understand the biological mechanisms relating to it. In models predicting CH_4 production, DMI is often used as the main predictor, but small improvements are obtained by including other variables, such as dietary composition, in the models (Ellis *et al*., 2007; Nielsen *et al*., 2013; Papers II and III). In some cases, no improvements were achieved by including other factors in the model (Mills *et al*., 2003), which also indicates the significant role of DMI in predicting CH_4 production.

When CH_4 production was determined *in vitro* with a modelling approach (Paper I), increasing the sample size from 300 to 1200 mg decreased CH_4 production from 8.0 to 6.1% of GE. Reduced CH_4 production can be attributed to reduced digestibility, changes in fermentation pattern and possible repartitioning of carbon between microbial cells and VFA production. In line with the *in vitro* study, CH_4-E/GE decreased as DMI per kg body weight increased (Paper II). The sensitivity analysis of the Karoline model (Paper IV) indicated that increased DMI had a negative effect on CH_4-E/GE. All findings relating the effect of DMI on CH_4 production (Papers I, II, III, IV) were in line with those reported in the literature indicating a decrease of CH_4 production per unit feed intake (Johnson & Johnson, 1995; Yan *et al*., 2000).

Reduced CH_4 production per unit intake with increased DMI can be partly explained by a decrease in OMD. The Karoline model predicted similar decreases in OMD with increased DMI to those observed in animal experiments (Yan *et al*., 2002; Huhtanen *et al*., 2009). Most of the decreases in CH_4 production per unit intake probably relate to improved efficiency of microbial protein synthesis (Paper IV). This has also been pointed out by

Russell *et al*. (1992), who stated that increased intake will lead to an increase in passage rate and, as a result, increased microbial cell yield per unit energy fermented. Because microbial cells are more reduced than carbohydrates (Hungate *et al*., 1971; Czerkawski, 1986; Van Soest, 1994), CH_4 production is likely to decrease with improved efficiency of microbial synthesis. It is possible that the decreases in CH_4 production observed in defaunated animals are at least partly related to improved EMPS in addition to reduced digestibility and changes in rumen fermentation pattern (Schönhusen *et al*., 2003). In *in vitro* studies, H_2 recovery has been incomplete and has shown wide ranges (78-96%), as reported by Demeyer (1991). Incomplete H_2 recovery is most likely associated with more reduced status of microbial cells compared with carbohydrates. Indeed, in the defaunation study by Schönhusen *et al*. (2003), CH_4 production was 42% greater in faunated than in defaunated animals, but changes in rumen fermentation pattern and digestibility accounted in total for about 60% of the difference. The most likely explanation is greater microbial synthesis in defaunated calves compared with faunated calves, as lower rumen ammonia N and higher D-alanine concentration also suggest.

Changes in fermentation pattern are one reason for reduced CH_4 production per unit DMI with increased intake. In Paper I, acetate decreased and propionate increased with increased sample size. This is in line with the findings of Sveinbjörnsson *et al*. (2006) from analysis of the Nordic data set. A decrease in the proportion of acetate and an increase in propionate with increased feeding level were reported by Schiemann *et al*. (1970) and Volden (1999). In Paper I the EMPS was not measured, but the distribution of carbon between microbial cells and VFA was most likely changed by increased sample size.

The models developed (Paper II) were based on linear functions. Because CH_4 production per unit intake decreases with increased intake, the overall relationship between intake and CH_4 production becomes curvilinear. Therefore, including a quadratic term for DMI effect in the model improved the fit. However, one disadvantage of linear modelling is that beyond the range of the intake data from which the model was developed, the estimates of CH_4 production could be biased. All published linear models have a positive intercept, which means predicting CH_4 production at zero intakes. One solution could be to use the quadratic term, but it might also have problems. For example, the quadratic model of Axelsson (1949) predicts maximum CH_4 production at DMI of 12.5 kg/d and declining values above this level. Non-linear modelling might be a better option for modelling intake effects. Axelsson (1949) was probably the first to present a non-linear model predicting CH_4 production. Applying his power function model to the data set used in

Paper III predicted CH_4 production reasonably well (mixed model RMSE of prediction, RMSPE=9.4%) with a small slope bias. Therefore, to develop models that can be applied beyond the range of DMI used for model development, a mixed non-linear modelling approach was used (Paper III, n=207). Non-linear models were more precise (smaller RMSE) than linear DMI models (Paper II). However, they were quite similar to the quadratic model (RMSE=25.5 for the non-linear model and 25.4 for the quadratic model). It appears that in model development, the differences between linear and non-linear models are small. However, applying linear models beyond the range of DMI from which the models were developed can result in serious bias. More data are required at higher intakes to evaluate the performance of the different (power, Mitscherlich) non-linear models at high DMI, where the models derived from the current data began to be separated (Paper III).

Mechanistic models can better handle wider ranges of DMI than empirical models. The sensitivity analysis (Paper IV) showed that the Karoline model predicted a similar decrease in CH_4-E/GE with increased feed intake as observed *in vivo* (*e.g.* Yan *et al*., 2000). The mechanistic model of Mills *et al*. (2001) predicted that CH_4-E/GE declined in a linear manner from 66 to 60 kJ/MJ as intake increased from 10 to 25 kg DM/d. This decline is much smaller than the results from respiration studies (Yan *et al*., 2000; Paper II). The decline in the proportion of CH_4-E/GE can be partly attributed to reduced diet digestibility and changes in rumen fermentation pattern with increased feed intake. As the residuals of CH_4 production were not significantly related to DMI (Paper V) and the Karoline model predicted OMD and EMPS accurately (Paper IV), the Karoline model has the potential to predict DMI responses to CH_4 production accurately.

5.2.2 Digestibility

Because CH_4 is only produced from OM fermentation in the rumen, it could be expected that CH_4 production is positively correlated with diet digestibility. A positive effect of increased digestibility on CH_4 production has also been reported in the literature. Models developed by Axelsson (1949) were probably the first to demonstrate the importance of digestibility in predicting CH_4 production. Axelsson (1949) reported that CH_4 production per unit DMI increased with an increased content of digested carbohydrates. Jentsch *et al*. (2007) showed a better relationship between CH_4 production and digestible nutrients compared with crude nutrients (R^2=0.90 vs. 0.86). Blaxter & Clapperton (1965) also showed positive effects of digestibility on CH_4-E/GE and found a strong interaction between feeding level and digestibility, with digestibility having a much stronger influence at low levels of intake.

Interactions were also significant in the current data set, but much smaller than in the study by Blaxter & Clapperton (1965). However, the model was not improved by including the interaction term and the variance inflation factors became unacceptably high (>100). Models developed by Yan *et al*. (2000) used digestible energy as an intake variable to predict CH_4 energy. In some cases, metabolisable energy intake has been used as a predictor of CH_4 production (Mills *et al*., 2003; Yan *et al*., 2010). The problem with this approach, when based on respiration chamber data, is that the estimates of metabolisable energy need data on CH_4 measurements first. In the empirical models developed in this thesis (Paper II), digestibility was estimated at maintenance level of feed intake rather than observed values. The advantage of OMD at maintenance is that it can be predicted with reasonable accuracy by using *in vitro* or NIRS predictions for forages and tabulated values for concentrates. In non-linear models (Paper III), OMD influenced the power (exponent) in both power and Mitscherlich models. An advantage of non-linear models is that the quantitative influence of OMD becomes greater with increased DMI (Paper III). It could be expected that a given change in OMD has a greater influence on CH_4 production at high compared with low intake levels, since increases in digestible OM intake increase with DMI. In Paper II, a positive relationship was achieved showing that increased digestibility increased CH_4-E/GE (0.076, kJ/MJ per g/kg). In the Karoline and other mechanistic models, digestibility influences CH_4 production via the amounts of fermented substrates (digestion rates, concentration of iNDF). In the Karoline model, iNDF concentration in the diet is the most sensitive factor influencing CH_4 production, followed by the rate of pdNDF digestion (Paper IV). The effect of digestibility on CH_4 production showed the same behaviour in all papers, indicating that digestibility has a positive effect on CH_4 production.

By expressing CH_4 production as a proportion of digestible energy, which is recommended to be used rather than CH_4-E/GE (Hristov *et al*., 2013a), the models developed (Paper II) showed that CH_4-E/DE tended to decrease with increased OMD. Similarly, Kennedy & Charmley (2012) reported a greater negative effect of digestibility on CH_4 energy as a proportion of digestible energy for tropical forages. As CH_4 production is generally related to digestible OM intake, a small reduction in CH_4 production as a proportion of digestible energy with increased digestibility could be due to a possible shift of digestion from the rumen to the hind-gut. This would change the rumen fermentation pattern towards increased propionate production (Johnson & Johnson, 1995). It is therefore recommended that more detailed information about the digestibility parameters of diets be included in papers reporting CH_4 production in ruminants.

5.2.3 Dietary fat

The inhibitory effect of fat in the diet on CH_4 production is well-established in the literature. Fat supplementation is consistently reported to decrease CH_4 production in individual studies (Beauchemin *et al*., 2009; Panyakaew *et al*., 2013) and in meta-analyses (Grainger & Beauchemin, 2011; Moate *et al*., 2011).

Dietary fat and the mechanisms involved in reducing CH_4 production are: 1) biohydrogenation of unsaturated fatty acids that will utilise H_2, 2) reduced supply of fermentable substrate, mainly carbohydrates, as a consequence of inclusion of fat in the diet and 3) the influence of dietary fat on fermentation pattern by favouring the production of propionate rather than acetate or butyrate. This may be partly related to the inhibitory effect of increased fat supplementation on rumen protozoa (Sutton *et al*., 1983; Testa, 1992). The proportions of acetate and butyrate are lower and that of propionate higher in defaunated animals than in faunated animals (Eugène *et al*., 2004). In all models developed in this thesis, fat had a negative coefficient (Paper II), indicating a negative effect on CH_4 production. Ether extract concentration was used in the model predicting CH_4-E/GE (Eq. 13 in Paper II), whereas in the model predicting total CH_4 production (Eq. 19 in Paper II) EE intake was used. In the non-linear model (Paper III), EE regulated the exponent and it showed a stronger quantitative effect with increased DMI. In the Karoline model, changes in the dietary fat concentration decreased CH_4 production as a consequence of reduced fermentable substrate, biohydrogenation and changes in the VFA pattern. In the sensitivity analysis (Paper IV), the effects of increased fat concentration on CH_4 production were similar to those observed *in vivo*. This, together with the absence of significant effects of dietary fat concentration on the residuals of the predictions of CH_4 production (Paper V), suggests that the Karoline model accurately predicts the responses of fat to CH_4 production. In empirical models, EE was used to describe dietary fat concentration, whereas in the evaluation of the Karoline model EE was converted to fatty acids using empirical equations. The use of fatty acids may be theoretically more correct, since forage EE contains compounds (*e.g*. waxes, chlorophylls and galactose) that are not true fatty acids (Van Soest, 1994). In addition, part of silage fermentation products are analysed as ether extract.

5.2.4 Dietary carbohydrates

It is often considered that increased concentrate proportion decreases CH_4 production, but substantial decreases have been observed, mainly for very high concentrate feedlot diets (Johnson & Johnson, 1995). Within normal ranges of concentrate, the effects are quite small. Ferris *et al*. (1999) reported a marginal

difference of between 37 and 59% proportion of concentrate on CH_4-E/GE. Lovet *et al*. (2003) did not find any significant reduction in CH_4-E/GE when concentrate proportion increased from 35 to 60%. Moss *et al*. (1995) reported increased CH_4-E/GE in sheep when grass silage was gradually replaced with barley, especially at lower feeding level. Because the objective was to develop prediction models for dairy cows, high concentrate diets were excluded from the data set to develop models predicting CH_4 production (Paper II). For predicting CH_4-E/GE (Paper II), the coefficient for NDF was only slightly greater than for NFC. However, the different statistical models can partly explain the discrepancy in the effects of carbohydrate composition on CH_4 production. The traditional approach using simple regression methods to integrate information across studies is statistically inaccurate and most likely results in erroneous conclusions (St-Pierre, 2001). When analysed with the fixed model regression in SAS (Eq. 13 in Paper II), the regression coefficient of NDF was greater than that of NFC (0.056 and 0.031, respectively), whereas with mixed model regression analysis the differences were much smaller (0.046 and 0.044, respectively). One explanation for the difference between the fixed and mixed models could be that the former is unable to correctly separate DMI and carbohydrate effects. Jentsch *et al*. (2007) showed greater effects of digestible crude fibre than digestible N-free extracts on CH_4 production when a fixed regression model was used. The effect of concentrate proportion on fermentation pattern was rather small, especially with grass silage-based diets (Paper II). The proportion of NFC in total carbohydrates [NFC/(NDF+NFC)] had a negative effect on the total CH_4 production (Paper II), but quantitatively the effects will be small within the ranges of practical dairy cow diets. Based on the common view in the literature, relatively small effects of dietary carbohydrate composition on CH_4 production could not be expected. However, the minor effect of dietary starch concentration on molar proportion of propionate with typical Nordic diets is consistent with CH_4 production data. In the analysis of the Nordic dairy cow data set, dietary starch concentration was not significantly related to molar proportion of propionate in rumen VFA (Sveinbjörnsson *et al*., 2006). In a later analysis based on 107 treatment means, Huhtanen *et al*. (2013b) found a quadratic relationship between dietary starch concentration and propionate, with the minimum at 200 g starch/kg DMI. Consistently in the data analysis, increasing the proportion of barley/wheat-based concentrate from 50 to 70% of diet DM had no influence in dairy cows (Murphy *et al*., 2000). Similarly, Jaakkola & Huhtanen (1993) did not observe differences in propionate proportion in growing cattle fed 25, 50 or 75% barley-based concentrate on a DM basis.

In the model predicting CH_4-E/GE, NDF and NFC were used to describe dietary carbohydrates, whereas the ratio was used in models predicting total CH_4 production (Papers II and III). In the non-linear models, the quantitative effect of a given change in carbohydrate ratio increases with DMI (Paper III). In the Karoline model, the effects of different carbohydrates on CH_4 production are regulated mainly by the proportions of fermented carbohydrates. The fermentation of carbohydrates is influenced by the dietary concentrations and digestion kinetic parameters (Paper IV). In the Karoline model, more propionate is produced from starch than NDF. Carbohydrate composition is influenced by forage:concentrate ratio and composition of both forage and concentrates (starchy vs. fibrous concentrates). Digestion rate of starch also influences CH_4 production (*e.g.* maize vs. barley) in the Karoline model (Paper IV), but dietary concentrations of NDF and starch and the proportion of concentrate were not significantly related to the residuals of CH_4 production. This suggests that the mechanism describing the effects of different carbohydrate sources on CH_4 production were reasonably well described (Paper V). However, in its current form the Karoline model cannot be used to predict CH_4 production for animals fed high concentrate feedlot diets. The fermentation equations need modification to take into account *e.g.* rumen pH effects (Bannink *et al*., 2006). Another option is to use empirical fermentation equations, as the prediction error is smaller than for stoichiometric equations (Sveinbjörnsson *et al*., 2006).

5.2.5 Other factors

The extent and type of silage fermentation can influence CH_4 production by two different mechanisms: changes in the rumen fermentation pattern and microbial cell synthesis. Concentration of total acids in forages had a negative effect on CH_4 production (P=0.02, Paper II). The model predicted decreases in CH_4 production with increases in the total acid concentration or with increases in the proportion of acetic acid in total acids (Paper IV). Numerically smaller CH_4-E/GE has also been reported for ensiled as opposed to dried grass forages (Ekern & Sunstøl, 1974). Predicted decreases in the efficiency of microbial N synthesis with increased acid concentrations in silage are consistent with experimental data (Jaakkola *et al*., 2006), which would decrease the contribution of microbial cells as a H_2 sink (Paper IV). However, both experimental evidence and model simulations suggest that increased silage fermentation within normal ranges decreases CH_4 production more than decreased microbial efficiency increases it.

5.3 Robustness of models in predicting CH_4 production

Models are developed based on a data set consisting of dietary and animal characteristics. However, evaluation of the models is always recommended when a new set of equations is being developed. The most suitable procedure for model evaluation is based on residual analysis, as described by St-Pierre (2003). Using a mixed model regression analysis can also be recommended, because ignoring the study effect leads to biased estimates of regression coefficients (St-Pierre, 2001). This is especially important when estimating the effects of dietary variables on CH_4 production.

In Paper I, the residual analysis showed that there were no mean or linear biases, indicating the accuracy of the gas *in vitro* system in measuring CH_4 production. The linear models developed in Paper II were also evaluated by cross-validation. The errors in cross-validation were only marginally greater than in development of the models from the whole data set, and neither the mean nor slope bias were significant. When evaluating the Karoline model (Paper V), the model slightly under-predicted CH_4 production ($P<0.05$) but the slope bias was not significant. The high coefficient of determination between predicted and observed CH_4 production (Paper V) shows the robustness of the Karoline model in predicting CH_4 production. Yan *et al*. (2000) developed a set of empirical equations and reported $R^2=0.92$, with a relatively small error in predicting CH_4 production. Compared with the study by Yan *et al*. (2000), DMI alone explained proportionately 0.85 of the variation in CH_4 production when estimated by the fixed model regression analysis in Paper II. Better predictions were obtained by the Karoline model (fixed model, $R^2=0.93$) (Paper V) compared with the study reported by Yan *et al*. (2000) or our linear models (Paper II). This indicates that the Karoline model is able to take into account factors other than intake in predicting CH_4 production.

The stoichiometric equations derived to estimate VFA are important in predicting CH_4 production. In the present study, the Karoline model predicted VFA proportions with reasonable accuracy (Paper V). The variation in individual VFA measured in studies (observed) was greater than predicted (Table 3), as frequently observed in other studies (Sveinbjörnsson *et al*., 2006). Alemu *et al*. (2011) demonstrated that the variation among stoichiometric models predicting VFA production has a strong influence on the accuracy of the predictions of enteric CH_4 production.

The greater variation in observed VFA could be partly due to between-laboratory variation in VFA analysis and different sampling schedules, and also to feeding levels, which will influence pH and consequently the proportion and concentrations of VFA (Table 3). In an analysis of the Nordic database using 107 diets (Sveinbjörnsson *et al*., 2006), the CV of measured acetate,

propionate and butyrate was 4.8, 12.0 and 13.5%, respectively, but when all major VFA were considered together as a stoichiometric relationship between CH_4 production and VFA the CV was smaller (5.5%), as shown in Table 3.

Table 3. *Mean values of VFA production (mmol/mol) and their relative variation within different experiments*

Reference	Acetate	CV[1]	Prop-ionate	CV	But-yrate	CV	CH_4VFA[3]	CV
Bannink *et al*. (2006)	624	5.9	214	13.5	123	10.6		
Cabezas-Garcia *et al*. (2013)	678	3.5	190	10.8	132	14.3	358	4.02
Huhtanen *et al*. (2010)[2]							352	5.1
Paper II	638	4.3	207	9.1	127	17.6	331	4.5
Sveinbjörnsson *et al*. (2006)	668	4.8	193	12.0	138	13.5	353	5.5

[1] CV: Coefficient of variation
[2] Individual VFA values were used to calculate CH_4VFA only.
[3] CH_4VFA: methane production (mmol/mol of VFA) predicted from VFA stoichiometry.

Comparison of the performance of different models in predicting CH_4 production is not so straight-forward, as the models are developed from different data sets. There are different criteria for model comparison; *e.g*. R^2 and RMSE. The RMSE, especially the relative error (RMSE/observed mean), is a better parameter for comparing models than R^2, which is highly dependent on the range of data. Errors in model development (RMSE) and model evaluation (RMSPE) may also differ, as the former does not have any mean or slope bias. The relative error of the Karoline model was similar to Eq. 19 in Paper II, but the relative prediction error was greater in the Karoline model because of minor mean and slope biases (Paper V).

In mixed model regression analysis, study is taken into account as the random variable reduces random variation (Table 4). Some factors that might relate to the random study effect are: 1) calibration of chambers, 2) random variation between animal groups and 3) methods of feed analysis (*e.g*. correction of silage VFA for volatile losses). Using treatment mean data in data sets basically decreases the standard error compared with individual data. It is possible that when the data are from single laboratories (Yan *et al*., 2000; Jentsch *et al*., 2007), the errors are smaller (Table 4) compared with models developed from data sets based on studies conducted in different environments. Using the laboratory as SUBJECT in the mixed model analysis can make the comparison of errors more unbiased. Relative RMSE was 9.2% and 8.8% and R^2 was 0.94 and 0.95 for the best linear and Karoline model, respectively. The error was smaller and R^2 value higher than in studies by Yan *et al*. (2000) and

Jentsch *et al*. (2007) based on data from a single laboratory. If the standard deviation of CH_4 production in the data set from which the model was developed is small, the relative error is likely to be small. However, R^2 may still not be high despite a small RMSE, as found *e.g*. in the study by Kirchgessner *et al*. (1991). Use of different methods for CH_4 measurements is another source of error. The greater relative error in the study by Ellis *et al*. (2007) can be at least partly due to the fact that the data set included studies in which the SF_6 technique was used for determination of CH_4 production (Table 4).

5.3.1 Empirical and mechanistic models

Empirical models are more applicable for predicting CH_4 production than mechanistic models, as fewer input variables are required. Empirical equations relate enteric CH_4 production to DMI and/or diet composition (Yan *et al*., 2000; Jentsch *et al*., 2007; Schils *et al*., 2013). Empirical models take into account most significant factors influencing CH_4 production, *e.g*. DMI, digestibility, fat and carbohydrates. With empirical models, the required input data are usually easily measured or estimated. These models can provide a rough estimate of CH_4 production using limited information about the animal or dietary factors (Schils *et al*., 2013). Empirical models developed in the present thesis predicted CH_4 production accurately and in some cases the error was smaller than that of mechanistic models (Table 4). Digested nutrients showed better prediction of CH_4 production in the empirical model developed by Jentsch *et al*. (2007) than crude nutrient intake (R^2=0.90 and 0.86, respectively) (Table 4). Non-linear modelling proved to have a smaller RMSE than linear modelling, and is recommended for use especially when the models are beyond the range of intakes in data sets used for model development (Mills *et al*., 2003).

Only a few mechanistic models predicting CH_4 production are described in the literature. The advantage of mechanistic modelling is that it allows the system behaviour to be better understood. Complicated biological pathways, rate of passage in the rumen, VFA production and the kinetic rate of digestibility for different feed fractions are the requirements for a mechanistic model to perform. Mechanistic models can also be used for screening different diets, and especially interactions between different dietary factors. The Karoline model showed potential to predict CH_4 production with reliable accuracy, clearly better than other mechanistic models in the literature (Table 4). When compared with a recent study using the Molly cow model (Gregorini *et al*., 2013), the Karoline model showed smaller relative prediction error.

One reason for the accurate estimates of CH_4 production by the Karoline model could be its high accuracy in predicting OMD or NDF digestibility (Paper IV). The Karoline model also considers the rumen as a two-compartment system, which results in less biased estimates of digestibility and consequently CH_4 production (Paper IV and Table 4).

The Karoline model predicted CH_4 production much better than the model based on DMI (R^2=0.93 compared with 0.85; Papers II and V), whereas in the recent paper by Nielsen *et al*. (2013) the model based on DMI alone predicted CH_4 production better than the model based on nutrients digested in the rumen (0.66 and 0.59, respectively). In the latter study, ruminally digested nutrients were estimated by the NorFor model (Volden & Larsen, 2011). The data set used in this thesis for model development included data on a wide range of diets (Papers II, III and IV) from studies conducted in a number of laboratories in Europe and North America. Both empirical and mechanistic models predicted CH_4 production at least as well as any published models. However, before final conclusions are drawn, the models should be compared using large data sets derived from studies conducted using a wide range of diets fed at different levels of intake.

Table 4. *Comparison of empirical and mechanistic modelling for predicting methane production in ruminants*

Reference	Method[1]	Animal[2]	Observation	Model[3]	Laboratory	Data[4]	FIXED model		MIXED Model	
							R^2	RMSE	R^2	Adj. RMSE
Axelsson (1949)	RC	D	175	E	4	I	0.75	0.131		
Axelsson (1949)	RC	D	176	E	4	I	0.80	0.121		
Ellis *et al*. (2007)	RC+SF_6	D	89	E	Many	T	0.65	0.256		
Ellis *et al*. (2007)	RC+SF_6	DB	172	E	Many	T	0.71	0.296		
Jentsch *et al*. (2007)	RC	DB	337	E	1	T	0.86			
Jentsch *et al*. (2007)	RC	DB	337	E	1	T	0.90			
Kirchgessner *et al*. (1991)	RC	D	153	E	1	I	0.66	0.077		
Mills *et al*. (2003)	RC	D	159	E	1	I			0.60	0.076
Moe & Tyrrel (1979)	RC	D	404	E	1	I	0.73	0.171		
Nielsen *et al*. (2013)	RC+SF_6	D	47	E	Many	T	0.77	0.138		
Yan *et al*. (2000)	RC	DB	322	E	1	I	0.89	0.118		
Empirical – Linear (Paper II)	RC	DB	184	E	Many	T	0.93	0.104	0.94	0.092
Empirical – Linear (Paper II)[5]	RC	DB	184	E	Many	T			0.98	0.048
Empirical – Mitscherlich (Paper III)	RC	DB	184	E	Many	T	0.92	0.106	0.97	0.051
Empirical – Power (Paper III)	RC	DB	184	E	Many	T	0.91	0.111	0.98	0.051
Benchaar *et al*. (1998)	RC	D	32	M	Many	T	0.70	0.199		
Mills *et al*. (2001)	RC	D	32	M	Many	T	0.76	0.154		
Mills *et al*. (2001)	RC	D	67	M	1	I	0.46	0.124		
Karoline model – (Paper V)	RC	DB	184	M	Many	T	0.93	0.101	0.95	0.088
Karoline model – (Paper V)[6]	RC	DB	184	M	Many	T			0.98	0.052

[1]RC: respiration, SF_6: sulphur hexafluoride.
[2]D: dairy cattle, B: beef cow.
[3]E: empirical, M: mechanistic.
[4]I: individual data, T: treatment mean data.
[5]Study as SUBJECT in the model.
[6]Study as SUBJECT in the model.

5.4 Between-laboratory variation

One difficulty in evaluating models predicting CH_4 production, especially mechanistic models, is identifying sources of errors in the models. The three main sources of errors in models are: 1) inadequate model structure, 2) inaccurate reference data and 3) inaccurate input data, especially for mechanistic models. It could be postulated that the model is not performing well, but variation due to the measurement method could also be an important source of error. Paper V showed that laboratories differed significantly in the residuals of CH_4 production when CH_4 production was scaled as L/kg DM. This variation could arise from calibrations not being made frequently enough on the respiration chambers.

Random variation between animals can also contribute to the study effects. For example, if the coefficient of variation between animals is 8% in CH_4-E/GE (Blaxter & Clapperton, 1965), random error in observed CH_4 production will be 4% when the measurements are based on four animals. This could be considered the minimum RMSE, provided that there are no calibration errors or errors in measurements of intake and variables included in prediction models.

6 Conclusions

Papers I-V in this thesis demonstrated that total dry matter intake is the main factor contributing to total methane production in dairy cows. It was found that increased sample size in an *in vitro* gas production system decreased predicted *in vivo* methane production. Increased sample size did not influence the rate of either methane or total gas production. A sample size of 1000 mg is recommended when using the *in vitro* system to predict CH_4 production *in vivo*. This recommended amount will also reduce the effects of blanks. Realistic values of predicted methane production compared with those measured in the gas *in vitro* system suggest that the method is a promising tool for evaluating diet effects, and especially for screening of feeds and additives. The empirical models developed showed that increased feed intake and inclusion of fat in the diet reduced methane as a proportion of gross energy, whereas organic matter digestibility and concentration of dietary carbohydrate were positively associated with methane energy. In linear models, total dry matter intake proved to be the driving factor influencing total CH_4 production. Non-linear models are biologically more valid when applied outside the range of data from which the model has been developed. Non-linear models are also flexible in that the exponents can be adjusted to account for the effects of diet composition. However, more data at higher intakes are required to evaluate the performance of the models at higher dry matter intakes. It can be concluded that a mixed non-linear modelling approach provides a more biologically sound basis for the development of empirical models predicting CH_4 production than linear models. The dynamic, mechanistic dairy cow model Karoline can predict accurately and precisely the effects of the level of feed intake and diet composition on diet digestibility, microbial protein synthesis and methane production. Sensitivity analyses suggested that the model predictions of methane production are sensitive to dietary variables associated with diet digestibility. It is therefore essential that accurate input data for

digestibility variables are used in model evaluations. Inaccurate input data for digestion variables can result in biased estimates of the amount of organic matter fermented in the rumen, and consequently biased predictions of methane production, even when the model structure is correct. Evaluation of the Karoline model showed that it is able to predict accurately observed methane production, with a small overall prediction error and mean bias without any slope bias. However, for accurate predictions of methane production using the Karoline model, accurate estimates of digestion kinetic parameters are recommended, particularly those of neutral detergent fibre and improvements in the VFA stoichiometric models taking into account the effect of pH.

In general, it was concluded that the empirical and mechanistic (Karoline) models developed in this thesis can be used in the dairy industry for developing appropriate feeding strategies to mitigate methane production. They can also be used by national inventories and advisory services for predicting methane production. The Karoline model could also be a useful tool for teaching purposes, to help students understand how the system behaves in simulating methane production in dairy cows.

7 Future perspectives

Models predicting the amounts of CH_4 produced by ruminants are more of an interest for inventories such as IPCC and for developing mitigation strategies. Emphasis should be placed on developing models predicting CH_4 production with minimum requirements needed as input variables, with the goal of achieving higher accuracy. For evaluating mitigation strategies, models that are able to predict feed intake with reasonable accuracy and that rely on input data available at the time of predictions should be developed. It would also be useful to develop ration formulation models, in which CH_4 production per unit product can be used as a constraint.

The model developed for predicting *in vivo* CH_4 production based on kinetic data from the *in vitro* gas system needs to be updated to include the effects of feeding level on microbial cell synthesis and thus take into account increased H_2 uptake by microbes. Adjustments to the partitioning of fermentable substrate between VFA, total gas production and microbial cells are also needed. The prediction results on CH_4 production obtained using *in vitro* gas production need to be evaluated against *in vivo* measurements of CH_4 production from respiration chambers.

To improve empirical models for predicting CH_4 production, it is recommended that more detailed information regarding digestibility parameters be given in published papers. Organic matter digestibility at the maintenance level of feeding and iNDF in forage are among the key variables required in both empirical and mechanistic models predicting CH_4 production. Concentrate ingredient composition should be reported to allow accurate estimates of OMD at the maintenance level of feeding. Because the extent and type of silage fermentation influence CH_4 production, it is also recommended that silage fermentation characteristics be reported in studies using silages, to allow adjustment of the models for silage fermentation products.

Further developments to the sub-model predicting VFA production and taking into account pH effects in the Karoline model might improve the predictions of CH_4 production. Applying empirical regression equations for predicting VFA in the Karoline model rather than stoichiometric equations could be worth testing. This could allow better predictions of VFA pattern when the fermentation pattern abruptly changes, as can occur at high levels of concentrate. The Karoline model could be revised to include the effects of other H_2 sinks (nitrates, sulphates) on CH_4 production. It would also be useful to compare the existing mechanistic model with large data sets from respiration chamber studies using the same input data. In addition to development of the mechanistic models themselves, special emphasis should be placed on the accuracy of input data, especially NDF digestion kinetic variables. It is possible that the model performance is constrained more by the accuracy of input data than by the model structure itself.

The empirical models developed can easily be introduced into practical ration formulation programmes for evaluating the effects of formulated diets on CH_4 production. Actually, the models developed in the present study have already been introduced into the development version of the Finnish ration formulation system. Methane production can be used as a constraint in ration formulation, allowing estimation of the cost of different mitigation strategies.
Because of rather limited potential for reducing CH_4 production per kg product by manipulating diet composition, overall ruminant production strategies should be evaluated by whole farm system (country) models. These models should also take into account CH_4 production from manure, cow longevity, optimal feeding intensity and integrated vs. specialised milk and beef production systems. Breeding for improved feed efficiency could indirectly decrease CH_4 production more per unit product than direct selection for CH_4 production, because this strategy can lead to reduced digestion efficiency. It is also important to evaluate the relationships between different factors influencing the concentration of metabolisable energy (digestibility, CH_4 production, urinary energy) and components of energy utilisation (maintenance requirement, efficiency of energy utilisation for milk production).

8 Popular scientific abstract

Methane is a potent greenhouse gas that originates from different sectors and is released to the atmosphere. If the amount produced is not reduced, the concentration in the atmosphere will increase, thereby contributing to global warming. Dairy cows are one of the main contributors to methane emissions. Food entering the stomach (rumen) of dairy cows is exposed to microbial digestion, which allows ruminants to digest feed resources that cannot be broken down by mammalian enzymes. The end-products of rumen fermentation are the gases hydrogen and carbon dioxide, volatile fatty acids and microbial cells. Hydrogen gas needs to be removed so that microorganisms are able to continue their work of fibre digestion. Therefore the rumen Methanogenesis combine hydrogen gas with carbon dioxide to create methane, which decreases the partial pressure of hydrogen gas. It is thus of interest to evaluate factors influencing methane production in the rumen, as well as measuring it in the laboratory. It would be ideal if methane production could be predicted by laboratory methods or models, as actual measurements of methane release are laborious and time-consuming.

The aim of this thesis was therefore to develop a laboratory method and models to predict methane production from dairy cows. An *in vitro* (laboratory) gas production method based on automated gas recordings and computer modelling approaches was developed. Increasing the sample size in this *in vitro* system had a negative effect on methane production per unit increase in sample size and increased the proportion of propionate (a volatile fatty acid) at the expense of acetate. Empirical models were developed to predict methane production *in vivo* (real life). According to the results obtained in this thesis, dry matter intake is the main factor determining total methane production in dairy cows. Total methane production increases with increased dry matter intake, but decreases per unit intake. The models developed in this thesis also showed the importance of dry matter intake, digestibility, dietary

concentrations of fibre carbohydrates, non-fibre carbohydrates and dietary fat in prediction of methane production.

Empirical models (based on nutrient intake and diet composition) are simpler than mechanistic models (based on mathematical descriptions of rumen fermentation and digestion processes), as they usually require fewer input variables. When the empirical and mechanistic (Karoline) models presented in this thesis were evaluated against measured methane production values published in the literature, both type of models showed only small errors in predicting methane production. The Karoline model was revised in this thesis to give better predictions than other mechanistic models in the literature.

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