To my parents, my sisters, my nephews

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# THE POTENTIAL OF BATCH IN VITRO SIMULATIONS AND MILK FATTY ACIDS TO ASSESS RUMEN METHANE MITIGATION

Thesis submitted in fulfillment of the requirements for the degree of Doctor (PhD) in Applied Biological Sciences

Dutch translation of the title

**MOGELIJKHEDEN VAN IN VITRO BATCH SIMULATIES EN MELKVETZUREN OM DE REDUCTIE VAN METHAANUITSTOOT DOOR HERKAUWERS IN TE SCHATTEN**

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| Adj $R_2$ | Adjusted coefficient of determination |
| BW | Body weight |
| $BW^{0.75}$ | Metabolic body weight |
| CCC | Concordance correlation coefficient |
| DHA | Docosahexanoic acid |
| DIM | Days in milk |
| DM | Dry matter |
| DMI | Dry matter intake |
| EPA | Eicosapentanoic acid |
| FA | Fatty acid |
| GA | Genetic algorithm |
| GC | Gas chromatography |
| GHG | Greenhouse gases |
| GPT | Gas production technique |
| IPCC | Intergovernmental Panel on Climate Change |
| MCFA | Medium chain fatty acids |
| MFA | Milk fatty acids |
| MLR | Multiple linear regression |
| MUFA | Mono-unsaturated fatty acids |
| OBCFA | odd- and branched-chain fatty acids |
| OM | Organic matter |
| PLS | Partial least squares |
| PUFA | Poly-unsaturated fatty acids |

| | |
|---|---|
| $R^2$ | Coefficient of determination |
| $R^2_{(m)}$ | Marginal coeffcient of determination |
| $R^2_{(c)}$ | Conditional coefficient of determination |
| RMSE | Root mean square error |
| RUSITEC | Rumen simulation technique |
| SD | Standard deviation |
| SEM | Standard error of the mean |
| $SF_6$ | Sulfur hexafluoride |
| SFA | Saturated fatty acids |
| TMR | Total mixed ration |
| UFA | Unsaturated fatty acids |
| Var PE | Variance of the prediction error |
| VFA | Volatile fatty acids |

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# INTRODUCTION

## GENERAL BACKGROUND

Global warming is one of the most important topics in environmental sciences in recent years and is directly linked with emissions of greenhouse gases (GHG). Human activities result in emissions of four long-lived GHG: carbon dioxide ($CO_2$), methane ($CH_4$), nitrous oxide ($N_2O$) and halocarbons (a group of gases containing fluorine, chlorine and bromine). Methane is not the most abundant GHG – $CO_2$ accounts for around 77% of the total anthropogenic GHG emissions – but it has a higher global warming potential than $CO_2$ (25 times that of $CO_2$ for a 100 years time horizon) (IPCC, 2007). Additionally, methane resides in the atmosphere during a shorter period than $CO_2$ and $N_2O$ (12 ± 3 years vs. *ca.*100 and 120 years, for $CO_2$ and $N_2O$, respectively), which opens opportunities to achieve an impact on GHG atmospheric concentrations by mitigating methane emissions in a relatively short-term period.

Methane emissions from agricultural activities represented 77% of the total Belgian methane emissions in 2010 (UNFCCC, 2011), with 68% of agricultural methane originating from enteric fermentation (UNFCCC, 2011). Enteric methane derives from the reduction of $CO_2$ with hydrogen during the microbial fermentation in the gastrointestinal tract, mainly in the rumen. Different strategies to reduce methane emissions have been assessed and described in several reports (Boadi et al., 2004; Patra, 2012). Increasing the productivity of animals has been, and in many situations still is, one effective way to decrease methane emissions per unit of product. This includes culling of unproductive animals (Patra, 2012) and, thus, reducing the total stock. Also a proper livestock management such as reducing the incidence of diseases and reproductive problems would increase productivity and ultimately decrease methane emissions (Eckard et al., 2010). Additionally, an improvement of the digestibility of the diets also contributes to reduce overall methane production (FAO-IDF, 2010) and has been

identified as the most effective measure for global reduction of GHG emissions from ruminants. In industrialized countries there has been a great improvement in digestibility for certain species (e.g. ryegrass), however, there is still some scope to improve digestibility of other species with drought resistant characteristics (e.g. tall fescue) (Aiken and Strickland, 2013) or to increase proportions of by-pass nutrients (e.g. starch, protein) from some feedstuffs. Furthermore, improving practices related to harvest time and prost-harvest management still can make an impact on diet digestibility (Hristov et al., 2013)

Hence, dietary interventions through e.g. supplementation of additives which allow reduction of methane production when relying on roughage-based diets are of particular interest. Some options like ionophore compounds, fat supplementation and some plant secondary metabolites, in association or not with some compounds aiming at sinking hydrogen, are among the strategies which show potential to reduce enteric methane (Patra, 2012).

The main constraint to apply methane mitigation strategies is that they are likely to increase the costs of production without any direct benefit for the farmer. From a nutritional view, decreasing methane emissions theoretically would be beneficial through enhancement of feed energy use (Johnson and Johnson, 1995), as methane is an energy loss, representing between 2 and 12% of the gross energy intake (Johnson et al., 1993). However, mitigation strategies are often associated with e.g. lower dry matter intake (DMI) or reduced digestibility resulting in no net productivity for the farmer. Therefore, financial incentives from governments could be an option to encourage farmers to adopt strategies that reduce methane production. However, to do that, governments need a simple and reliable estimation methodology to assess methane emissions at farm level as well as their decrease when mitigation strategies are applied. Such a methodology should allow the assessment of methane production on a routine basis. Routine measurements – along with accuracy – are actually the key for a methodology to be adopted as the tool to monitor herds for methane emissions.

## Methane measurements techniques

There are different equipments to estimate methane emissions, however, most of them are not applicable under practical conditions. Enclosure techniques, tracer gas and tunnels are among those techniques and are briefly described below.

Enclosure techniques such as whole animal chambers, ventilated hoods and face masks have been used effectively to collect most of the available information concerning methane emissions from cattle (Johnson and Johnson, 1995). Chamber systems were originally meant to study the energy metabolism of cattle. The principle of the open circuit enclosure techniques is that air is circulated around the animal's head, mouth, and nose and exhaled breath from the animal is collected. Methane output is estimated by quantifying the volume of ventilated air and its methane concentration (Storm et al., 2012). Chambers are regarded as the most reliable method for measurement of methane emissions from ruminants. They also allow to observe patterns of methane production throughout the day. However, some studies report that animals stress from being confined in the chambers, which might represent a drawback of the system, as dry matter and water intake, and as a consequence milk and methane production might be affected (Storm et al., 2012).

Ventilated hoods follow the same principle of the chambers. This technique involves the use of an air-tight box that surrounds the animal's head (Johnson and Johnson, 1995). Compared to the whole chamber system, the headbox is a less expensive option. However, it has the disadvantage of not measuring all hindgut methane (Johnson and Johnson, 1995), which is, nevertheless, a minor fraction of which still the majority is exhaled via the breath.

Face masks have been also proposed to quantify enteric methane (Liang et al., 1989), following the same principle of the ventilated hood. The main disadvantage of the system is the inability of the animal to eat and drink, questioning the reliability of the measurements

(Liang et al., 1989). Furthermore, the facemask, compared with chamber methods, underestimates heat production and likely $CH_4$ as well by an average of 9% (Liang et al., 1989).

Another common technique to estimate methane production is the tracer technique. Isotopic and non-isotopic tracers such as [$^3$H–] methane, [$^{14}$C–] methane or $C_2H_6$ are used as tracer gas (Storm et al., 2012). However, the gas most commonly used across scientific studies is the sulphur hexafluoride ($SF_6$) gas. The main feature of the latter method is the ability to be used in free ranging cattle (Storm et al., 2012). The basic idea of the method is that methane emission can be estimated if the emission rate of a tracer gas from the rumen is known and its concentration, as well as that of methane, in a sampling canister is determined (Johnson and Johnson, 1995). For this purpose a non-toxic, physiologically inert, stabile gas is needed. Additionally, the gas should mix with rumen gas in the same way as methane.Permeation tubes filled with $SF_6$ and with a previously known diffusion rate are placed in the rumen of an experimental animal. Capillary tubing is placed at the nose of the animal and is connected with an evacuated collection canister, which sample the exhaled gas. The concentration of $SF_6$ and $CH_4$ in the canister is determined by gas chromatography.

The main drawbacks of the method are related with the accuracy of the rate of release of $SF_6$ from the permeation tubes deposited in the rumen (Pinares–Patiño, 2008). Given that permeation rate does not remain constant over time (Lassey et al., 2001; Pinares–Patiño, 2008), the changes in this rate are particularly important in trials taking place over long periods of time after the tubes' calibration (Pinares–Patiño, 2008). Moreover, there is no consensus on how the $SF_6$ technique compares with respiration chambers, with some studies finding a slightly lower emission (5-10%) and others finding slightly higher emissions with the tracer technique (See e.g. Storm et al., 2012). What has been generally recognized is that both within as well as between animal variation is much higher in experiments with the $SF_6$

technique than in chambers (Pinares-Patiño et al., 2011), which also means that more animals would be needed to identify differences between treatments in experiments with the tracer technique compared with the chambers.

Other methodologies have been proposed in recent years like using open-path laser measurements (Mcginn et al., 2011), and measuring the ratio of $CO_2$ to $CH_4$ to estimate the latter (Madsen et al., 2010). These methods could be feasible in practice. However, the accuracy of these techniques still has to be proved. Furthermore, similar to other techniques, some sophisticated and expensive equipment might be needed and this might impair their applicability on a routine basis.

Finally, another recent study has proposed the estimation of methane emissions based on air sampling from eructation during milking (Garnsworthy et al., 2012). Even though the measurements by this technique correlated good ($R^2$ = 0.79) with daily methane output measured in chambers, the technique is not able to estimate daily methane emissions by itself, however, the technique detects differences in $CH_4$ emissions of cows on feeding regimens that induced high or low methane outputs and it offers a high level of replication as needed for statistical analysis (Garnsworthy et al., 2012).

## Mathematical models

In general, there is still a gap between the current techniques to estimate methane and routine measurements. Scientists have always been aware that expensive equipment and poor practicality are the main shortcomings of the existing techniques to be applied on a large scale. That is why mathematical models to estimate methane have been developed.

These models are generally divided into two groups: empirical models, which directly relate the nutrient intake to methane production; and mechanistic models, which estimate methane

emissions based on detailed modeling of the fermentation processes occurring in the rumen (e.g. feed degradation and formation of VFA).

***Empirical models***

Empirical models are usually linear equations based on intake of certain nutrients (e.g. Moe and Tyrrell, 1979; Eq (1)) and/or on their digestibility (e.g. Blaxter and Clapperton, 1965; Eq (2)). Nonlinear empirical models also have been proposed to predict methane (e.g. Ellis et al., 2009; Eq. (3)). However, the model most commonly used to estimate national inventories of methane is the Tier II of the IPCC (Eq. (4), which estimates methane emissions as a constant proportion of the gross energy intake and a default conversion rate depending on the type of animal (e.g. lactating cow, steer, heifer).

Moe and Tyrrell, 1979:

$CH_4$ (Mcal/d) = 0.439 + 0.273 SR + 0.512 Hem + 1.393 Cel (1)

Where

SR = digestible soluble residue (kg/d)*
Hem = digestible hemicelluloses (kg/d)
Cel = digestible cellulose (kg/d)

* Calculated by subtracting crude protein and ether extract from the neutral-detergent solubles

The equation was developed from data of 404 total energy balance trials with Holstein cows, where methane production in all trials was measured in open circuit respiration chambers.

Blaxter and Clapperton, 1965:

$CH_4$ (kcal/100 kcal feed) = 1.30 + 0.112 D - L (2.37 -0.05D) (2)

Where

D = digestibility of energy at the maintenance level of feeding (%), and
L = level of feeding as a multiple of the maintenance level

The equation was developed from 20 studies with sheep and cattle including 55 diets, where methane production was measured using close circuit respiration equipment.

Ellis et al., 2009

$CH_4$ (MJ/d) = $10.8 \times [1 - e^{\{- [-0.034 \times (NFC/NDF) + 0.228] \times DMI\}}]$ (3)

Where

NFC = non fiber carbohydrate [100 – (crude protein % + fat % + NDF% + ash %] (kg/d)
NDF = neutral detergent fiber (kg/d)

DMI = dry matter intake (kg/d)

Developed from 872 $CH_4$ data points of published and unpublished data from 12 studies with beef cattle conducted at the University of Alberta and at the Lethbridge Research Centre of Agriculture and Agri-Food Canada (Alberta, Canada). In seven of the studies methane was measured by calorimetry, one with the $SF_6$ technique and 4 by hood calorimetry.

IPCC, Tier II:

$CH_4$ (kg/yr) = [Intake (MJ/day) × *Ym* × (365 days/yr)] / [55.65 MJ/kg of methane] (4)

Where

*Ym* is the methane conversion rate expressed as a fraction of the gross energy intake (GEI) (i.e., the fractional loss of GEI as combustible $CH_4$ in %)

Examples of *Ym* (%)

- Feedlot receiving 90% or more concentrate: 3.0 ± 1.0
- Dairy cows and their young: 6.5 ± 1.0
- Other cattle and buffalo fed low-quality crop residues: 6.5 ± 1.0
- Other grazing cattle and buffalo: 6.5 ± 1.0
- Lambs (< 1 year old): 4.5 ± 1.0
- Mature sheep: 6.5 ± 1.0

Feed-based models are popular convenient tools to estimate methane emissions on a regular basis, but they need to have a precise record of individual feed consumption and feed composition. Furthermore, these models might not be applicable when mitigating additives are added to the diet, because these additives are usually included in small amounts not affecting DMI or the chemical composition of the diet. Additionally, they do not consider changes in the rumen microbial system, which are likely to occur when additives to mitigate methane are applied. Finally, these models are not able to show differences in digestibility between animals, therefore, the variability between animals in their natural ability to produce methane cannot be assessed with these models, which is an important feature when aiming at selecting for animals with lower methane emissions.

***Mechanistic models***

Mechanistic models have been developed to consider the changes taking place in the rumen, which are supposed to accompany changes in methane production. Mechanistic models

estimate $CH_4$ production in the rumen based on the hydrogen balance which represents sources and sinks of rumen hydrogen. MOLLY is a model developed at the university of California, Davis (Baldwin, 1995). The model uses stoichiometric coefficients to calculate the conversion rates of starch, soluble carbohydrates and amino acids into individual VFA. The model assumes that a fraction of the hydrogen produced in the rumen is used to support microbial growth, for biohydrogenation of unsaturated fatty acids and for production of glucogenic VFA (e.g. propionate, valerate), while the remaining hydrogen is used to produce $CH_4$ from $CO_2$ reduction (Baldwin, 1995).

In another popular model (COWPOLL), Mills et al. (2001) used the principles of Baldwin (1995) to include rumen and hindgut $CH_4$ production to the model of Dijkstra et al. (1992). In this model VFA molar proportions are estimated based on the stoichiometry developed by Bannink et al. (2006). Different to MOLLY, COWPOLL considers three microbial pools (amylolytic, fibrolytic and protozoa) instead of one.

***Remarks***

Empirical models still represent a rather practical tool to estimate methane emissions, however their predictions might not be as accurate as those from mechanistic models. Alemu et al. (2011) used a dataset from 8 studies to predict methane using the IPCC Tier 2 approach and compared it with methane predicted from mechanistic-model predicted VFA (Bannink et al., 2006; Murphy et al., 1982; Nozière et al., 2010 and Sveinbjörnsson et al., 2006). The IPCC Tier 2 approach overestimated $CH_4$ production and its RMSE was higher than that of Bannink et al. (2006), Nozière et al. (2010) and Murphy et al. (1982) (Table I). Furthermore, Ellis et al. (2010) after evaluating the IPCC Tier 2 model against a set of independent data concluded that this model does not fully describe changes in dietary composition, which

limits its usefulness when estimating impacts on varying nutritional strategies on $CH_4$ emissions.

Table I. Comparison of stoichiometric VFA models in estimating methane production ($n$ = 18). Adapted from Alemu et al. (2011)

| | IPCC Tier 2 | Murphy et al., 1982 | Bannink et al., 2006 | Sveinbjörnsson et al., 2006 | Nozière et al., 2010 |
|---|---|---|---|---|---|
| Predicted (MJ/d) [a] | 24.4 | 21.4 | 21.5 | 24.8 | 22.5 |
| RMSE (MJ/d) | 3.7 | 2.8 | 2.2 | 3.8 | 2.5 |
| RMSE (%) | 16.4 | 12.2 | 9.8 | 16.7 | 11.2 |
| $R^2$ | n.d. | 0.19 | 0.51 | 0.35 | 0.36 |

[a] Measured $CH_4$ production = 22.6 MJ/d
n.d. = not determined

The VFA model of Murphy et al. (1982) was developed using data generated mainly from beef cattle and sheep by dividing the data set into a forage based group (>500 g forage/kg DMI ) and a concentrate group (>500 g concentrate/kg DMI). Substrate compositions of the diet were divided into soluble carbohydrate, starch, cellulose, hemicellulose and protein. A single model parameter was assigned to the portion of substrate incorporated into microbes from all substrate types. The model estimated stoichiometric coefficients to describe partitioning of fermented carbon into individual VFA for the type of substrate fermented fraction (Alemu et al., 2011).

Bannink et al. (2006) gathered literature data on lactating dairy cattle reporting the calculated rumen true digestibility of dietary substrate and VFA molar proportions. Similar to Murphy et al. (1982), Bannink et al. (2006) also organized diets based on forage proportion, similarly divided de components of DM into starch, cellulose, hemicellulose, crude protein (CP)'. Additionally, a dietary fraction was included not accounted for by component analysis, which was considered mainly rapidly fermentable or soluble (non-starch) carbohydrates, although it also included analytical error. The model assumed a fixed proportion of each substrate being converted into microbial biomass (Alemu et al., 2011).

The model of Sveinbjörnsson et al. (2006) is similar to that of Bannink et al. (2006) but also includes some dietary factors as additional explanatory variables. The data set originated from a Nordic database of dairy cow digestion studies. Input variables for the model are rumen degraded forage NDF (fNDF), concentrate NDF (cNDF), starch, CP, lactate and the remaining fraction of organic matter (DM – ash – starch – CP – fNDF – cNDF – lactate − VFA). Additionally, correction factors were included in the model based on concentrate ether extract content and feeding level (Alemu et al., 2011).

Finally, the model of Nozière et al. (2010) used an empirical approach to estimate production of total VFA and the proportion of individual VFA in the rumen based on a meta-analysis of literature data. Digestible NDF, digestible OM, ruminal starch digestibility (g/kg starch intake) and DM intake (kg/d/100 kg body weight, BW) were included as covariates (Alemu et al., 2011).

The downside of mechanistic models is that even though they should be able to reflect changes occurring in the rumen provoked by supplementation with $CH_4$ mitigation additives, required inputs of these models are mostly not easily available from most rumen digestion studies (Alemu et al., 2011), consequently decreasing their applicability at a large scale. Moreover, Ellis et al. (2008) stated that mechanistic models to predict $CH_4$ still can be improved by including other factors like competition for substrate between methanogens and bacteria, estimation and influence of rumen pH, protozoa and supplemental fat, and a better representation of postruminal digestion.

### *In vitro* techniques

*In vitro* gas production methods to estimate $CH_4$ are useful techniques to screen many samples in a short period, being less expensive and allowing to control more precise experimental conditions than *in vivo* trials. Gas production techniques allow for a direct measurement of $CH_4$ production, which is a major advantage as compared with *in vitro*

methods based on gravimetric measurements. Furthermore, gas production techniques have the advantage that gas measurements focus on the appearances of fermentation products (soluble but not fermentable products do not contribute to gas production) (Makkar, 2004).

In general, an efficient laboratory method should be reproducible and should correlate well with actually measured *in vivo* parameters (Getachew et al., 1998). For some parameters this holds true, Menke et al. (1979) using the *in vitro* gas measurements and chemical composition (data from 89 experiments), found a high precision ($R^2$ = 0.98; S.D. = 0.25) in prediction of *in vivo* organic matter digestibility (reviewed by Getachew et al., 1998). Similarly, dry matter intake has also significantly correlated with *in vitro* gas measurements (Blummel and Becker, 1997). Particularly, the *in vitro* gas production from NDF (prepared by refluxing each roughage with neutral-detergent solution) correlated better and explained more (82% vs. 75%) of the variation in DMI than the values obtained from the incubation of whole roughages (Blümmel and Becker, 1997).

More specifically on methane, Blümmel et al. (2005) found that total daily $CH_4$ production, calculated from *in vitro* fermentation characteristics (true degradability, VFA ratio and efficiency of microbial protein synthesis) and OM intake, explained to a large extend variation in $CH_4$ measured in open circuit respiration chambers ($R^2$ = 0.89). The study of Blümmel et al. (2005) was done with 15 cereal straws which both were fed to sheep and incubated in syringes for 96 h using the Hohenheim gas production system. In this experiment, no additive to decrease $CH_4$ was tested, and the variation in $CH_4$ production reflected changes in rumen degradability as straws studied were treated to improve degradability, which would be reflected both in the *in vivo* $CH_4$ emissions and in the substrate disappearance *in vitro*, which was the basis for the $CH_4$ calculations. This was addressed by Bhatta et al. (2007), who fed five different diets individually to four non-lactating Holstein cows and $CH_4$ output was measured by the $SF_6$ technique. The same diets were tested in both

the rumen simulation technique (RUSITEC; Kajikawa et al., 2003) and the gas production technique (Menke and Steingass, 1988). The RUSITEC measurements underestimated $CH_4$ production (ml/g DM) by 68% and did not correlate well with the $SF_6$ measurements (0.17). The gas production performed better, underestimating *in vivo* measurements by 8% but highly correlating ($r$ = 0.75 and 0.94 after 24 and 48 h, respectively). Another study of Bhatta et al. (2008), $CH_4$ output from japanese goats, measured in respiration chambers, was compared with $CH_4$ produced during the Hohenheim *in vitro* gas technique. The study had similar findings, concluding that the $CH_4$ produced in this *in vitro* technique is reflective of *in vivo* conditions.

From the studies above it might be concluded that $CH_4$ measurements from the gas production *in vitro* method correlate well with *in vivo* measurements and could be effectively used as a tool to rank feeds for their $CH_4$ production potential. However, it has to be noted that none of the studies reported a comparison of $CH_4$ outputs from diets including mitigating additives. These additives are generally added in small amounts and are expected to decrease $CH_4$ emissions without drastically affecting parameters like DMI and OM digestibility (*in vivo*), or VFA production and gas production (*in vitro*) (with the possible exception of propionate precursors e.g. fumarate).

Moreover, it is not always the case that additives producing an effect on *in vitro* $CH_4$ production show an effect *in vivo* (See review of Patra, 2012). Furthermore, it is difficult to effectively convert doses used *in vitro* to *in vivo*. *In vitro* techniques are still important tools to test mitigating additives, but to better rely on their results as decision makers for *in vivo* $CH_4$ mitigation, the systems have to be optimized.

## PROPOSED APPROACHES TO TACKLE THE CURRENT DIFFICULTIES TO ESTIMATE METHANE

We propose two ways to deal with the disadvantages found in the former approaches to estimate $CH_4$: 1) optimization of *in vitro* techniques to better reflect *in vivo* conditions when $CH_4$ mitigating additives are tested; 2) a biomarker milk-based approach relying on a model based on the milk fatty acid profile. These two approaches are further described below.

### *In vitro* optimization

Over the years, many *in vitro* studies have tested substances to reduce $CH_4$ emissions. The results range from very strong inhibitions of *in vitro* $CH_4$ to no effects of the product tested. As Flaschowsky and Lebzien (2009) outlined, it is extremely difficult to extrapolate from *in vitro* measurements to *in vivo* situations in ruminants, or to field conditions, because the relationship between $CH_4$ produced *in vivo* and *in vitro* is very poor (Moss and Givens, 1997) (Figure I).

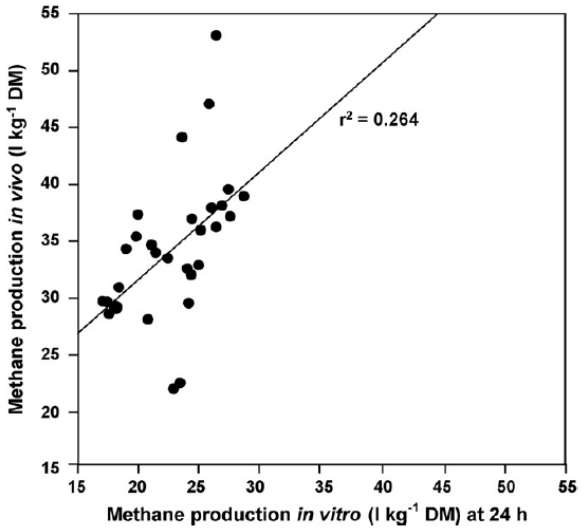


Figure I. Relationship between methane produced *in vivo* and *in vitro* from a range of diets. Taken from Flachowsky and Lebzien (2009)

Therefore this section mainly aims at modifying the normal *in vitro* methodologies, so that they better reflect *in vivo* conditions, especially when additives to decrease $CH_4$ are tested.

This should be done by considering some factors present on farm but normally not considered *in vitro*. The rule in most *in vitro* studies is to test more than one dose identifying at what point changes begin to occur. However, other factors like choice of incubation substrates, interaction between substrates or between additives and substrates, duration of the incubation, moment of additive supplementation among others often have been ignored. These factors should be considered at the time of setting an *in vitro* trial in order to make the techniques more specific for each additive or at least for each type of additive according to their nature (e.g. liquid, solid, soluble, volatile) and their mode of action (e.g. protozoa inhibitors, archaea inhibitors, propionate precursors).

*In vitro* techniques, like continuous cultures, in which some animal characteristics (e.g. rumen outflow rate, diurnal variation in rumen pH) can be imitated are important techniques to simulate rumen fermentation, however the limited amount of samples that can be tested in such a system make them not suitable for screening purposes, which is the main scope of this dissertation. Therefore, we keep the focus on routine techniques like the *in vitro* batch system, which was described by e.g. Van Nevel and Demeyer (1977) and consists of incubating a given feedstuff in gastight incubation flasks containing a buffer solution and rumen fluid in a certain proportion which might vary from 1:1 to 10:1. Further, anaerobiosis, proper temperature, suitable pH and adequate buffering are important factors to simulate the fermentation. Methane production during fermentation of a substrate is measured in a gas sample by means of diverse techniques (e.g. gas chromatography, infrared).

Moreover, as mentioned before, "translation" of *in vitro* dose responses to *in vivo* is not straightforward, which might be related to a limited number of doses tested, acute effect of additives after 24 h and diversity within one single additive. Therefore, we aim at exploring variations in the *in vitro* estimation of $CH_4$ by looking for additive × substrate interactions, by changing the moment of addition before the incubation's start, by increasing the incubation

time and by constantly supplying new additive and substrate for longer periods. Finally, this additive will be supplied *in vivo* to animals in which $CH_4$ emissions are monitored and *in vitro* results will be compared with *in vivo* results.

### Biomarker approach

A major advantage of the "biomarker" approach is its integration of variation in $CH_4$ emissions due to additives as well as their interactions with animal and dietary factors. Biomarkers in milk are targeted because milk represents an easy-to-take/handle sample that can be analyzed on a routine basis. Milk biomarkers currently available are e.g. urea and acetone, which give an indication of surplus of degradable protein and risk of occurrence of ketosis, respectively.

Recently, milk fatty acid (MFA) profiles gained interest as biomarkers as milk fat contains a rich spectrum of fatty acids originating from several processes, some of which reflect rumen metabolism. Milk FA arise from both preformed FA absorbed in the intestine and *de novo* synthesis from rumen acetate and butyrate (Chilliard et al., 2009) and to a limited extent using propionate as the initial precursor. Therefore, a relationship between $CH_4$ and MFA can be assumed based on the common biochemical pathways between $CH_4$, acetate, and butyrate in the rumen (Chilliard et al., 2009). An approach based on biomarkers on animal level would allow either a direct quantification of $CH_4$ emissions or the estimation of a relative change in emissions compared with standard situations (e.g. no mitigation strategy applied).

In light of the above, this PhD dissertation was divided in two major experimental parts: 1) a first one related the optimization of *in vitro* techniques, i.e. Chapter 1a and 1b; and 2) a second part on the link between MFA and *in vivo* $CH_4$ emissions, i.e. Chapters 2 to 4. In short, interactions between dietary additives and substrates on *in vitro* $CH_4$ inhibition were tested in **Chapter 1a**; modifications in the *in vitro* set up to better reflect *in vivo* conditions were

studied in **Chapter 1b**; the potential of OBCFA to predict calculated $CH_4$ emissions and the comparison of three modeling techniques were discussed in **Chapter 2**; the relationships between MFA and $CH_4$ was investigated by using a meta-analysis approach on literature data and were presented in **Chapter 3**; finally, models to predict $CH_4$ were developed from a database from 9 experiments with dairy cattle and were presented in **Chapter 4**.

# CHAPTER 1A. INTERACTIONS BETWEEN METHANE MITIGATION ADDITIVES AND BASAL SUBSTRATES ON *IN VITRO* METHANE AND VFA PRODUCTION

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Castro-Montoya, J.M. et al. 2012. Interactions between methane mitigation additives and basal substrates on in vitro methane and VFA production. Anim. Feed Sci. Technol. 176, 47–60.

## ABSTRACT

Seven feed additives (*i.e.,* quillaja saponins, fumaric acid, garlic oil, fish oil, cinnamaldehyde, monensin, medium chain fatty acids (MCFA)), were evaluated for their effects on $CH_4$ inhibition *in vitro* in combination with four substrates: concentrate (CON), grass silage (GS), maize silage (MS) and the mixture of CON+GS+MS (300:350:350 (dry matter, DM), MIX), all feeds regularly used in dairy cattle feeding. Substrates and additives were incubated in a batch incubation system containing buffered rumen fluid for 24 h. Cinnamaldehyde had an interaction with substrate for $CH_4$ inhibition, volatile fatty acid (VFA) production and inhibition of $CH_4$ relative to VFA. For fumaric acid, interactions occurred for $CH_4$ relative to VFA. Fish oil, quillaja saponins and MCFA had additive × substrate interactions for inhibition of $CH_4$ and VFA production, but they had no interactions for $CH_4$ relative to VFA. Garlic oil had no interaction with substrates for $CH_4$ production and $CH_4$ relative to VFA, but had interactions for VFA production. Monensin had substrate × additive interactions for $CH_4$ and VFA production and $CH_4$ relative to VFA. Monensin and quillaja saponins were more effective at inhibiting $CH_4$ production when combined with GS and MS than with CON. Fish oil had higher inhibition when combined with CON and GS than with MS. The MCFA had higher inhibitions when combined with MS and were lowest with CON, the combination with GS was intermediate and differed from both other substrates. Cinnamaldehyde and MCFA decreased, whereas fumaric acid increased, total VFA production. No other additive affected total VFA production. As a general $CH_4$ mitigation strategy, fumaric acid, garlic oil and fish oil were better in combination with CON. Monensin was more effective in combination with GS, and quillaja saponins were more effective when combined with MS. Cinnamaldehyde and MCFA strongly inhibited fermentation which

impaired appropriate evaluation of the most promising combination. Despite additive × substrate interactions, $CH_4$ and VFA production in incubations with CON, GS and MS did not differ from the weighted average of incubations with single substrates with or without additives. Hence no synergism between additive and substrate combinations seemed to exist. Results clearly indicate interactions between additives and substrates. However, it is unlikely that this interaction is the origin of often variable results among *in vitro* studies.

## INTRODUCTION

In recent years many researchers have been involved in identifying enteric $CH_4$ mitigation strategies. Options such as immunisation, biological control, probiotics, elimination of rumen protozoa, manipulation of dietary ingredients and mitigation through dietary additives have been considered (Patra, 2012). Various mechanisms of $CH_4$ inhibition have been studied, such as inhibition of protozoa (*e.g.*, saponins, in some cases poly- and mono-unsaturated fatty acids (PUFA and MUFA)) (Beauchemin et al., 2008; Prins et al., 1972); stimulation of propionate (*e.g.*, fumaric acid) (Asanuma et al., 1999); reduction of hydrogen production (*e.g.*, monensin, PUFA and medium chain fatty acids (MCFA), cinnamaldehyde) (Chen and Wolin, 1979; Freese et al., 1973; McAllister et al., 1996), direct inhibition of methanogens (*e.g.*, garlic oil) (Busquet et al., 2005a). However, as additives have different modes of action to inhibit $CH_4$, the response of each dietary additive could vary depending on the basal diet as different feedstuffs induce different conditions during rumen fermentation.

*In vitro* rumen fermentation techniques are tools for routine screening of large numbers of samples and to gain insight on the fermentation process as affected by different conditions (*e.g.*, additives). However, *in vitro* results of dietary additives are not always consistent among experiments. Differences in fermentation substrates among *in vitro* experiments might be partly responsible. Moreover, *in vitro* studies with a single substrate do not allow assessment of substrate-additive interactions. Some *in vitro* studies combined additives with

single substrates such as grain meals (*e.g.*, Callaway and Martin, 1996; Carro and Ranilla, 2003, Pelikaan et al., 2011) or hay (*e.g.*, Goel et al., 2009; Lourenço et al., 2008; Wang et al., 2000). Other studies used a mixed basal substrate such as alfalfa hay (*e.g.*, Busquet et al., 2005a; Wang et al., 2000) or grass hay (*e.g.*, Guo et al., 2008; Hu, 2005; Lila et al., 2003) combined with a concentrate. Few reports exist in which effects of additives were studied in combination with different substrates within a single experiment. Fumaric acid was examined with different concentrate:forage ratios (García-Martínez et al., 2005) and with different grain meals (Carro and Ranilla, 2003). Machmüller et al. (2001) investigated effects of MCFA combined with basal diets high or low in fiber. Monensin was examined with corn meal or timothy hay (Russell et al., 1988) and with corn meal or soybean hulls (Pellikaan et al., 2011). But no reports on other additives are available. Even fewer studies (Lee et al., 2003) exist in which the synergistic effect of combining different substrates or feed ingredients on $CH_4$ production was studied.

This study aimed to evaluate *in vitro* effects of $CH_4$ mitigation additives with different working mechanisms and their possible interaction with feed compounds regularly used in dairy feeding. Further synergism of these interactions was examined by combining feed compounds.

## MATERIALS AND METHODS

### Experiment

Seven additives each at two concentrations were incubated in a batch system with 3 single substrates (Table 1.1) and their mixture in a 7x2×3 factorial design.

## Materials

The materials were quillaja saponins (Sigma-Aldrich, Steinheim, Germany), monensin (Monensin sodium salt, 90-95%. TLC. Sigma-aldrich, Steinheim, Germany), garlic oil (Sigma-Aldrich, Steinheim, Germany), fish oil (Nutrition Sciences, Drongen, Belgium), fumaric acid (UCB, Brussels, Belgium), cinnamaldehyde (FLUKA, Buchs, Germany) and a source of MCFA (Nutrition Sciences, Drongen, Belgium). The main active compounds of the garlic oil were diallyl disulfide, diallyl trisulfide and allyl sulfide, with a guaranteed contents ranging between 300-500 g/kg, 100-130 g/kg and 50-130 g/kg, respectively. The source of MCFA contained caprilic acid (C8:0, 312 g/kg FA), capric acid (C10:0, 200 g/kg FA) and lauric acid (C12:0, 485 g/kg FA). Fish oil contained 193 and 111 g/kg FA of EPA and DHA, respectively. Additives were added to incubation flasks as a solution of di-ethyl ether (Garlic oil, fish oil, cinnamaldehyde and MCFA), water (quillaja saponins) or ethanol (monensin) or were weighed (fumaric acid). Additive concentrations were based on previous *in vitro* studies (see Table 1.2).

Table 1.1. Chemical composition[a] of concentrate (CON), grass silage (GS) and maize (MS) (g/kg DM, except DM: g/kg)

| | CON | GS | MS |
|---|---|---|---|
| Dry matter | 891 | 331 | 398 |
| Crude protein | 178 | 181 | 62.0 |
| Crude fat | 38.4 | 39.9 | 34.9 |
| Organic matter | 903 | 824 | 956 |
| Starch | 97.4 | --- | 327 |
| Sugars | 99.0 | 8.2 | --- |
| Neutral detergent fiber [b] | 187 | 368 | 434 |
| Acid detergent fiber [c] | --- | 224 | 257 |
| Lignin(sa) | --- | 17.9 | 24.4 |

[a] - One representative sample of the concentrate batch and of each silage (GS, MS) was taken for analysis of chemical composition
[b] assayed with a heat stable amylase and expressed exclusive of residual ash
[c] expressed exclusive of residual ash

Table 1.2. Additives and doses applied to the incubation medium.

| | mg/ml medium | mg/g substrate DM |
|---|---|---|
| Quillaja saponins | 0.5 | 50 |
| | 1 | 100 |
| Garlic oil | 0.15 | 15 |
| | 0.3 | 30 |
| Fish oil | 1 | 100 |
| | 2 | 200 |
| Cinnamaldehyde | 1.5 | 150 |
| | 3 | 300 |
| Fumaric acid | 1.17 (10 mM) | 117 |
| | 2.33 (20 mM) | 233 |
| MCFA | 0.6 | 60 |
| | 1.2 | 120 |
| Monensin | 0.0015 (2.5 µM) | 0.15 |
| | 0.003 (5 µM) | 0.3 |

The four substrates were grass silage (GS), maize silage (MS), concentrate (CON) and combinations of these substrates in a proportion of 350:350:300 on a dry matter (DM) basis (MIX). The chemical composition of the substrates is in Table 1.

### *In vitro* batch incubation

The rumen fluid was collected before the morning feeding from three rumen fistulated sheep. The sheep were fed hay *ad libitum* and had free access to drinking water. Fistulation of the sheep had been approved by the ethical commission of the Institute for Agricultural and Fisheries Research (ILVO), Belgium (EC 2009, 114). The rumen fluid obtained from the sheep was brought in insulated flasks for transport and was mixed, homogenized and filtered through a sieve with a pore size of 1 mm under continuous $CO_2$ flushing and kept in a water bath at 39°C to be used as the source of inoculum.

The *in vitro* batch incubation method was as described by Fievez et al. (2005). Briefly, 250 mg of the dried corresponding substrate was incubated in 120 ml capacity gastight incubation flasks flushed with $CO_2$ having 20 ml of a phosphate buffer (/L distilled water: 28.8 g $Na_2HPO_4 \cdot 12H_2O$; 6.1 g $NaH_2PO_4 \cdot H_2O$; 1.4 g $NH_4Cl$, flushed with $CO_2$ for 1 h and adjusted

to pH 6.8) and 5 ml rumen fluid in a batch culture incubator (Edmund Bühler Gmbh, Hechingen, Germany). Before adding the buffer solution, a freshly prepared solution of each additive was added to each flask to reach the desired concentration in 25 ml of the medium. Solutions were prepared so that the maximum amount to be added to the flask would not exceed 250 µl, which had been shown to guarantee normal fermentation (*i.e.*, similar fermentation of substrate with and without di-ethyl ether). Fermentation flasks without additives, but containing 250 mg of the corresponding substrate, were used as a control.

After 24 h at 39°C, flasks were removed from the incubator, placed in ice water to stop the fermentation and sampled for gas, pH and VFA determination. A 2 ml portion of filtered mixed rumen fluid was acidified immediately with 0.2 ml of formic acid to prevent fermentation (as the 0 h sample).

After 24 h of incubation, the gas phase was analyzed for $CH_4$ using a micro-GC equipped with two gas chromatographic modules and a thermal conductivity detector (3000 micro-GC, Agilent, USA). Ethane ($C_2H_6$; 1 ml/flask) was used as the internal standard and argon as a carrier gas for both columns.

After opening the incubation flask, pH was measured (Hanna Instruments, Temse, Belgium), and 2 ml of incubation medium were collected and acidified with 200 µl of formic acid which contained the internal standard (10 mg 2-Ethyl butyric acid/ml formic acid). After 15 min centrifugation at 4°C and 22,000x*g*, supernatant was filtered and an aliquot transferred into a 1.5 ml glass vial. Samples were stored at 4°C until VFA analysis using gas chromatography on a Shimadzu 2010 (Shimadzu Corporation, ‘s-Hertogenbosch, The Netherlands) equipped with a Nukol column (30 m × 0.25 mm × 0.25 µm, Supelco) with a flame ionization detector. Briefly, 0.5 µl of sample was injected with the carrier gas $N_2$, the injector temperature was 250°C and the inlet pressure 52.7 kPa. The temperature program was 90°C at the start of the

injection, increasing 20°C/min until 160°C (kept for 8.5 min), increasing 10°C/min until 170°C (kept for 2). The detection temperature was 250°C.

### Chemical analyses

The chemical composition of substrates was determined according to European and ISO standard methods. Samples of grass and maize silage were dried in a forced air oven at 65°C. Subsequently, silages and concentrate samples were ground with a knife-mill (Brabender, Duisburg, Germany, 1 mm sieve) for further analysis. Residual moisture was determined by oven drying at 103°C for 4 h (71/393/EEC). Nitrogen was determined following Kjeldahl (ISO 5983-2) with crude protein calculated as N x 6.25. Organic matter was obtained by incineration at 550°C for 4 h (ISO 5984), and crude fat was extracted with petroleum ether (ISO 6492). Neutral detergent fiber was determined with the filter bag method using α-amylase and sodium sulfite (Van Soest et al. 1991). Acid detergent fiber was also determined with the filter bag method, whereas lignin(sa) was sequentially obtained after treatment with 720 g sulfuric acid/L with water (Van Soest et al., 1991). Sugars were extracted with 400 g ethanol/L with water and analyzed with the Luff-Schoorl reagent (71/250/EEC). Starch was determined with an enzymatic method (NNI, 1974).

### Statistical Analysis

The design was a 7×2×3 factorial with 7 additives, 2 doses of each additive and 3 substrates. Each combination of substrate + additive was replicated twice/batch and repeated thrice (runs). The average of run and replicates (6 observations) were subjected to statistical analysis, as runs were considered analytical replicates (Udén et al, 2012). Hence, the factorial design did not allow assessment of the three-way interaction, but did allow assessment of all two-way interactions among substrates, additives and their doses (*e.g.*, means of additives were replicated in substrates and in doses), giving a minimum of two degrees (substrate x

dose interaction) of freedom for Tukey's comparison test for the interactions. Other interactions had 3 (additive x dose interaction) and 7 (substrate x dose interaction) degrees of freedom, respectively. Accordingly, the model used for the statistical analysis was:

$$Y_{ijk} = \mu + \beta_i + \beta_j + \beta_k + (\beta_i \times \beta_j) + (\beta_i \times \beta_k) + (\beta_j \times \beta_k) + \xi_{ijk},$$

where: $Y_{ijk}$ = observation, $\mu$ = population mean, $\beta_i$ = substrate effect (i = 1 to 3), $\beta_j$ = additive effect (j = 1 to 7), $\beta_k$ = dose effect (k = 1 to 2), $(\beta_i \times \beta_j)$ = interaction effect between substrate and additive, $(\beta_i \times \beta_k)$ = interaction effect between substrate and dose, $(\beta_j \times \beta_k)$ = interaction effect between additive and dose, and $\xi_{ijk}$ = residual error. Differences among LS means were evaluated using Tukey's multiple comparison test. Significances were declared at $P<0.05$, whereas tendencies were declared at $P<0.10$.

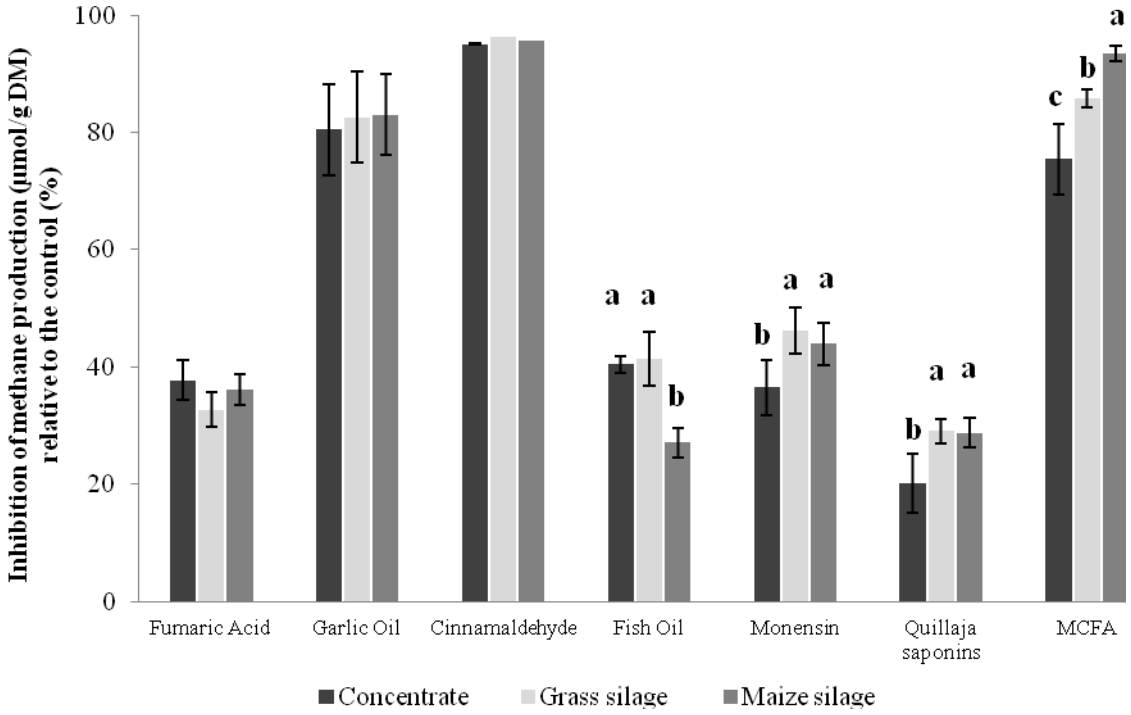
To assess synergistic effects among substrates, the two-sided Welch's t-test was completed to determine differences between calculated and observed VFA or $CH_4$ production for MIX. The coefficient of determination ($R^2$), the slope (ideally one) and the intercept (ideally zero) were used to evaluate the match between calculated and observed values.

## RESULTS

Our main focus was on effects of additives combined with substrates on $CH_4$ and total VFA production. Therefore, although individual VFA production and proportions were determined, these results are not presented. Moreover, as interaction effects of substrate x additive are the main focus, only main effects of the full factorial model are presented.

Inhibition of $CH_4$ ($P=0.007$) and changes in VFA production relative to control ($P<0.001$) differed among substrates. However, differences disappeared when expressing $CH_4$ inhibition relative to VFA. All additives at the concentrations applied differed from their respective control value for all three parameters in Figures 1.1 to 1.3 ($P<0.001$). Doses showed effects on inhibition of both $CH_4$ production and $CH_4$ relative to VFA ($P<0.001$), but not for VFA

production. Substrate × additive interactions occurred for inhibition of $CH_4$ production and $CH_4$ relative to VFA ($P$<0.05) and a tendency occurred for VFA production ($P$=0.07). Substrate × dose interactions did not occur for any of the three parameters. Finally, additive × dose interactions occurred for all parameters ($P$<0.05).




a, b, c superscripts over the bars indicate significant substrate × additive interactions ($P$<0.05)

All additives at the concentrations applied differed from the respective control value ($P$<0.05)

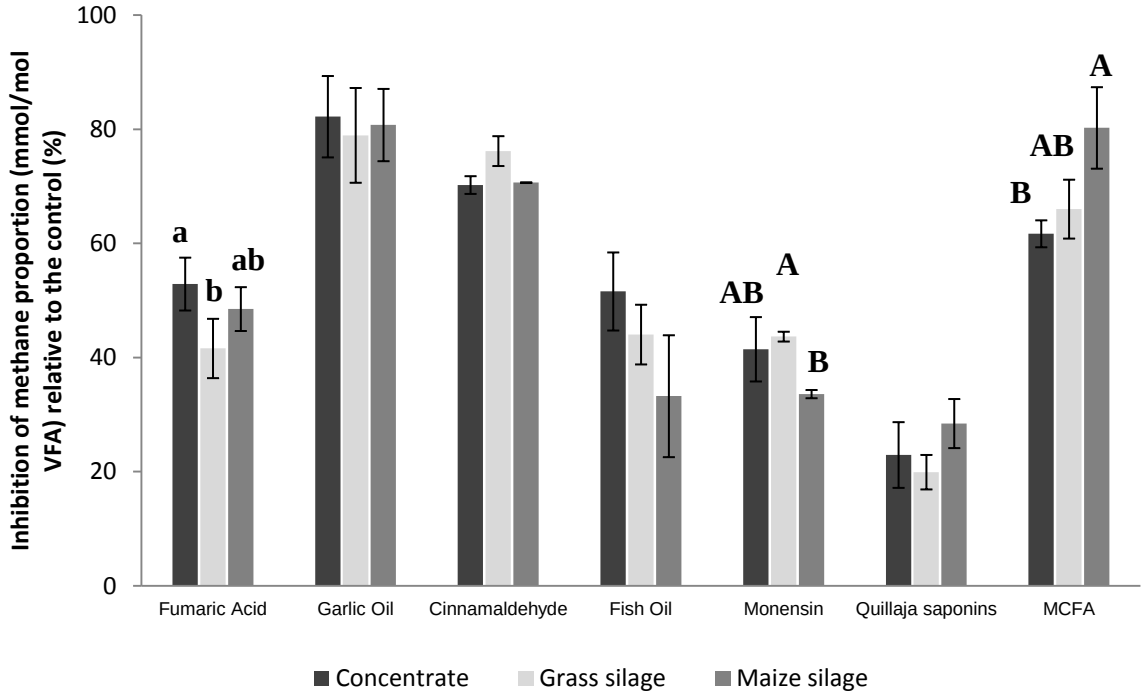
Figure 1.1. Inhibition (expressed relative to control) of *in vitro* $CH_4$ production (µmol/g DM) through supplementation of additives at high or low doses (Table 1.2) to either a standard dairy concentrate (CON), grass silage (GS) or maize silage (MS)

### Effects of additives on $CH_4$ inhibition, VFA production and $CH_4$ relative to VFA and rtio of C2 to C3

At the concentrations applied, all additives decreased $CH_4$ production (µmol/g DM; Figure 1.1) and $CH_4$ relative to VFA (mmol/mol VFA; Figure 1.2). Compared with control, garlic oil, cinnamaldehyde and MCFA inhibited $CH_4$ production by more than 70% ($P$<0.001); fumaric acid, fish oil and monensin decreased $CH_4$ by 25 to 50% ($P$<0.001) and quillaja saponins decreased $CH_4$ production by ~25% ($P$<0.001) (Figure 1.1).

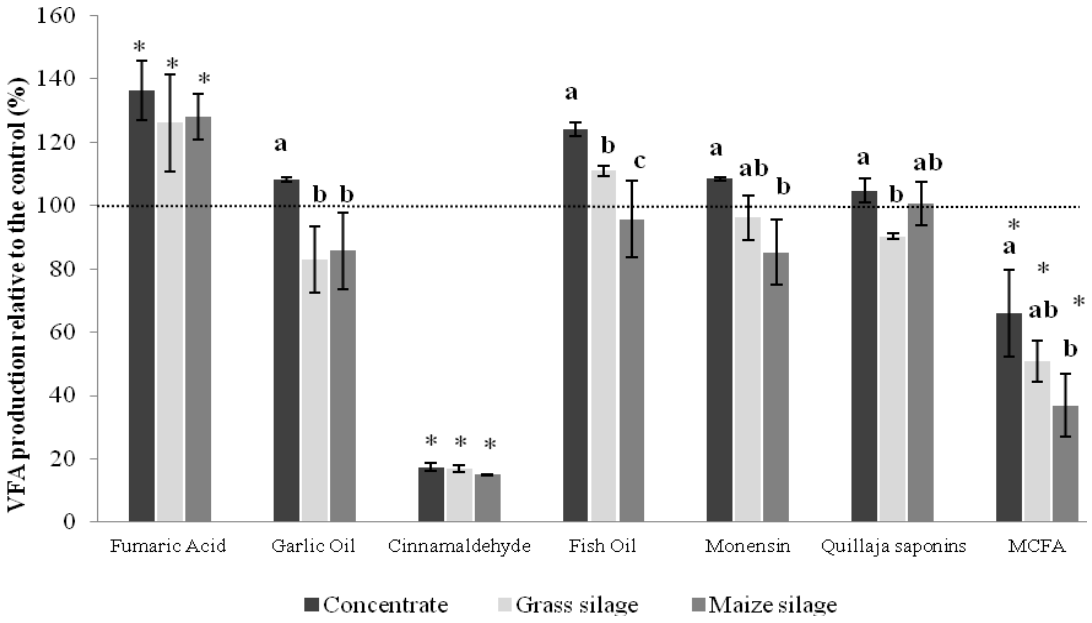
In contrast, not all additives changed VFA productions (Figure 1.3). In general, VFA production increased when fumaric acid was added to each substrate ($P$<0.05). Whereas, cinnamaldehyde and MCFA decreased total VFA production ($P$<0.05), there were no effects for the other additives at the concentrations applied.

Furthermore, fumaric acid was the only additive decreasing the ratio of C2 to C3 in combination with any of the substrates. Garlic oil, monensin and QS, also decreased C2:C3 but only in combination with concentrate. On the other hand, MCFA decreased the ratio of C2 to C3 in combination with grass silage. Whereas, fish oil did not affect C2:C3 with any substrate. Cinnamaldedyde, strongly inhibited production of all VFA.




a, b, c superscripts over the bars indicate significant substrate × additive interactions ($P$<0.05)
A, B superscripts over the bars indicate a tendency for significant substrate × additive interaction ($P$<0.10)
All additives at the concentrations applied differed from the respective control value ($P$<0.05)

Figure 1.2. Inhibition (expressed relative to control) of *in vitro* $CH_4$ proportion (mmol/mol VFA) through supplementation of additives at high or low doses (Table 1.2) to either a standard dairy concentrate (CON), grass silage (GS) or maize silage (MS)




a, b, c superscripts over the bars indicate substrate × additive interactions ($P$<0.05)
Stars above the bars indicate differences with the respective control (indicated by the dotted line at the 100% mark) ($P$<0.05)

Figure 1.3. Standardized (to the respective control value) *in vitro* VFA production through supplementation of additives at high or low doses (Table 1.2) to either a standard dairy concentrate (CON), grass silage (GS) or maize silage (MS)

## Interactions between additives and substrates on $CH_4$ inhibition

A comprehensive qualitative summary is in Table 1.3. Substrate × additive interactions on $CH_4$ and total VFA production could be divided into four effects being: 1) a group without interaction, 2) a group with an interaction on only one parameter, 3) a group with an interaction on both absolute $CH_4$ and VFA production and, 4) a group having an interaction on all three parameters.

### *Group 1. No substrate × additive interaction*

The effect of cinnamaldehyde did not differ among substrates, likely the result of inhibition of the fermentation process as reflected in the strong reduction of VFA production.

Table 1.3. Overview of substrate [a] × additive interactions on VFA production, inhibition of $CH_4$ production and inhibition of $CH_4$ relative to VFA (qualitative summary of Figures 1.1 to 1.3).

| | Relative VFA production [b] | | Inhibition $CH_4$ | | Inhibition $CH_4$ relative to VFA | |
|---|---|---|---|---|---|---|
| | Largest | Smallest | Largest | Smallest | Largest | Smallest |
| Cinnamaldehyde [c] | | | | | | |
| Quillaja saponins | CON | GS | GS, MS [d] | CON | | |
| Fumaric acid | | | | | CON | GS |
| MCFA | CON | MS | MS | CON | MS† | CON |
| Fish oil | CON | MS | CON, GS [d] | MS | | |
| Garlic oil | CON | GS, MS [d] | | | | |
| Monensin | CON | MS | GS, MS [d] | CON | GS† | MS |

† Tendency ($P$<0.10)

[a] Substrate: CON = concentrate, MS = maize silage, GS = grass silage.

[b] Relative VFA production $= \frac{\text{mol VFA supplemented incubation}}{\text{mol VFA of the corresponding non-supplemented control}}$

[c] Cinnamaldehyde did not have any substrate × additive interactions

[d] substrates not differing within a category but differing from the substrate in the other category

***Group 2. Substrate × additive interaction on one parameter***

Fumaric acid was the only additive with no interactions with substrates on both $CH_4$ and VFA production. When $CH_4$ was expressed relative to VFA, fumaric acid inhibited $CH_4$ (mmol/mol VFA) more in combination with CON *versus* GS ($P$=0.04). No differences occurred between CON and MS and between GS and MS. However, garlic oil was the only additive which had interaction effects on VFA but not on $CH_4$ production or $CH_4$ relative to VFA. Total VFA production was higher for CON than for GS ($P$<0.001) and MS ($P$=0.001).

***Group 3. Substrate × additive interaction on $CH_4$ and VFA production***

Fish oil and quillaja saponins had interaction effects on inhibition of $CH_4$ production and production of VFA, but had no effect on $CH_4$ relative to VFA, indicating the interaction mainly was caused by differences in total fermentation. Fish oil stimulated higher VFA

production when added to CON and GS than when combined with MS ($P$<0.001), which resulted in a higher $CH_4$ inhibition in combination with CON and GS than in combination with MS ($P$<0.001).

Quillaja saponins had higher VFA production in combination with CON *versus* GS ($P$=0.03), but not compared with MS, whereas no differences occurred between GS and MS. Consequently, quillaja saponins had higher $CH_4$ inhibition in combination with both GS and MS than in combination with CON ($P$<0.05).

***Group 4. Substrate × additive interaction on $CH_4$ production, VFA production and $CH_4$ relative to VFA***

Monensin had higher $CH_4$ inhibition in combination with GS and MS than in combination with CON ($P$<0.05). Monensin combined with CON had higher total VFA than when combined with MS ($P$=0.005), but there was no difference between GS and CON and GS and MS. Monensin tended to inhibit $CH_4$ relative to VFA more when combined with GS than in combination with MS ($P$=0.09).

By adding MCFA, total VFA production for CON was higher than for MS ($P$=0.007), but not different from GS. There were no differences between GS and MS. Due to their stronger effect on fermentation, MCFA combined with MS caused higher $CH_4$ inhibition than in combination with GS ($P$=0.006), and this also had a higher inhibition than combined with CON ($P$<0.001). Inhibition of $CH_4$ relative to VFA tended to be higher for MS than for CON ($P$=0.09).

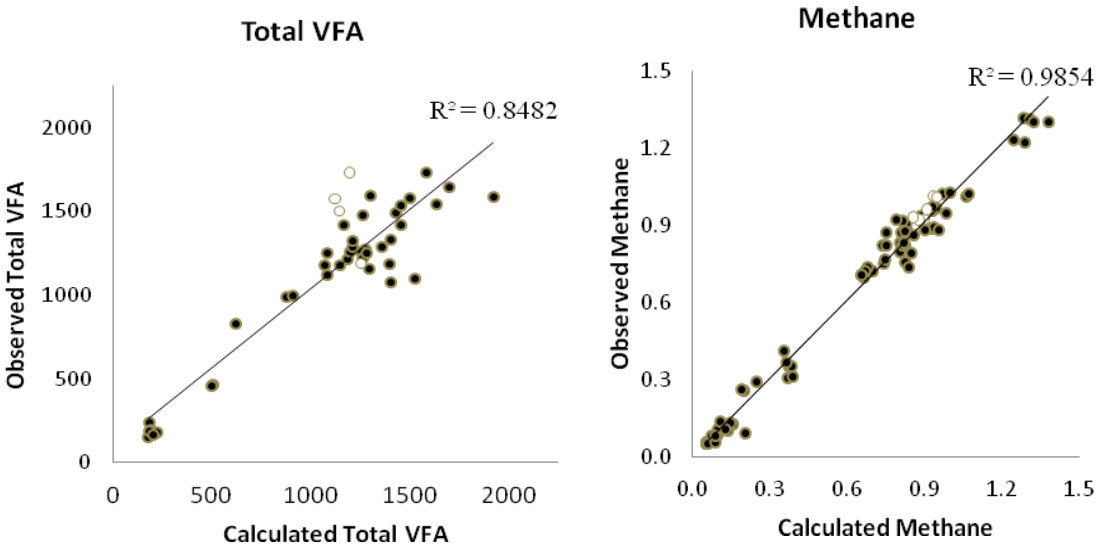
## Interaction between substrate and dose response on $CH_4$ inhibition and VFA production

Cinnamaldehyde did not have any dose effect for any of the parameters. For all the other additives, the higher dose caused higher inhibition of $CH_4$ production compared with the

lower dose. However, not all additives had a dose effect on VFA production or $CH_4$ relative to VFA. Fumaric acid, MCFA and garlic oil had differences between both doses for VFA production, with the former causing higher VFA production with the higher dose and the two latter having the opposite. Furthermore, quillaja saponins, fumaric acid and garlic oil also had a dose response for $CH_4$ relative to VFA with, in all cases, the higher dose causing higher inhibition than the lowest dose. Monensin had a tendency ($P$=0.09) to higher inhibition of $CH_4$ relative to VFA at the higher dose.

**Synergistic effects between substrates**

Synergistic effects among substrates were examined by comparing observed and calculated outputs of the combination of the substrates (MIX). Calculated outputs of MIX were estimated from the weighed mean of results from the three individual substrates (*i.e.*, CON, GS, MS). Figure 1.4 shows the strong relationship between calculated and observed data for both parameters. A strong relationship occurred between actually measured and calculated total VFA production ($r^2$ = 0.85) and for $CH_4$ production ($r^2$ = 0.98). One and zero fell within the 95%confidence interval of the slope and the intercept, respectively, for both $CH_4$ and VFA production. For total VFA, only addition of monensin at 0.015 mg/ml showed differences between observed and calculated data ($P$<0.05). Fumaric acid at 1.17 mg/ml were the only calculated observations differing from observed $CH_4$ production ($P$<0.05).




Empty symbols correspond to observations with differences between calculated and observed values ($P$<0.05)
Calculated data from individual incubations with either CON, MS and GS.

Figure 1.4. Calculated *versus* observed total VFA (µmol) and $CH_4$ production (µmol/g DM) of MIX (CON + GS + MS, 30:35:35)

## DISCUSSION

Doses applied for each additive were based on previous *in vitro* studies showing positive effects on $CH_4$ mitigation with the additives examined: Asanuma et al. (1999) used fumaric acid at 2.33 and 4.5 mg/ml incubation medium (20 and 30 mM); Busquet et al. (2005a) applied garlic oil at 0.3 mg/ml incubation medium; Pellikaan et al. (2011) used cinnamaldehyde at a concentration of 2.5 mg/ml incubation medium; fish oil was applied by Fievez et al. (2003) at concentrations ranging from 0.25 to 2.5 mg/ml incubation medium; Castro-Montoya et al. (2011) incubated quillaja saponins at concentrations varying between 0.5 and 1.25 mg/ml incubation medium; Callaway et al. (1997) added monensin to an alfalfa substrate at concentrations from 0.01 to 10 µM incubation medium. Goel et al., (2009) tested C10:0 at 0.6 mg/ml incubation medium, whereas Dohme et al. (2001) studied effects of the medium chain fatty acids C8:0, C10:0, C12:0 and C14:0 at a concentration of 50 g/kg DM (~ 0.62 mg/ml incubation medium).

Dietary additives to mitigate enteric $CH_4$ production have different working mechanisms (*e.g.*, inhibition of protozoa, stimulation of propionate, reduction of hydrogen production, direct inhibition of methanogens). The effectiveness of these additives might be strongly linked to the ruminal fermentation conditions induced by dietary ingredients, such as nutrient concentrations and pH. However, interactions among additives and feedstuffs in the basal diet have often been poorly described in previous studies. In our study, additives with potential to mitigate $CH_4$ were examined *in vitro* in combination with feedstuffs commonly used in dairy cattle diets.

**Substrate × additive interactions on $CH_4$ inhibition and VFA production.**

Even though there were differences between the doses of each additive on $CH_4$ inhibition, both doses of all additives caused a decrease in $CH_4$ production compared to control. It is clear that effects of additives on rumen fermentation and $CH_4$ mitigation vary among reported study. Different incubation substrates might be at the origin of inter-experimental variation in responses to additives. In our study we obtained information on how additives interact with substrates in order to better understand and interpret results of other studies.

For additives not having substrate × additive interactions of biologic relevance (*i.e.*, cinnamaldehyde, fumaric acid, garlic oil), differences between our and literature data, and among literature data, cannot be explained by differences due to the substrates incubated. Hence, there might be other reasons for the inconsistent results among studies. For additives with interactions, other factors (e.g. source of rumen fluid, dose of the additive, source and physical form of the additive, ratio of rumen fluid to buffer) could play a role in the lack of match among studies, but a larger variation could be due to different responses depending on type of substrate.

Our results were compared with previous studies (Figures 1.5 to 1.10) where the concentrations of the additives are presented relative to the milliliters of rumen fluid in the

incubation media (*e.g.*, mg/ml rumen fluid) to allow better comparison among short term incubations. A summary of the studies in Figures 1.5 to 1.10 is in Table 1.4.

***Cinnamaldehyde***

At concentrations of 1.5 and 3 mg/ml, inhibition of $CH_4$ production was 90 to 95%. Similarly, by adding cinnamaldehyde at 2.5 mg/ml to either soybean hulls or maize, Pellikaan et al. (2011) reported inhibitions of $CH_4$ production of close to 100% (Figure 1.5) during the first 24 h of a 72 h incubation run. The high doses applied in that study strongly inhibited fermentation regardless from substrate, which reduced the possibility to assess substrate × additive interactions upon addition of cinnamaldehyde. Remarkably, in the study of Pellikaan et al. (2011), complete $CH_4$ inhibitions occurred during first the 30 h regardless of substrate. However, $CH_4$ production from soybean hulls was ~65% after 72 h, suggesting some adaptation. Nevertheless, this adaptation seemed substrate dependent since $CH_4$ was completely inhibited for maize. No other studies determining effects of cinnamaldehyde on $CH_4$ production were located.

***Fumaric acid***

For each of the substrates, fumaric acid decreased $CH_4$ production and, in most cases, increased total VFA production. Previous studies have also shown inhibition of $CH_4$ production (Figure 1.5) and increases in VFA production in batch cultures upon addition of fumaric acid or its salts (Asanuma et al., 1999; López et al., 1999; Newbold et al., 2005).

Increases in VFA production are likely the result of conversion of fumaric acid to propionate and acetate. The amounts added in our study account for 250 and 500 µmol of fumaric acid and, even though complete conversion of it to VFA is not certain, this helps explain the increases in total VFA when fumaric acid was combined with CON, GS and MS, where increases were 240 to 480 µmol.

Newbold et al. (2005) found stronger $CH_4$ inhibition by fumaric acid than by sodium fumarate, which might help explain the larger $CH_4$ inhibitions in our study compared with others (Figure 1.5). Moreover, Newbold et al. (2005) and Ungerfeld et al. (2007, meta-analysis) did not find an interaction on $CH_4$ production between the forage to concentrate ratio and fumarate, which we confirmed, where no substrate × additive interaction occurred. In our study, there was no difference in VFA production among substrates and, similarly, substrates did not differ among them on $CH_4$ inhibition. However, fumaric acid was the only additive with differences among substrates for $CH_4$ relative to VFA, likely the result of combination of both a numerically higher $CH_4$ inhibition and VFA production for CON. Nevertheless, no consistent difference in forage to concentrate proportions occurred when combining studies in which fumarate was added (Figure 1.5).

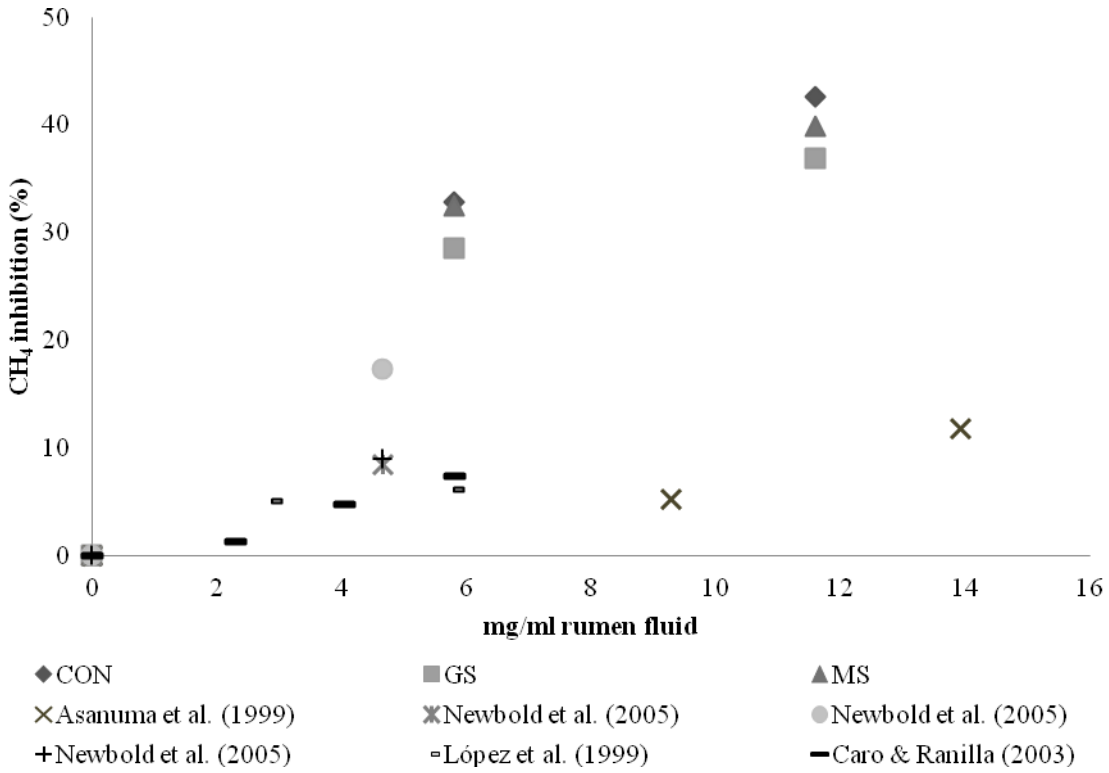



Figure 1.5. Inhibition (%) of *in vitro* $CH_4$ production by fumaric acid or sodium fumarate in combination with concentrate (CON), grass silage (GS) and maize silage (MS) from current study and inhibitions reported in previous studies

Table 1.4 Overview of studies used to compare with own results in Figures 1.5 to 1.10

| Study [a] | Source | Substrate | Buffer [b] | Incubation system [c] |
|---|---|---|---|---|
| Fumaric acid | | | | |
| Asanuma et al., 1999 | Disodium salt | Tymothy/alfalfa hay : concentrate (1:3) | 1:3 | Batch |
| López et al., 1999 | Disodium salt (Sigma Chemical Co., UK) | Grass hay : concentrate (1:1) | 1:4 | Batch |
| Caro and Ranilla., 2003 | Disodium salt (Sigma, Spain) | Corn meal | 1:4 | Batch |
| Newbold et al., 2005 (1) | Fumaric acid (Sigma Chemical Co., UK) | Alfalfa hay : concentrate (3:1) | 1:4 | Batch |
| Newbold et al., 2005 (2) | Disodium salt (Sigma Chemical Co., UK) | Alfalfa hay : concentrate (3:1) | 1:4 | Batch |
| Newbold et al., 2005 (3) | Disodium salt (Sigma Chemical Co., UK) | Alfalfa hay : concentrate (1:3) | 1:4 | Batch |
| Garlic oil | | | | |
| Busquet et al., 2005a | Pancomsa SA ( France) | Alfalfa hay : concentrate (1:1) | 1:4 | Batch |
| Patra et al. 2006 (1) | Garlic extract in ethanol | Wheat straw : concentrate (1:1) | 1:3 | Batch |
| Patra et al., 2006 (2) | Garlic extract in methanol | Wheat straw : concentrate (1:1) | 1:3 | Batch |
| Fish oil | | | | |
| Fievez et al., 2003 (1) | High EPA + DHA. Pronova Biocare, Sandefjord, Norway | Grass hay | 1:4 | Batch (two steps) |
| Fievez et al., 2003 (2) | Low EPA + DHA. Technology Laboratory of the Danish Ministry of Fisheries | Grass hay | 1:4 | Batch (two steps) |
| Medium chain fatty acids | | | | |
| Dohme et al., 2001 (1) | C10:0 | Forage: concentrate (3:1) | 8:1 | RUSITEC |
| Dohme et al., 2001 (2) | C12:0 | Forage: concentrate (3:1) | 8:1 | RUSITEC |
| Machmüller et al., 2002 | C12:0 (Fluka Chemie AG, Switzerland) | Forage: concentrate (3:1) | 8:1 | RUSITEC |
| Soliva et al., 2003 | C12:0 (Fluka Chemie AG, Switzerland) | $H_2$:$CO_2$ (4:1) | 1:2 | HGT |
| Goel et al., 2009 | C10:0 (Vitamex Inc., Belgium) | Concentrate | 1:4 | Batch |
| Quillaja saponins | | | | |
| Pen et al., 2008 | Liquid extract. Mitsuba Trading Co. Ltd., Japan | Oat hay + concentrate (1:1) | 1:1 | Continuous |
| Castro-Montoya et al., 2011 | Purified powder. Spray dried, Desert King, USA | Grass hay + concentrate (7:3) | 1:3 | HGT |
| Monensin | | | | |
| Russell et al., 1988 (1) | ----- | Corn meal | 1:3 | Batch |
| Russell et al., 1988 (2) | ----- | Tymothy hay | 1:3 | Batch |
| Callaway et al., 1997 | Sigma Chemical Co. (MO, USA) | Alfalfa | 1:2 | Batch |
| Busquet et al., 2005a | Sigma Chemical (MO, USA), | Alfalfa hay : concentrate (1:1) | 1:4 | Batch |

[a] Numbers between parenthesis indicate different observations in the same study.
[b] Ratio of rumen fluid to buffer solution in the incubation medium.
[c] Incubation system: HGT = Hohenheim gas test; RUSITEC = Rumen simulation technique.

***Garlic oil***

Methane production decreased by ~70 and 90% at garlic oil concentrations of 0.15 and 0.3 mg/ml, respectively. Similarly, Busquet et al. (2005a) found strong reductions of $CH_4$ production by adding garlic oil, while Patra et al. (2006) also found inhibitions ranging from 20 to 60% by adding ethanol and methanol garlic extracts (Figure 1.6). However, the $CH_4$ inhibitions in our study were much higher. It is unlikely that these differences are due to differences in substrates, since substrate × additive in our study were not biologically relevant. Consistent with our findings, *in vitro* studies of Busquet et al., (2005a; 2005b) did not find negative effects of garlic oil on total VFA production.

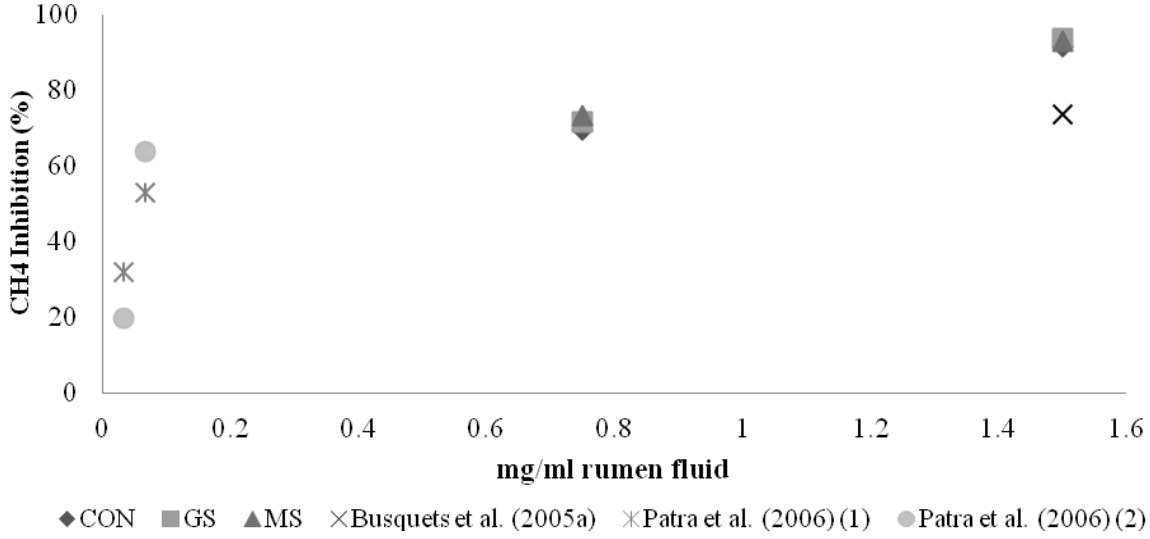



Figure 1.6. Inhibition (%) of *in vitro* $CH_4$ production by garlic oil and garlic extract in combination with concentrate (CON), grass silage (GS) and maize silage (MS) from the current study and inhibitions reported in previous studies.

***Fish oil***

The fatty acids responsible for $CH_4$ are *n*-3-eicosapentanoic acid (EPA) and *n*-3-docosahexanoic acid (DHA; Fievez et al., 2003). These fatty acids represent 193 and 111 g/kg FA of EPA and DHA, respectively, in the fish oil which we used. The oil contained 310 g/kg

FA of saturated fatty acids, 254 g/kg FA of MUFA and 132 g/kg FA of other PUFA. Fievez et al. (2003) studied effects of two sources of fish oil differing in concentrations of EPA and DHA combined with hay in a two steps *in vitro* batch system of 48 h. The high EPA (181 g/kg FA) and DHA (119 g/kg FA) source of fish oil caused a stronger reduction of $CH_4$ than the low EPA-DHA (EPA, 54 g/kg FA; DHA, 75 g/kg FA) source, but both caused larger $CH_4$ inhibitions than those observed in our study (Figure 1.7). Fievez et al. (2003), in agreement with our findings, did not find effects of fish oil on total VFA production. No other *in vitro* studies were identified where fish oil was used to inhibit methanogenesis.

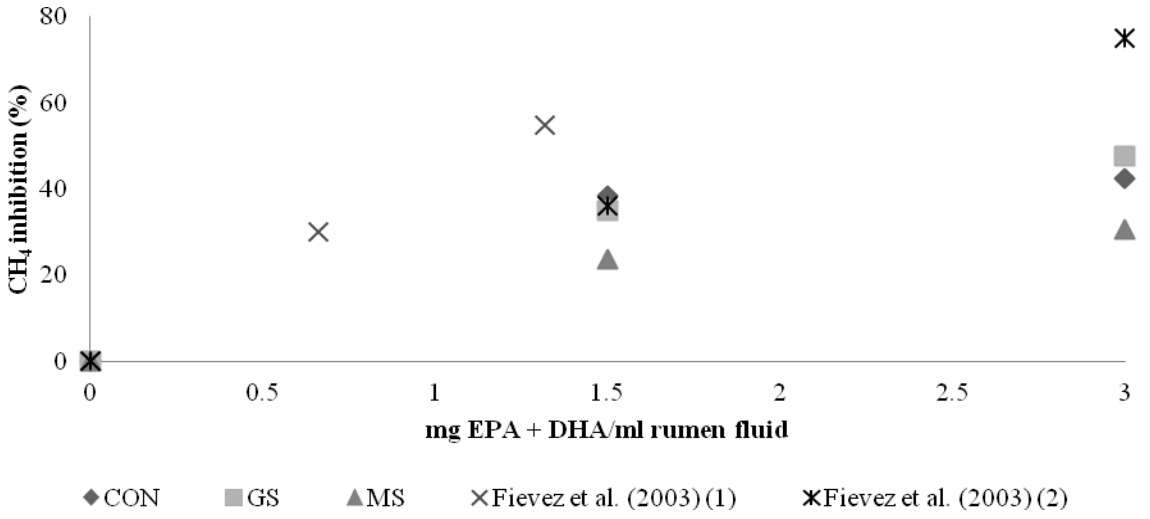
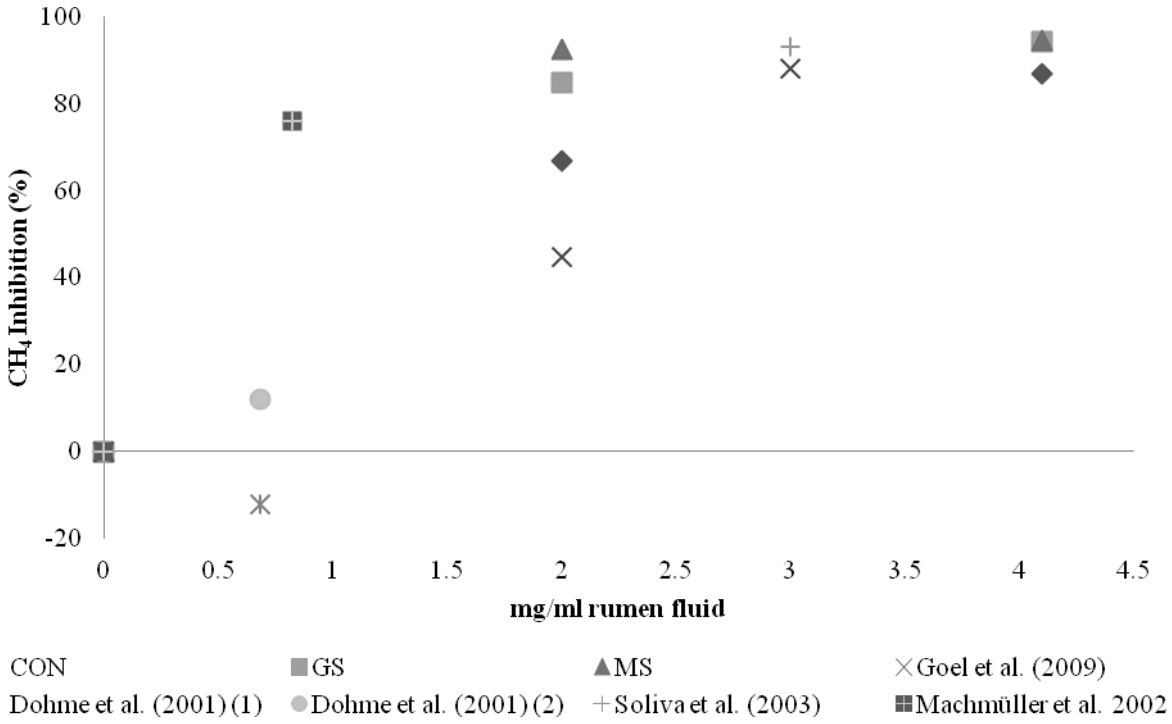



Figure 1.7. Inhibition (%) of *in vitro* $CH_4$ production by EPA and DHA from fish oil in combination with concentrate (CON), grass silage (GS) and maize silage (MS) from the current study and inhibitions reported in previous studies

***Medium chain fatty acids***

The MCFA mixture contained mainly caprilic acid (C8:0, 312 g/kg FA), capric acid (C10:0, 200 g/kg FA) and lauric acid (C12:0, 485 g/kg FA). Addition of MCFA at 0.6 and 1.2 mg/ml incubation medium decreased $CH_4$ production by ~80% but also reduced VFA production. Dohme et al. (2001) found that C12:0 and C14:0 decreased $CH_4$ production by ~18% without effects on VFA production, whereas C8:0 and C10:0 did not elicit any effect on

methanogenesis. Conversely, Goel et al. (2009) found reductions of 44 and 88% by using C10:0 at 0.4 and 0.6 mg/ml incubation medium and a 23% decrease of VFA production. Other studies with a Hohenheim *in vitro* system (Soliva et al., 2003) or a RUSITEC (Soliva et al., 2004) found that combinations of lauric and myristic acids decreased $CH_4$ production by 50% or more without affecting total VFA when C12:0 was in proportions of 33%, or higher, incubation medium.




Dose of MCFA for CON, GS and MS as C10:0 + C12:0

Figure 1.8. Inhibition (%) of *in vitro* $CH_4$ production by medium chain fatty acids in combination with concentrate (CON), grass silage (GS) and maize silage (MS) in the current study and inhibitions reported in previous studies. Dose of MCFA for CON, GS and MS as C10:0 + C12:0

In our study, inhibition of VFA production by MCFA was higher in combination with MS, followed by GS, and lower in combination with CON. The pattern of VFA inhibition was clearly reflected on $CH_4$, where the order of inhibition was MS > GS > CON. Machmüller et al. (2003) found that MCFA have more potential to inhibit methanogenesis in combination with concentrate rich diets *in vivo*, noting that fatty acids probably attach more readily to feed particles, and less to methanogens, when MCFA are combined with fiber rich diets. While our

results contrast to findings of Machmüller et al. (2003), the strong inhibition of fermentation in our study impaired our ability to draw firm conclusions. However, overall, based on previous and current results (Figure 1.8), inhibition of rumen $CH_4$ production with MCFA largely depends on the source (*i.e.*, proportions of individual MCFA) and dose applied.

***Quillaja saponins***

Figure 1.9 is a comparison of the three substrates in our study and two other studies where quillaja saponins were tested for effects on $CH_4$ production. In Castro-Montoya et al. (2011) quillaja saponins decreased $CH_4$, but the inhibitions were lower than in our study. However Pen et al. (2008) did not find any effect of quillaja saponins on $CH_4$ production. Furthermore, quillaja saponins do not affect total VFA production (Castro-Montoya et al., 2011; Pen et al., 2008), which implies that the digestibility of substrate is not affected by these compounds. This might be related to the considerable proportion (300 to 500 g/kg DM) of concentrate in the substrate of the latter two studies. Indeed VFA production by quillaja saponins was lower in combination with GS, than with CON, and numerically lower than in combination wih MS. These effects might be related to their adverse effects on cellulolytic bacteria, protozoa and fungi (Holtshausen et al., 2009; Kamra et al., 2008), whose inhibition decreases fermentation of substrate and reduces availability of hydrogen for methanogenesis. Indeed, inhibition of cellulolytic activity would be more likely in a fibrous than a starch rich substrate.

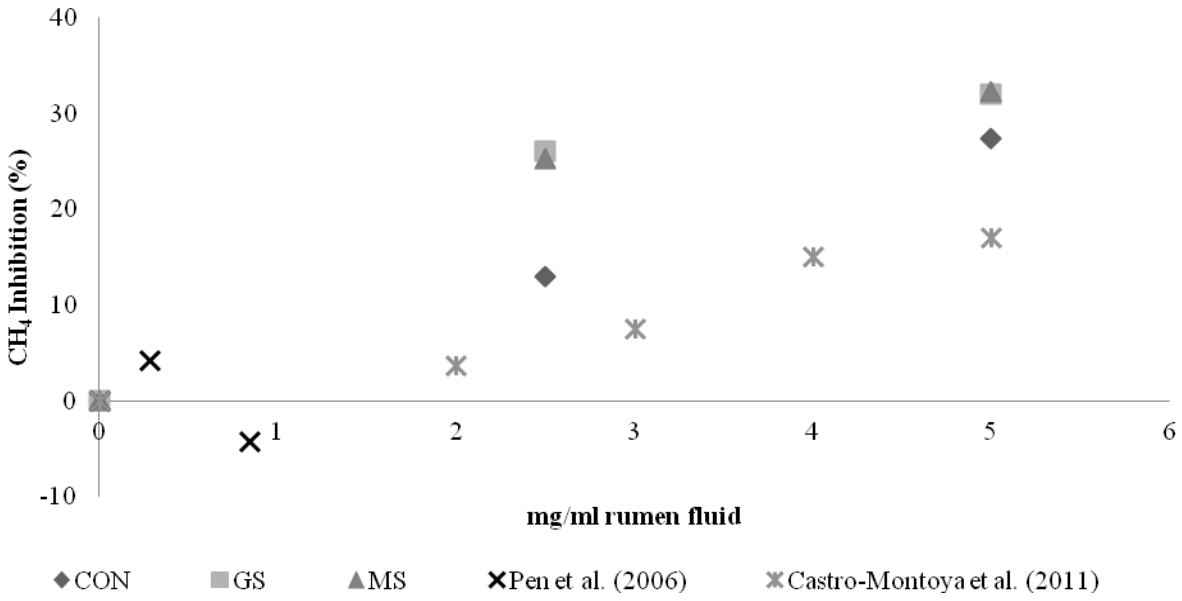



Figure 1.9. Inhibition (%) of *in vitro* $CH_4$ production by quillaja saponins in combination with concentrate (CON), grass silage (GS) and maize silage (MS) from the current study and inhibitions reported in previous studies

***Monensin***

Monensin at 0.0015 (2.5 µM) and 0.003 (5 µM) mg/ml incubation medium inhibited $CH_4$ production by 30 to 50% without effects on VFA production, which is consistent with other studies (Figure 1.10; Callaway et al., 1997; Russell et al., 1988) although the magnitude of the response differed. Similar to saponins, monensin inhibits cellulolytic activity, but also enhances amylolytic activity, which leads to decreasing availability of $H_2$ to $CH_4$ formation. In our study, VFA production was higher when monensin combined with CON than with MS, while GS did not differ from any other substrate. Correspondingly, monensin caused higher $CH_4$ inhibition in combination with GS and MS than with CON. It is possible that the negative impact of monensin on cellulolytic activity was higher in both silages where cellulolytic bacteria play a more important role than in a starchy substrate. In contrast, in the study of Russell et al. (1988), the $CH_4$ reduction was stronger when supplementing corn grain compared with hay.

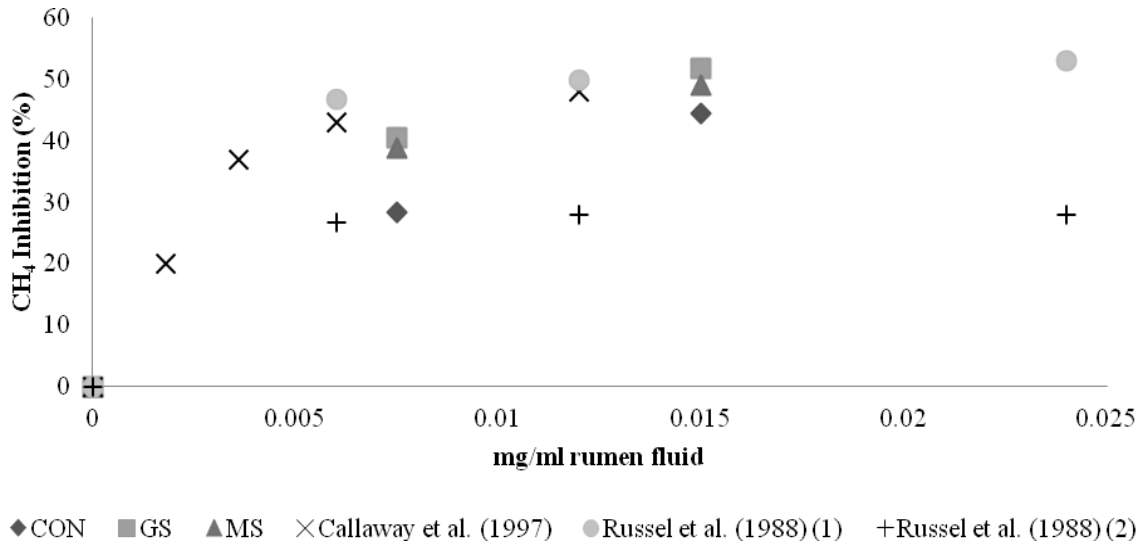



Figure 1.10. Inhibition (%) of *in vitro* $CH_4$ production by monensin in combination with concentrate (CON), grass silage (GS) and maize silage (MS) from the current study and inhibitions reported in previous studies

In our study effects of additives on $CH_4$ inhibition were strongly linked to their effects on VFA production, suggesting that the additives interact with the substrate by modifying the fermentation, rather than directly affecting $CH_4$ formation. In most cases, effects of the additives on VFA production was larger for CON and smaller for MS. This is in line with a generally larger inhibition of $CH_4$ production for silages compared with CON.

Overall, for fumaric acid (higher inhibition of $CH_4$ relative to VFA), garlic oil (higher VFA production) and fish oil (highest inhibition of $CH_4$ production and highest VFA production) the combination with CON was the one with the highest potential to inhibit $CH_4$ emissions. For monensin, GS was the substrate causing the best response for $CH_4$ mitigation (*i.e.*, higher VFA production, higher inhibition of $CH_4$ production and a tendency to higher inhibition of $CH_4$ relative to VFA). Maize silage was the substrate with the best results in combination with quillaja saponins, because it caused the largest $CH_4$ production inhibition without decreasing VFA production compared with both control and CON. For MCFA and cinnamaldehyde, due to the very strong inhibition of fermentation, it is hard to assess which combination would be better as a $CH_4$ mitigation strategy.

This study shows that additives respond differently depending on substrate. However, when comparing our data with literature data, general conclusions on the nature of the substrate × additive interactions are difficult to draw. This might be due to specificities of the substrates used in each of the studies, which impair generalization in, for example, terms of concentrate to forage proportions.

**Synergistic effects between substrates**

In cattle industry, diets tend to be well balanced in terms of protein, energy, fiber and micronutrients in order to support a healthy rumen environment and to maximize microbial protein synthesis. There is no such thing as an "ideal" diet which can be fed to every animal, not even within the herd of a single farm due to variations in individual animal characteristics and performance. This makes it difficult to design dietary strategies to decrease $CH_4$ production, since different diets will induce different conditions in the rumen and, in some cases, these conditions are suggested to influence the way the additive affects methanogenesis. It would be interesting to identify the additive/substrate combinations which cause higher responses in $CH_4$ inhibition to help target the ideal additive depending on the dominant diet ingredient.

We found a strong relationship between measured and calculated total VFA production ($R^2$ = 0.85), and between measured and clculated $CH_4$ production ($R^2$ = 0.98), which indicates that there were no synergistic effects between substrates and that effects of an additive combined with a mixed substrate *in vitro* is the result of addition of effects of that additive combined with the substrates. These results agree with findings of Lee et al. (2003) in not showing differences between actual and predicted values of $CH_4$ production of 28 combinations of 9 feeds. Even though Lee et al. (2003) did not add a $CH_4$ mitigation additive, their study suggested that there is no interaction effect among substrates. We can now extend that finding to additive/substrate combinations, even when additive responses vary by substrate, which

would allow identification of the most promising additive to supplement mixed diets based on data of single substrate × additive combinations for the type of substrates.

## CONCLUSIONS

It was shown *in vitro* that additives with different modes of action to inhibit $CH_4$ production interact with substrates with different characteristics. It is not possible to classify and group the additives used and, therefore, it is difficult to draw a general conclusion on which type of additive (*e.g.*, propionate precursors, inhibitors of $H_2$ production) should be combined with a particular substrate. However the absence of synergistic effects among substrates suggests that, in practical conditions, an additive can be used in combination with a particular substrate/feed that maximizes $CH_4$ inhibition.

# CHAPTER 1B. MODIFICATIONS TO *IN VITRO* BATCH INCUBATIONS ATTEMPTING TO BETTER REFLECT IN VIVO MITIGATION OF RUMEN METHANE: A CASE STUDY WITH A BLEND OF ESSENTIAL OILS

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Redrafted after:

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## ABSTRACT

The effects of Agolin Ruminant, a blend of essential oils, on methane emissions were investigated in two *in vivo* experiments during six weeks with dairy and beef cattle, and in four *in vitro* experiments. In the *in vivo* experiment with dairy cattle, 0.2 g/d of essential oils (*ca.* 2 ppm *m/v*) tended to decrease the daily methane emissions (g/d) and methane relative to dry matter intake (g/kg DMI) by 15 and 14%, respectively, after 6 weeks of supplementation, but no difference was observed for methane relative to milk production (g/kg milk). In the *in vivo* experiment with beef cattle, the daily methane emissions and methane relative to DMI did not change through addition of the blend of essential oils (0.2 g/d, equivalent to *ca.* 4 ppm, *m*/*v*). The *in vitro* experiments attempted to replicate the results observed *in vivo.* A decrease in methane production of 15% was observed in 24 h batch incubations, but only at very high concentrations (50 ppm, *m/v*). Methane was inhibited to a similar extent (17%) when somewhat lower amounts of essential oils (30 ppm) were applied in a gas production technique (GPT) system. Nevertheless, both concentrations are considerably higher than those applied under *in vivo* conditions. A longer contact time between the essential oils (15 and 30 ppm) and the feedstuff (essential oils added *ca*. 16 h prior the start of the incubation) did not elicit any effect on $CH_4$ production and was not different from addition immediately prior to the start of the incubation. A longer incubation time and regular supply of both substrate and additive in a consecutive batch incubation system with transfers every 24 h for 96 h or transfers every 48 h for 14 d did not induce $CH_4$ inhibition at lower doses of essential oils (5, 15 and 30 ppm, *m/v*) and hence, were not able to replicate *in vivo* results. In this comparative study modifications to the *in vitro* set-up did not allow to cancel out large differences in the essential oils doses needed *in vitro* to provoke similar $CH_4$ inhibitory effects as observed *in*

*vivo*. This might be due to differences in the mode of action of the essential oils *in vitro* and *in vivo*, which merits attention for future research.

## INTRODUCTION

The rising interest in decreasing methane ($CH_4$) emissions from ruminants and the interest in finding alternatives to antibiotics has prompted the research on plant derived compounds to modulate ruminal fermentation. In general, a large number of plant compounds are screened, usually through batch in vitro tools. Promising compounds are later validated in vivo.

Essential oils are plant secondary metabolites believed to have potential as dietary additives, due to their strong antibacterial properties (Burt, 2004). Essential oils inhibited the energy metabolism of *Streptococcus bovis* and *Selenomonas ruminantium* (Evans and Martin, 2000) and the growth of *Methanobrevibacter smithii*, a rumen Archaea (McIntosh et al., 2003). The latter is of major interest given worldwide attempts to decrease $CH_4$ from ruminants.

Essential oils have been studied both *in vivo* and *in vitro*, but effects on $CH_4$ were variable, which may be linked to the large diversity in the nature of these compounds (Calsamiglia et al., 2007). Hence, routine screening is required to assess the effectiveness of numerous essential oils and blends at different doses. *In vitro* batch incubation systems are practical tools for this purpose. However, in many cases promising in vitro results cannot be replicated *in vivo* (Flachowsky and Lebzien, 2012). Another challenge is the transformation of effective *in vitro* concentrations towards in vivo doses. Obviously, there are clear differences between *in vitro* and in vivo systems (see e.g. review by Getachew et al. 2004). Therefore, in vitro batch systems should improve to better simulate in vivo responses while maintaining its routine nature. Given the current interest on rumen $CH_4$ production, the focus of this study is to explore different adjustments to a standard in vitro batch system to better simulate in vivo responses of $CH_4$ production to addition of a blend of essential oils.

## MATERIALS AND METHODS

Procedures with animals were approved by the ethical commission of the Institute for Agricultural and Fisheries Research (ILVO), Belgium (EC 2011, 154).

### Materials

The blend of essential oils is a commercially available product (Agolin Ruminant) provided by AGOLIN SA (Bière, Switzerland) contained 20% of active compounds, mainly coriander oil, geranyl acetate and eugenol. From here on when referring to doses and concentrations we will mean the amount of essential oils supplemented.

### *In vivo* experiments

*Methane measurements.* Enteric methane was measured in weeks 0, 2, 4 and 6 by keeping the animals in individual open circuit chambers (De Campeneere and Peiren, 2012). Measurements in week 0 (no blend of essential oils) were considered as control for this experiment. Each measuring period lasted from Tuesday morning until Friday morning.

Through ventilation, a slight negative atmospheric pressure was generated inside the chamber to avoid leakage of air to the outside of the chamber. Each chamber had a volume of 12.3 $m^3$, this volume was completely exchange d by the ventilation system (400 $m^3/h$) approximately every 2 minutes. Air samples were taken from the outlet of the chamber through a tube connected to an eight-channel multi sampler directly attached to the gas analyzer (infrared laser optical-feedback cavity-enhanced absorption spectrometer). Methane was determined every second. Each channel was monitored during 180 seconds. The average of the last 60 seconds was used as a data point. Background air outside the chambers was also sampled and analyzed by two additional channels.

***Experiment with dairy cattle***

Four multiparous lactating Holstein dairy cows with an average body weight of 603 kg (± 70.0) and being 296 (± 97.6) days in milk at the start of the experiment were used for the *in vivo* measurements. The experiment ran over a period of six weeks and was performed between October and December 2011.

Before the start of the experiment, cows had *ad libitum* access to a mixture of grass silage, maize silage and soybean meal and were supplemented with concentrate (Table 1.5). The proportions during this period were equal to those during the experiment. Two weeks before the start of the experiment a fixed amount (95% of the *ad libitum* intake of the cow with the lowest intake) was offered daily until the end of the measurements. This was done to avoid confounding effects of treatment and advanced lactation stage on feed intake and passage rate in the rumen. The proportions of feedstuffs along with their chemical composition are presented in Table 1.5. Cows were fed and milked twice a day at 0730 AM and 1730 PM and had free access to drinking water at every time throughout the experiment.

***Experiment with beef cattle***

Four Belgian Blue beef heifers with an average body weight of 484 kg (± 111.3 kg) at the start of the experiment were used for the *in vivo* measurements. The six-weeks experiment was performed between October and December 2012.

Two weeks before the experiment, the animals were fed maize silage *ad libitum* and supplemented with concentrate. The proportions (Table 1.5) during this period were equal to those during the experiment. Similar to the experiment with dairy cattle, two weeks before the start, a fixed amount (95% of the *ad libitum* intake of the heifer with the lowest intake) was offered daily until the end of the experiment. The amounts and proportions of feedstuffs along

with their chemical composition are presented in Table 1.5. Heifers were fed once daily at 0730 AM and had free access to drinking water throughout the experiment.

When the animals were not in the chamber they were housed and tied in a stall next to the room were the chambers were located. They were fed and milked under the same regimen as when being in the chamber.

*Blend of essential oils supplementation.* For both beef and dairy cattle the blend of essential oils was supplemented daily after the first gas measurements of week 0. Each animal received a daily dose of 1 g of the essential oils' blend, delivering 0.2 g of essential oils, which was mixed in 1 kg of concentrate using an industrial bakery mixer during *ca.* 2 minutes. The mix was stored at ambient temperature and the daily portion of 1 kg was fed to the animals either divided into two portions for the dairy cattle or as a single feeding for the beef cattle.

Table 1.5. Ingredient and chemical composition of the experimental diets (g/kg DM) as offered to both dairy cows and beef heifers

| | Dairy cattle | | | | Beef cattle | |
|---|---|---|---|---|---|---|
| | Grass silage | Maize silage | Con-centrate | Soybean meal | Maize silage | Con-centrate |
| Dietary proportion (g/kg DM) | 460 | 370 | 120 | 50 | 500 | 500 |
| Chemical composition (g/kg DM) | | | | | | |
| Dry matter (g/kg fresh matter) | 313 | 376 | 877 | 862 | 373 | 876 |
| Crude protein | 171 | 83 | 189 | 516 | 71 | 212 |
| Crude fat | 43 | 29 | 31 | 33 | 38 | 79 |
| Crude ash | 100 | 41 | 84 | 64 | 65 | 76 |
| Sugars | 25 | 0.4 | 105 | 109 | n.d. | 83 |
| Starch | n.d. | 418 | 219 | n.d. | 382 | 34 |
| Organic matter | 900 | 959 | 916 | 936 | 965 | 924 |
| Organic matter digestibility (g/kg DM)[a] | 799 | 787 | 898 | 907 | 753 | 812 |

[a] *In vivo* organic matter digestibility estimated from cellulose digestibility

***In vitro experiments***

Substrates

Three batches of each concentrate, grass silage and maize silage were used as substrates for the *in vitro* experiments. The chemical composition of the feedstuffs is presented in Table 1.6. The first series of experiment 1 was performed using two different feed mixes as substrates: concentrate + maize silage (50:50, DM basis) and concentrate + grass silage + maize silage (30:35:35, DM basis), whereas in all of the other *in vitro* experiments only the latter substrate was used.

Table 1.6. Chemical composition of the substrates used for *in vitro* experiments (g/kg DM)

| | Concentrate : GS :M S | | | Concentrate : MS | | |
|---|---|---|---|---|---|---|
| | 1 | 2 | 3 | 1 | 2 | 3 |
| Crude protein | 151 | 163 | 155 | 130 | 148 | 136 |
| Crude fat | 57.9 | 52.6 | 41.1 | 56.5 | 51.8 | 41.6 |
| Crude ash | 86.4 | 85.4 | 73.1 | 65.7 | 56.2 | 58.5 |
| NDF | 490 | 486 | 492 | 448 | 397 | 369 |
| ADF | 258 | 270 | 224 | 225 | 215 | 166 |
| Lignin | 41.5 | 51.7 | 34.3 | 42.9 | 40.3 | 24.6 |

Experiment 1. Standard 24 h batch incubations with increasing essential oils concentrations

In a first series of batch incubations the blend of essential oils was incubated during 24 h (Castro-Montoya et al., 2012) delivering essential oils concentrations of 0, 0.25, 0.5, 1, 2, 5, 15 and 30 ppm (volume basis) in triplicate. In a second series of these standard incubations the concentration range was extended to 0, 5, 10, 15, 20, 25, 30, 35, 40, 45 and 50 ppm.

Experiment 2. Standard 24h incubation with varying moment of essential oils addition

In this incubation set-up, which was the same as for experiment 1, the effect of the moment of addition of the aqueous solution with essential oils was assessed. In the standard set-up the additive is added to the *in vitro* batch system shortly before the start of the incubation. This was compared with the addition of the additive the afternoon before the start of the

experiment (*ca*. 16 h earlier). This comparison was made using essential oils at levels of 15 and 30 ppm during 24 h.

Experiment 3. Gas production technique, 72 h

The blend of essential oils was incubated at concentrations of 15 and 30 ppm during 72 h with $CH_4$ measurements after 2, 4, 8, 12, 24, 30, 36, 48, 58 and 72 h using the gas production technique (GPT) (Cone et al., 1996).

Experiment 4. Consecutive batch incubation

In a first series within the consecutive batch incubations (Theodorou et al., 1984) the blend of essential oils was tested at two concentrations (15 and 30 ppm) with transfer every 24 h for a total incubation time of 96 h. In a second series of consecutive batch incubations, the incubation time was extended to 14 days with transfers every 48 h. The blend of essential oils was tested at three concentrations (5, 15 and 30 ppm).

For experiments 1, 2 and 3, incubations were done in triplicates within one run. The first series of experiment 4 included 6 replicates divided in two runs in different weeks. The second series of experiment 4 included 5 replicates within the same run.

Methane formation was measured in all experiments, whereas total VFA was measured in the first series of experiment 1, in experiments 2 and 3, and in the second series of experiment 4.

***Standard in vitro 24 h batch incubation***

The rumen fluid was collected before the morning feeding from three rumen fistulated sheep. The sheep were fed hay *ad libitum* and had free access to drinking water. Fistulation of the sheep had been approved by the ethical commission of the Institute for Agricultural and Fisheries Research (ILVO), Belgium (EC 2009, 114). The rumen fluid obtained from the sheep was brought in insulated flasks for transport and was mixed, homogenized and filtered

through a sieve with a pore size of 1 mm under continuous $CO_2$ flushing and kept in a water bath at 39 °C to be used as the source of inoculum.

The *in vitro* batch incubation method was as described by Fievez et al. (2005). Briefly, 250 mg of the dried corresponding substrate was incubated in 120 ml capacity gastight incubation flasks flushed with $CO_2$ having 20 ml of a phosphate buffer (per liter of distilled water: 28.8 g $Na_2HPO_4$ $12H_2O$; 6.1 g $NaH_2PO_4$ $H_2O$; 1.4 g $NH_4Cl$, flushed with $CO_2$ for 1 h and adjusted to pH 6.8) and 5 ml rumen fluid in a batch culture incubator (Edmund Bühler Gmbh, Hechingen, Germany). Before adding the buffer solution, a freshly prepared aqueous solution of Agolin Ruminant was added to each flask to reach the desired concentration in 25 ml of the medium. Fermentation flasks without additives, but containing 250 mg of the corresponding substrate, were used as a control. After 24 h at 39 ◦C, flasks were removed from the incubator, placed in ice water to stop the fermentation and sampled for gas, pH and VFA determination.

***Gas production technique (GPT)***

Gas production incubations were performed in the facilities of the Animal Nutrition Group, Department of Animal Sciences of Wageningen University (The Netherlands) following the procedure of Cone et al. (1996). Rumen fluid was collected from two lactating rumen cannulated Holstein Friesian cows, 2 h after the morning feeding. Rumen fluid from both cows was combined and stored in warm insulated flasks filled with $CO_2$, filtered through two layers of cheesecloth, and mixed (1:2, v/v) with an anaerobic buffer/mineral solution as described by Cone et al. (1996). The blend of essential oils was incubated with 0.5 g DM of substrate in triplicate in 60 ml buffered rumen fluid in 250 ml bottles modified for gas sampling as described by Pellikaan et al. (2011) in a shaking water bath at 39 °C and gas production was recorded for 72 h. Gas production measurements were corrected for blank gas productions (i.e., gas productions in buffered rumen fluid without sample). At the end of the

incubation time the flasks were opened and 2 ml of the incubation medium was sampled, acidified and prepared for VFA analysis.

***Consecutive batch incubation***

The effect of essential oils on $CH_4$ formation was tested using a modification of the consecutive batch culture technique first described by Theodorou et al. (1984). The first incubation run was prepared as described in section 2.3 and incubated for 24 h. The following incubation flasks contained 250 mg of substrate, essential oils and 20 ml of phosphate buffer plus clarified rumen fluid (3:1, *v/v*). Each incubation flask received 5 ml inoculum from the previous incubation. For the first series of experiment 4, three transfers were done for a total of 96 h incubation. In the second series of experiment 4, this methodology was adapted to allow for transfers of inoculum every 48 h for a total of 14 days incubation time.

***Analysis of metabolites during in vitro incubations***

Methane analysis

At the end of the incubation, the gas phase was analyzed for $CH_4$ using a micro-GC equipped with two gas chromatographic modules and a thermal conductivity detector (3000 micro-GC, Agilent, USA). Ethane ($C_2H_6$; 1 ml/flask) was used as the internal standard and argon as a carrier gas for both columns (Hassim et al., 2010).

Volatile fatty acids analysis

After opening the incubation flask, pH was measured (Hanna Instruments, Temse, Belgium) and 2 ml of incubation medium were collected and acidified with 200 µl of formic acid which contained the internal standard (10 mg 2-ethyl butyric acid/ml formic acid). After 15 min centrifugation at 4 °C and 22000 × ***g***, the supernatant was filtered with glass wool and an aliquot was transferred into a 1.5 ml glass vial. Samples were stored at 4 °C until VFA

analysis using gas chromatography on a Shimadzu 2010 (Shimadzu Corporation, 's-Hertogenbosch, The Netherlands) equipped with a Nukol column (30 m × 0.25 mm × 0.25 µm, Supelco) with a flame ionization detector as described by Castro-Montoya et al. (2012).

Methane measurements for gas samples from the GPT technique

Using a gas-ight syringe (Hamilton 1701N, point style 5 needles, 51 mm; Hamilton, Bonaduz, Switzerland) an aliquot of 10 µl was sampled sequentially from the headspace gas of each bottle at 2, 4, 8, 12, 24, 30, 36, 48, 58 and 72 h and analyzed for $CH_4$. Methane was determined using a gas chromatograph (GC8000Top, CE Instruments, Milan, Italy) fitted to a flame ionization detector, using a packed column (PorapakQ, 6 m × 1/8 in., 50–80 mesh, Grace/Alltech, Lexington, Kentucky, USA) with nitrogen as carrier gas (100 kPa) and an oven temperature maintained at 60 °C (Pellikaan et al. 2011).

**Statistical analysis**

For the *in vivo* experiments the main effect of addition of essential oils was tested using the MIXED procedure of SAS with repeated measurements in time and using an autoregressive covariance structure according to:

$$Y_i = \mu + \beta_i + \xi_i$$

where $\mu$ = the overall mean; $\beta_i$ = the effect of essential oils addition; and $\xi_i$ = the error term. Orthogonal contrasts analysis was performed to look for differences between no-addition and addition of essential oils (Week 0 vs. Week 2-4-6) and polynomial contrasts were performed to look for significant linear and quadratic effects over time once essential oils were supplemented (Week 2-4-6). Significances were declared at $P < 0.05$ and tendencies at $P < 0.1$.

In experiment 1 (first series) the main effect of essential oils was tested by the GLM procedure of SAS, according to:

$$Y_{ij} = \mu + \beta_i + \beta_j + (\beta_i \times \beta_j) + \xi_{ij}$$

where $\mu$ = the overall mean; $\beta_i$ = the effect of the $i^{th}$ essential oils concentration; $\beta_j$ = the effect of the $j^{th}$ substrate; $(\beta_i \times \beta_j)$ = the interaction effect between substrate and concentration and $\xi_i$ = the error term.

In the second series of experiment 1 and in experiments 3 and 4 (both series) the main effect of essential oils was tested by the GLM procedure of SAS, according to:

$$Y_i = \mu + \beta_i + \xi_i$$

Where $\mu$ = the overall mean; $\beta_i$ = the effect of the $i^{th}$ essential oils; and $\xi_i$ = the error term.

Effects of concentration of essential oils with $P < 0.1$ were characterized using orthogonal contrasts testing the probability of linear responses. Coefficients for polynomial contrasts were calculated for the unequally spaced treatments using the ILM procedure of SAS. Differences between means were evaluated using Tukey's multiple comparison test.

In experiment 2 the main effect of time of addition of essential oils was tested according to:

$$Y_i = \mu + \beta_i + \xi_i$$

where $\mu$ = the overall mean; $\beta_i$ = the effect of time of addition; and $\xi_i$ = the error term.

## RESULTS

### In vivo experiments

#### *Dairy cattle*

Data of week 4 are not presented for this experiment as they were unreliable in that period due to technical problems. All analysis and interpretations are therefore based on results of week 0, 2 and 6. Table 1.7 shows daily milk production and milk composition. Throughout the experiment the animals consumed 15.7 kg of DM daily (grass silage: 7.2 kg, maize silage: 5.8 kg, concentrate: 1.9 kg, and soybean meal: 0.8 kg). Dry matter intake was kept at 95% of the lowest *ad libitum* intake during the eight weeks and hence remained constant according to the intended experimental design. Milk production showed a decline at the end of the experiment. Similarly, fat, protein and lactose in milk did not show variations during the experimental period.

Table 1.7. Milk production and milk composition of dairy cows supplemented with a blend of essential oils

| | Week | | | | SEM | P values | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | | Week 0 vs. Week 2 to 6 | Contrast[a] L | Q |
| Milk production (kg/d) | 19.5 | 19.8 | 17.9 | 16.9 | 0.727 | 0.21 | 0.03 | 0.50 |
| Milk composition (g/100 g milk) | | | | | | | | |
| Milk fat | 4.75 | 4.80 | 4.80 | 4.80 | 0.093 | 0.71 | 0.79 | 0.82 |
| Protein | 3.41 | 3.28 | 3.50 | 3.59 | 0.077 | 0.78 | 0.04 | 0.05 |
| Lactose | 4.54 | 4.42 | 4.43 | 4.44 | 0.054 | 0.12 | 0.95 | 0.99 |

Absolute daily enteric $CH_4$ production (g/d) and $CH_4$ relative to DMI (g/kg DM) and to milk production (g/kg milk) are presented in Table 1.8. By comparing the effects of addition of essential oils versus no addition (week 0 vs. week 2 to 6)**,** tendencies were observed for a lower $CH_4$ production in g/d and g/kg DM when the essential oils were supplemented in the diet ($P$ = 0.07). The decreases in $CH_4$ production accounted for 15% (g/d) and 14% (g/kg

DMI) at the end of the experiment. These differences disappeared when $CH_4$ was expressed relative to milk production. However, a comparison in these units may be flawed in the current experimental set up, testing the supplementation effect in time, which coincides with decreasing milk production due to progress in lactation. Furthermore, during the supplementation period of Agolin Ruminant, a tendency ($P$ = 0.09) for a linear decrease in time of daily $CH_4$ production (g/d) was observed. This effect was not observed when $CH_4$ was expressed as g/kg milk.

***Beef cattle***

The daily dry matter intake by beef cattle was 8.36 kg, divided in 4.33 kg from maize silage and 4.03 kg from concentrate. Absolute daily enteric $CH_4$ production (g/d) and $CH_4$ relative to DMI (g/kg DM) are presented in Table 1.8. No differences were observed in daily $CH_4$ production, neither when $CH_4$ was corrected for DMI. During the supplementation period of essential oils there were neither linear nor quadratic effects over time.

Table 1.8. Methane production of dairy cows or beef heifers supplemented with a blend of essential oils

| | Week | | | | SEM | P Values | | |
|---|---|---|---|---|---|---|---|---|
| | 0 | 2 | 4 | 6 | | Week 0 vs. Week 2 to 6 | Contrast[a] L | Q |
| *Dairy cattle* | | | | | | | | |
| g/d | 283 | 252 | --- | 241 | 9.07 | 0.07 | 0.09 | --- |
| g/kg DM | 18.0 | 16.1 | --- | 15.5 | 0.61 | 0.07 | 0.10 | --- |
| g/kg milk | 14.6 | 13.1 | --- | 14.9 | 1.71 | 0.64 | 0.19 | --- |
| *Beef cattle* | | | | | | | | |
| g/d | 142 | 126 | 136 | 128 | 8.74 | 0.29 | 0.90 | 0.21 |
| g/kg DM | 17.1 | 15.1 | 16.3 | 15.3 | 1.06 | 0.23 | 0.90 | 0.20 |

[a] Linear (L) and quadratic (Q) effects over time tested for weeks 2 to 6

### *In vitro* experiments

In the first series of experiment 1, none of the concentrations at which the essential oils were tested showed any effect on $CH_4$ formation ($P > 0.05$) in combination with any of the two substrates (Figure 1.11). Similarly, total VFA (ranging from 1742 to 1958 mmol/flask) and individual VFA proportions were not affected by the addition of the essential oils (data not shown). The substrate effect was significant for $CH_4$ formation ($P < 0.001$), with the Concentrate + Maize silage substrate producing more $CH_4$ than the Concentrate + Maize silage + Grass silage substrate, but differences disappeared when $CH_4$ was expressed relative to total VFA production.

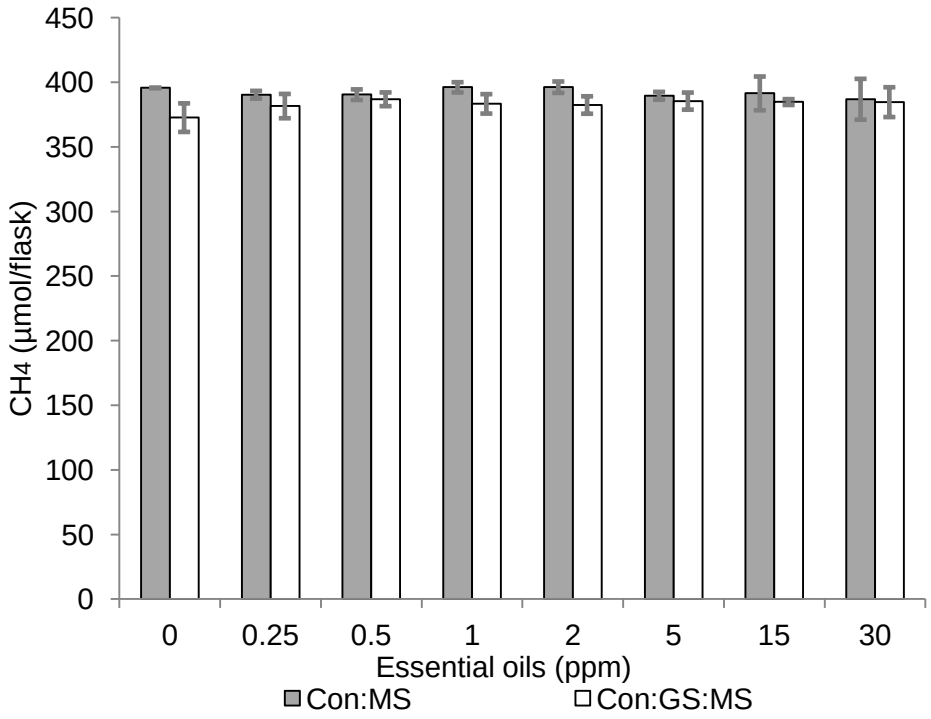



Figure 1.11. *In vitro* methane (µmol/flask) production after 24 h batch incubation with a blend of essential oils. Experiment 1, first series. Con:MS = Concentrate + Maize silage (50:50, DM basis). Con:GS:MS = Concentrate + Grass silage + Maize silage (30:35:35, DM basis)

Moreover, there were no interaction effects of substrate and of essential oils. Total VFA was not affected by substrate type or interaction between substrate and essential oils ($P > 0.05$).

Given the absence of essential oils × substrate interactions all subsequent experiments were performed using the concentrate + grass silage + maize silage substrate.

In the second series of experiment 1, essential oils were supplemented at higher concentrations (Figure 1.12) showing a tendency to decrease the *in vitro* $CH_4$ formation with increasing essential oils concentrations (P = 0.07), with concentrations of 225 and 250 ppm showing a decrease relative to the control of 8.9% and 15.6%, respectively.

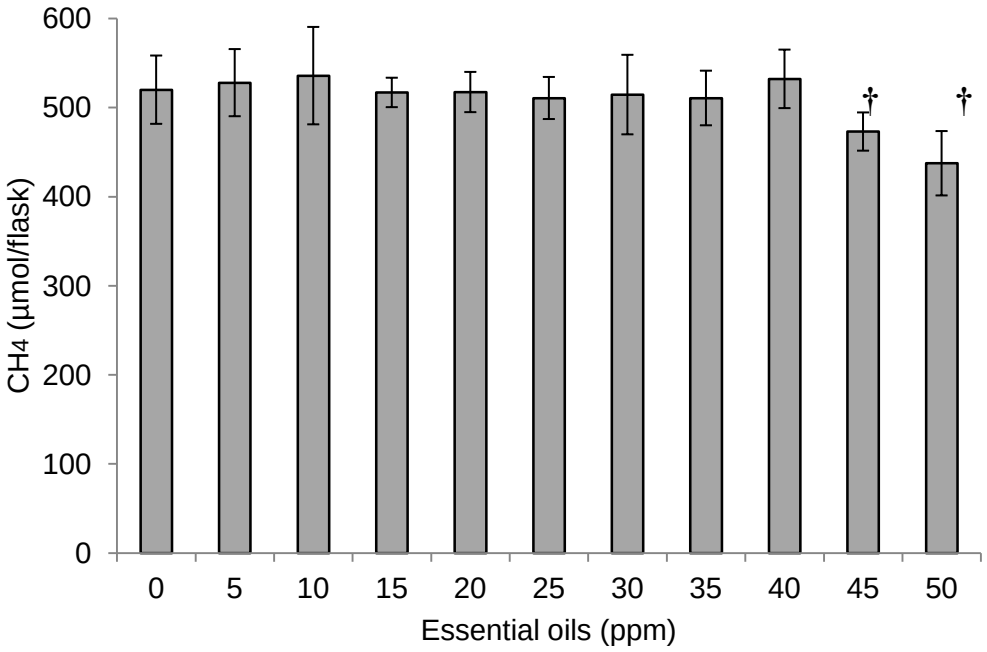



Figure 1.12. *In vitro* methane (µmol/flask) production after 24 h batch incubation with a blend of essential oils. Experiment 1, second series. († = tendency to differ from the 0 ppm)

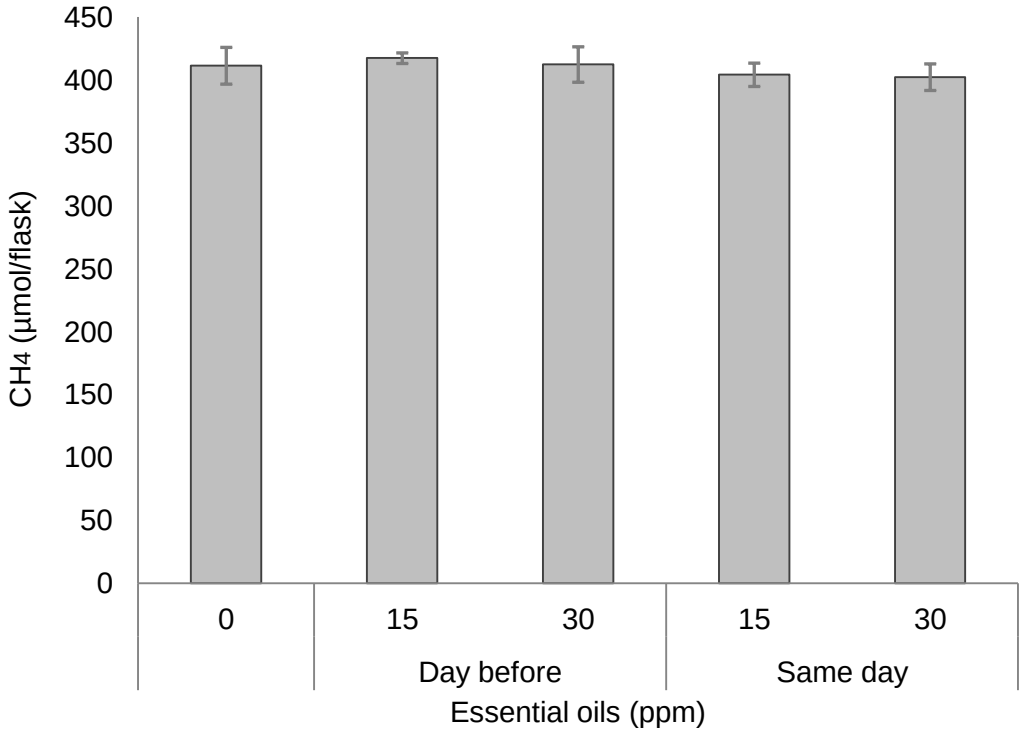
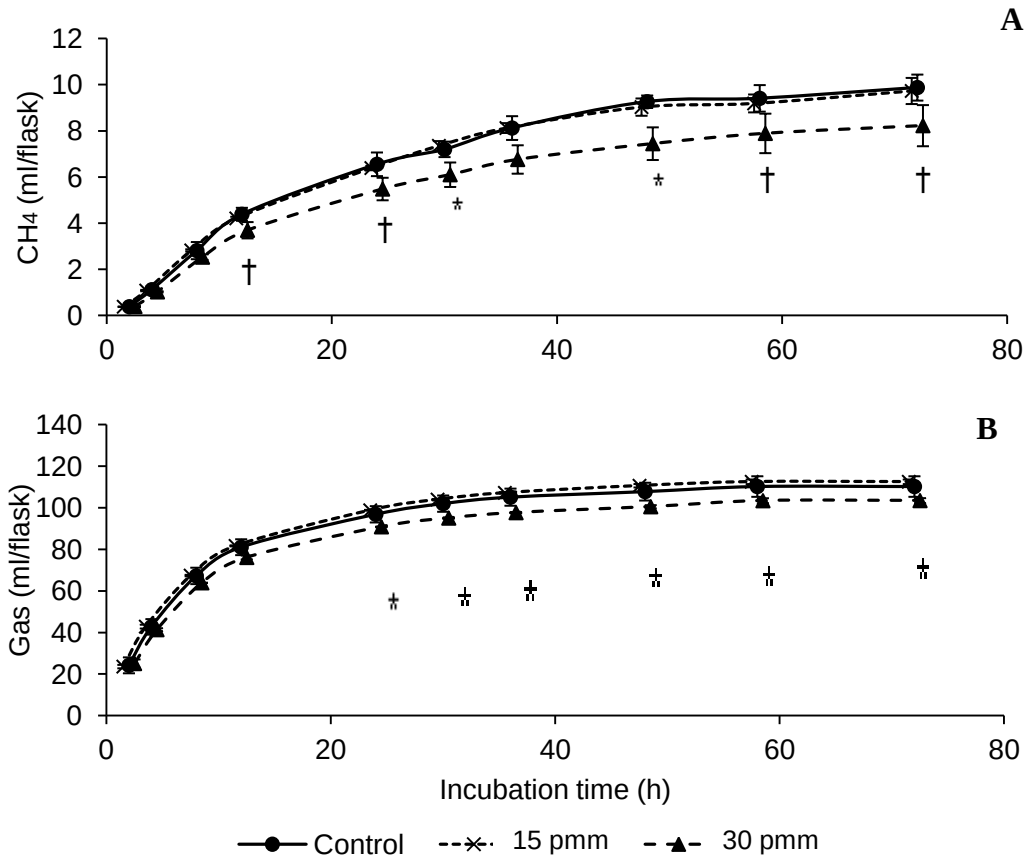



Figure 1.13. *In vitro* methane (µmol/flask) and total VFA (mmol/flask) production after 24 h batch incubation with a blend of essential oils added one day before or on the same day of the incubation. Experiment 2

In experiment 2 there was no difference for $CH_4$ formation and total VFA between the addition of essential oils one day before the start of the incubations or addition on the same day (Figure 1.13).

In experiment 3 the GPT system was used to test for midterm effects (72 h) of essential oils on *in vitro* $CH_4$ formation (Figure 1.14). Methane formation (ml/flask) decreased through addition of essential oils at 150 ppm after 30, 36 and 48 h and tended to decrease at 12, 24, 58 and 72h (Figure 1.14A). These decreases were accompanied by decreases in gas production (ml/flask) from 24 h onwards (tendencies at 58 and 72h) (Figure 1.14B). Hence, differences in $CH_4$ production disappeared when $CH_4$ was corrected for gas production (ml $CH_4$/ml gas), although tendencies for lower relative $CH_4$ production remained for the 150 ppm

concentration after 12 and 48 h of incubation. Total VFA production after 72 h was not affected by the essential oils (3525, 3787 and 3408 mmol/flask for 0, 75 and 150 ppm, respectively; $P > 0.05$).




Stars (*) indicate significant differences (P < 0.05), crosses (†) indicate tendency (P <0.1)

Figure 1.14. Methane production (ml/flask, A) and total gas production (ml/flask, B) during 72 h incubation in a gas production technique with a blend of essential oils. Experiment 3

In the first series of experiment 4, with sequential transfer every 24 h for a total incubation time of 96 h there was no effect of essential oils on $CH_4$ formation (Figure 1.15A; $P < 0.05$). Both for the control as well as the two essential oils concentrations, $CH_4$ formation strongly decreased after the first transfer and it kept on decreasing, albeit slightly, up to 96 h.

In the second series of experiment 4, where the transfer interval was extended to 48 h and the total incubation time was 14 days, there was no effect of essential oils on $CH_4$ formation for any of the concentrations studied (Figure 1.15B; $P < 0.05$).

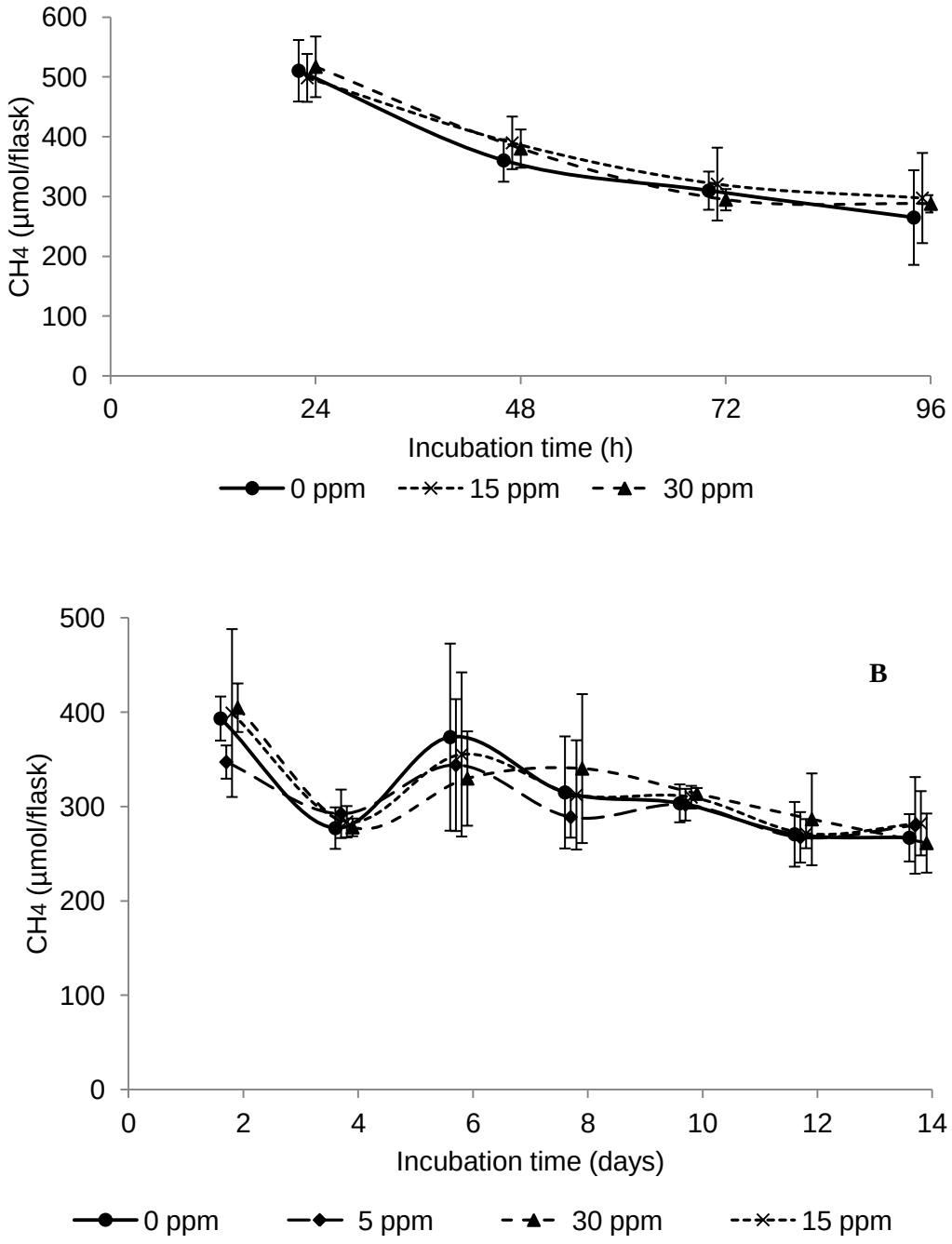



Figure. 1.15. *In vitro* methane (µmol/flask) production from consecutive batch incubations after 96 h with transfers every 24 h (A) and after 14 days with transfers every 48 h (B) with a blend of essential oils. Experiment 4

## DISCUSSION

To our knowledge this is the first study reporting the effects of a blend of essential oils containing corianderoil, gernaylacetate and eugenol on $CH_4$ emissions from dairy and beef cattle combined with extensive *in vitro* experiments. Few *in vivo* studies have explored the effects of essential oils on $CH_4$ emissions. Moreover, these few studies showed inconsistent results, which are summarized in Table 1.9.

Table 1.9. Summary of previous studies supplementing essential oils to decrease methane emissions

| Author | (Source of) Essential oil | Animal | Basal diet [a] | Doses | | | Change on $CH_4$ (%)[b] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | | g/d | mg/kg BW | g/kg DMI | g/d | g/kg DMI | g/kg BW |
| Beauchemin and McGinn (2006) | Essential oils and spice extract[c] | Beef heifers | Barley silage (75) + Steam rolled barley (25) | 1.0 | 3.85 | 1.39 | +2.4 | +7.8 | n.r. |
| Mohammed et al. (2004) | Horseradish oil | Holstein steers | Sudan grass (60) + Concentrate (40) | 2.74 | 19.4 | 0.7 | n.r. | –19* | n.r. |
| Klevenhusen et al. (2011) | Garlic oil | Sheep | Hay (50) + Concentrate (50) | 5.31 | 64.0 | 5.00 | –9.0 | –2.4 | –7.9 |
| Wang et al. (2009) | Ropadiar | Sheep | Hay (75) + Concentrate (25) | 0.21 | 4.68 | 0.182 | –12* | n.r. | n.r. |

[a] Proportions (DM basis) reported between brackets
[b] Change from the respective control treatment. Stars denote significant differences from the control
[c] Mixture of thymol, limonene and guaiacol (Castillejos et al., 2005)
n.r.: Not reported

Two studies with large ruminants showed opposite results: Beauchemin and McGinn (2006) did not find any effect of a blend of essential oils and spice extract (Crina Ruminants; Akzo Nobel Surface Chemistry S.A.) on $CH_4$ output, whereas Mohammed et al. (2004) reported a 19% decrease in $CH_4$ relative to DMI when supplementing the animals with horseradish oil.

Two other studies with small ruminants again had contrasting results: Klevenhusen et al. (2011) did not find any effect of garlic oil on $CH_4$, while Wang et al. (2009) found that ropadiar decreased daily $CH_4$ by 12% relative to its control.

Nevertheless, comparison across studies are disputable, as none of the essential oil sources was tested twice, which confirms that the variability inherent to these compounds, is a decisive factor in the testing of them. Diet is another factor differing between studies, as none of the experimental diets was the same.

In this study the blend of essential oils was fed to the animals at 1 g/d, delivering 0.2 g of essential oils which equates to 0.332 and 0.402 mg/kg BW for dairy and beef cattle, respectively (12.8 and 24.2 mg/kg DMI for dairy and beef cattle, respectively), a dose much lower than the dose provoking effects in previous studies. After 2 weeks of supplementation daily emissions had decreased by 11% for dairy cattle and up to 15% decrease was observed after 6 weeks of supplementation. Conversely, the blend of essential oils did not decrease $CH_4$ emission in beef cattle, even though the doses fed to the latest was up to 2-fold higher than the doses fed to the of dairy cattle (relative to BW, rumen volume and DMI). The reasons for this discrepancy are not clear. The main differences between both experiments are related to the feeding management of the additive and the basal diet. The additive was distributed (once daily to the beef cattle and divided in two feedings for the dairy cattle). The seperation of the additive feeding might have kept the concentration of the essential oil blend higher in the rumen for a longer time in dariy as compared to beef cattle. Another difference was the basal diet (grass silage, maize silage, concentrate and soybean meal *vs*. maize silage and concentrate, for dairy cows and beef cattle, respectively). It is also important to notice that there was a greater variation in the data set of the beef cattle experiment as compared with the variation of the dairy cattle experiment (coefficient of variation of 25 and 12%, respectively), which might have prevented the detection of statistical differences (numerical decreases in

daily $CH_4$ and $CH_4$ relative to DMI). Essential oils are very diverse in composition, nature, active compounds and, hence, activity (Calsamiglia et al., 2007). Therefore, it might not be appropriate to compare the effect of the blend used here on $CH_4$ with the effect shown by other essential oils. Nevertheless, it is still remarkable to reach decreases in $CH_4$ at such small doses. Furthermore, no adaptation of the rumen microorganisms to the blend of essential oils seemed to occur, as it was capable to reduce $CH_4$ emissions as soon as two weeks after starting its supplementation and more importantly, these decreases were sustained over the 6 weeks of the experiment.

Milk production decreased throughout the experiment, and as a result $CH_4$ in g/kg milk returned to the levels of week 2 by the end of the experiment. However, the milk production decrease most likely was the result of a normal decline in production due to lactation stage rather than a negative impact of the essential oils supplementation. The experiment was not designed to assess the effects of essential oils on milk production. Other milk components like fat, protein and lactose did not change when the blend of essential oils was supplemented.

Simultaneous to the *in vivo* experiments a first *in vitro* experiment was designed to mimic the effects of essential oils in an *in vitro* system. A first series of concentrations (experiment 1) included levels around the theoretical concentration of essential oils in the rumen when fed at the suggested dose. The dose of 0.2 g/d administered *in vivo* is equivalent to *ca.* 2 and 4 mg/L for dairy and beef cattle, assuming a rumen volume of 100 and 50 L, respectively, and disregarding the passage rate, or 2 and 4 ppm (*m/v*) for dairy and beef cattle, respectively. Furthermore, depending on the type of additive, there might be interaction effects between substrates and additives on $CH_4$ production (*in vitro*, Castro-Montoya et al., 2012). As different diets were used during both *in vivo* experiments, the first series of experiment 1 was performed using two substrates, reflecting the diets used *in vivo*.

None of the concentrations used in experiment 1, first series, had an effect on $CH_4$ or total VFA production, even though the highest concentration used was 15 times higher than the *in vivo* dose. Hence, higher concentrations were used in a second series of incubation in this experiment.

Higher concentrations of essential oils (experiment 1, second series) showed a tendency to decrease $CH_4$ production at 45 and 50 ppm (numerical reduction of 9 and 16% of $CH_4$ was observed relative to the control). These reductions in $CH_4$ production are comparable with those observed *in vivo*, however, at much higher concentrations.

Previous studies with other essential oils have needed even higher concentrations to achieve inhibition of $CH_4$ *in vitro*. Evans and Martin (2000) found that thymol strongly inhibited *in vitro* $CH_4$ production when added at a concentration of 400 ppm. This inhibition of $CH_4$ was accompanied by a strong decrease in the concentrations of acetate and propionate in the incubation medium, which indicates that the fermentation itself was inhibited. When thymol was incubated at a concentration of 200 ppm or lower there were no effects on $CH_4$, acetate and propionate concentrations. Similarly, Busquets et al. (2005) found that garlic oil and diallyl disulfide decreased *in vitro* $CH_4$ production when applied at a concentration of 300 ppm after 17 h *in vitro* batch incubation. However, total VFA production was also decreased by these components. Conversely, when both essential oils were added at lower concentrations (30 ppm) there was no negative effect on the fermentation (no $CH_4$ measurements done).

Our results and results of previous studies suggest that high concentrations of essential oils should be added to the incubation medium to observe inhibitory effects on *in vitro* $CH_4$. However, these high concentrations not only increase the risk of impairing the fermentation, but also are not in agreement with effective concentrations supplemented *in vivo* in the current study. Knowing that the blend of essential oils was effective in decreasing $CH_4$ production at

a theoretical concentration of 2 ppm, we decided to further explore the effects of this blend *in vitro* and some mismatches between *in vitro* and *in vivo* systems that might account for this dose difference between *in vitro* and *in vivo* experiments.

It has always been clear that *in vitro* systems do not completely reflect what happens in the rumen. However, these systems, especially short term batch incubations, have been regarded as good screening tools for additives and feedstuffs, although *in vitro* responses are often stronger or observed at lower doses as compared with *in vivo* conditions (e.g. Fievez et al., 2003). Hence, the lack of *in vitro* response at doses up to 30 ppm (*m/v*) and the modest response (–9 and –15%) at elevated concentrations of 45 and 50 ppm (*m/v*) is the current study is surprising given a $CH_4$ reduction of about 15% induced by extremely low *in vivo* doses (0.2 g/d ≈ 2 ppm, *m/v*). Therefore, several options were considered in an attempt to optimize *in vitro* simulation.

A first adjustment was related to the timing of the additive supplementation (Personal communication from James Newbold, IBERS, Wales). The normal practice *in vitro* is to add a freshly prepared solution of an additive shortly before the start of the simulation. Conversely, the regular practice *in vivo* is mixing the additive with the feed, generally the concentrate, prior to feeding, allowing some contact between the additive and the feed and exposure to the environment for some time before being ingested.

Therefore, in experiment 2, our hypothesis was that essential oils may have $CH_4$ inhibitory effect at lower doses when being in contact with feedstuffs and the environment from the afternoon before the start of the incubation. In some other studies (not reporting rumen $CH_4$ production) such a supplementation procedure has been applied for another essential oil mixture (Crina®) (Castillejos et al., 2005). The concentrations used for the subsequent experiments were 15 and 30 ppm. The addition of essential oils the day before the incubation

did not elicit any effect on $CH_4$ and total VFA production compared with the addition shortly before the start of the fermentation. These results disregard the hypothesis that the time of addition of essential oils has an influence on the *in vitro* fermentation.

Incubation time is another factor playing a role in *in vitro* batch systems, most of which have an incubation period of about 24 h. However, *in vivo* conditions allow for a longer interaction between rumen microbes and the additive, when the latter is supplemented e.g. daily. Longer exposure periods may result in adaptation of rumen microbes to a certain product, minimizing its effects. On the other hand, it is possible that some products do not provoke acute effects at lower doses, but modification of the microbial population and changes in the fermentation may occur through longer exposure to these lower doses. Therefore, in experiment 3, we hypothesized that a longer exposure time might be needed when applying lower doses of essential oils. In a first attempt to test this, we used the gas production technique (GPT) which is a routine *in vitro* system with a longer incubation period (72 h). Methane and gas production were monitored after 2, 4, 8, 12, 24, 30, 36, 48, 58 and 72 h. Methane and total gas production decreased when supplying the higher dose of essential oils from 30 or 24 h onwards, respectively. Although essential oils inhibited $CH_4$ production (17%) at a lower dose in the current GPT set up, it may be surprising that this inhibitory effect already occurs after 30 h of incubation and $CH_4$ production already tended to be lower after 24 h of incubation, whereas essential oils at a dose of 30 ppm did not reduce methanogenesis in the 24 h batch incubation of experiments 1 and 2.

Some dissimilarities exist between both systems. Different to the GPT, batch culture systems do not allow the release of produced gases which might impair microbial fermentation (Getachew et al., 1998). Moreover, the ratio of rumen fluid to buffer (1:2 vs. 1:4, for GPT and batch system, respectively) might lead to a faster depletion of the buffer in the GPT as compared to the batch system, and a concomitant decrease in pH, which might be inhibitory

for some microbes (Getachew et al., 1998), and could have resulted in a higher sensitivity to essential oils in the GPT system. The difference in the ratio of rumen fluid to buffer might have played a role in relation to the abundance of microorganisms in the incubation medium. It can be hypothesized that a higher microbial mass in the incubation medium (i.e. GPT) might require a higher amount of additive to elicit an effect on the microbial activity and the concomitant changes in fermentation, including $CH_4$ production. However, it also could be argued that a more diluted rumen fluid opens the possibility for a faster growth of rumen microorganism, not only increasing their activity, but also allowing for the growth of those microorganisms less sensitive to the effects of the additive. In this perspective, an incubation medium with less microbial mass would require a higher amount of the additive to observe an effect.

Furthermore, microbial populations in rumen fluid from different species (sheep fed hay and cows fed grass silage, maize silage and concentrate, for batch systems and GPT, respectively) can have different capacities to degrade plant secondary compounds (Frutos et al., 2004; Kronberg and Walker, 1993), mycotoxins (Kiesling et al., 1984) and anti-nematodes/cestodes (Beretta et al., 1989). The donor animal was another difference between the batch and the GPT systems that could have influenced the fermentation. Microbial populations in different ruminant species can have different activity (e.g. Hervas et al., 2005) and even though there is no information on essential oils, there is plenty of information regarding this topic with other compounds (e.g. Kronberg and Walker, 1993; Kiesling et al., 1984; Frutos et al., 2004). The diet of the donor animal is also a factor. A study of Khiaosa-ard et al. (2011) compared the adaptation of donor cows to an alpine forage or to lowland forage and observed different fermentation patterns regardless of the substrate incubated with either of the rumen fluid sources. In our study it is clear that the diet fed to the donor animals in the GPT system is more representative of the *in vivo* conditions. Furthermore, the choice to collect rumen fluid

prior to morning feeding is related to preventing the rumen fluid to supply too much feed particles, while collection shortly after feeding may allow a more active pool of rumen microbes (Kiesling et al., 1984).

As the GPT still may show limitations to assess long term effects (e.g. single supply of additive, exhaustion of fermentation substrate and buffering capacity), in experiment 4, we used a consecutive batch incubation technique, originally proposed by Theodorou et al. (1984) and recently used by Morgavi et al. (2013). It has been found that in the consecutive batch incubations protozoa tend to decline with each transfer and might not survive beyond a fifth transfer (Theodorou et al., 1984). However, fibrolytic and sacharolytic bacteria, as well a methanogens do survive and proliferate in this system (Theodorou et al., 1987). Provided that the mode of action of essential oils is more related to their toxicity to gram-positive and gram-negative bacteria (Calsamiglia et al., 2007), and probably direct toxicity to archaea (Mohammed et al., 2004), than to any effect on protozoa, the incubation medium in this technique should still contain a representative microbial pool to test for the effects of essential oils on the fermentation and $CH_4$ production.

The consecutive batch incubations have the advantage of allowing for a daily supply of both substrate and additive, as under *in vivo* conditions. This more dynamic system would also allow for the growth of rumen microbes (only 5 ml aliquot from the previous incubation is transferred to the new one). After 3 transfers and 96 h incubation essential oils did not have any effect on $CH_4$ production when supplied daily at doses of 15 and 30 ppm. It is worth noticing that $CH_4$ production continuously decreased with each transfer, and seemed to stabilize between the incubations after 72 and 96 h. Such a decrease also has been observed by Morgavi et al. (2013). It was expected that with this consecutive system, lower doses of essential oils could inhibit rumen methanogenesis through longer exposure of microorganisms to the additive. It might be possible that essential oils needs an even longer

exposure before inhibition of $CH_4$ production occurs. Moreover, the decrease in $CH_4$ production in the control incubations through inoculum transfer every 24 h may have affected the microbial community which may have masked the additive's effects.

Indeed, in the original study, Theodorou et al. (1984) reported that for 24 h transfers the fermentation stabilized only after the sixth transfer. Conversely, when transfers were done every 48 h and every 72 h the fermentation stabilized after 3 and 1 transfers, respectively. Taking this into account, in the second series of experiment 4 48 h transfers have been chosen. The latter were preferred over the 72 h transfers because 72 h was the incubation time of the GPT in experiment 3, thought to have a greater risk of depletion of the substrate. The 48 h transfers resulted in a more stable fermentation from the third transfer onwards, and gave the possibility to extend the whole incubation time up to 14 days, which corresponds with the first $CH_4$ registration *in vivo* and after which $CH_4$ inhibition was observed. Additionally, a lower concentration of essential oils (5 ppm) was included in this experiment assuming that a longer incubation time could allow observing effects of essential oils at lower concentrations. However, the addition of essential oils did not have any effect on $CH_4$ at any point of the incubation time and for none of the doses.

The inhibitory effects of essential oils observed *in vivo* were not replicated *in vitro*, stressing once again the differences between both systems and the need to make *in vitro* systems more reflective of *in vivo* conditions. In this study we started with a standard batch incubation system for 24 h and we observed decreases in $CH_4$ similar to those *in vivo* but at a much higher concentrations (45 ppm and 50 ppm vs. 2 ppm for *in vitro* and *in vivo*, respectively) (Experiment 1). In the current study, we explored some adaptations to the *in vitro* system in order to somewhat better resemble *in vivo* conditions, while maintaining the routine nature of the batch *in vitro* systems (i.e. continuous culture systems were not considered here). The addition of essential oils ca. 16 h prior the start of the incubations did not show any difference

with the addition right before the start (Experiment 2). Similarly, by extending the time of exposure of microbes to essential oils as well as ensuring a constant supply of both substrate and additive, no inhibition of $CH_4$ was observed (Experiment 4). Decreases in $CH_4$ production were observed at 30 ppm by using the GPT system (Experiment 3). Despite adaptations to the *in vitro* conditions studied here, in any case, $CH_4$ inhibition only occurred at a much higher concentration than under *in vivo* conditions. There is no clear explanation for this, which means that *in vitro* systems still have to be adapted to better reflect *in vivo* conditions on supplementation with essential oils. Essentially, the *in vitro* simulations used here assess a direct effect of the additive on microbial fermentative process taking place in the rumen. As outlined by Benchaar and Greathead (2011) there is little information about the fate of essential oils and their compounds in the gastro-intestinal tract and the mechanisms proposed for the disappearance of essential oil terpenes from the digestive tract include among others absorption across the ruminal wall into the blood system and later excretion in the urine (Michiels et al., 2008; Malecky et al., 2009), although these compounds may have provoked effects on the intermediary metabolism of the animal before being excreted. Eventually, *in vivo* depression of rumen $CH_4$ production trough essential oils was indirect through a host-microbe interaction. These effects are not simulated through the current *in vitro* set up and potentially should be emphasized in the future.

## CONCLUSIONS

The blend of essential oils decreased $CH_4$ emissions from dairy cattle at a lower dose as compared with the doses of other essential oils reported in literature, and the decrease was sustained for the six weeks of the experiment. However, there was no effect of the blend of essential oils when supplemented to beef cattle. Interestingly, the decreases observed in dairy cattle were not replicated *in vitro*, even though diverse adjustments of the batch incubation system were done to better simulate the *in vivo* conditions. It seems that *in vitro* there was a

need for very high concentrations of this particular blend of essential oils before $CH_4$ inhibition was observed. If *in vitro* batch incubations are to be used as screening tools for additives to decrease $CH_4$ production, the system still needs to be improved in order to avoid ruling out promising products based only on their observed effects in *in vitro* batch cultures.

# CHAPTER 2. RELATIONSHIP BETWEEN ODD- AND BRANCHED-CHAIN FATTY ACID PROFILES IN MILK AND CALCULATED ENTERIC METHANE PROPORTION FOR LACTATING DAIRY CATTLE

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## ABSTRACT

The purpose of this study was to explore the relationships between odd- and branched-chain fatty acids (OBCFA) in milk with calculated enteric $CH_4$) production for lactating dairy cows using multiple linear regression (MLR), partial least squares regression (PLS) and a genetic algorithm approach (GA). A dataset collected from 13 experiments containing 224 paired observations of measured acetate, propionate and butyrate proportions in rumen fluid VFA and 7 measured OBCFA was used. Methane proportion (mmol/mol VFA) was calculated from acetate, propionate and butyrate and expressed relative to the sum of these volatile fatty acids (VFA). Calculated $CH_4$ production was related to milk OBCFA using MLR and PLS, resulting in a linear prediction model. The GA approach resulted in a model that predicted rumen VFA proportions of total VFA from milk OBCFA. Methane proportion was calculated from predicted acetate, propionate and butyrate proportions in total VFA based on rumen stoichiometry and compared with $CH_4$ proportion calculated from measured VFA proportions. The prediction error was low (*i.e.*, root mean square error < 5%), and the models captured up to 66% of the variance in the data and the concordance correlation coefficient was close to 0.8. However, the variance of the prediction error was less than 40% of the variance of the calculated $CH_4$ proportion. Seven milk OBCFA were initially considered as predictors, from which branched-fatty acids *iso* C14:0 and *iso* C16:0 were positively related to calculated enteric $CH_4$ production and odd-fatty acids (*i.e.*, C15:0 and sum of C17:0 and C17:1 *cis*-9) were negatively related to it in the MLR and PLS models. Relationships in this large data set identify the most relevant OBCFA in milk as potential predictors of rumen methanogenesis.

## INTRODUCTION

As outlined in the Introduction section, a number of studies have simultaneously reported changes in $CH_4$ output and milk fatty acids (MFA) profiles upon supplementation with diverse additives (e.g. Johnson et al., 2002; Odongo et al., 2007; Hristov et al., 2009). However Chilliard et al. (2009) were the first evaluating the use of milk FA composition to predict $CH_4$ emissions (g/d). These authors used multiple linear regression to develop two equations from an experiment studying the effect of 3 different physical forms of linseed fatty acids on dairy cow performance, MFA secretion and $CH_4$ output (Martin et al., 2008). Their first equation [$R^2 = 0.95$; Eq. (5)] includes 5 predictive variables, among which, according to the authors, 3 are not surprising and their inclusion was explained as follows: forage intake, reflecting the organic matter fermented that follows the acetate–$CH_4$ pathway; palmitic acid, which is related to *de novo* synthesis (therefore from acetate), although less strongly than shorter chain fatty acids; and *trans*-16+*cis*-14 18:1, which comprises an intermediate of linolenic acid hydrogenation. The authors found the inclusion of *cis*-9 C14:1 isomer (negatively related to $CH_4$) and of *n-6* C18:2 (positively related to $CH_4$), somewhat unexpected. Chilliard et al. (2009) also developed a second equation by including only the first 3 predictive parameters (each significant at $P < 0.002$) Eq. (6), and the result was simpler but as well performing ($R^2 = 0.93$):

$$CH_4\ (g/d) = (9.46 \times C16{:}0) - (97.6 \times \textit{trans}\text{-}16 + \textit{cis}\text{-}14\ C18{:}1) + (13.3 \times \text{Forage intake}) - (78.3 \times \textit{cis-9}\ C14{:}1) + (77.4 \times \textit{n-6}\ C18{:}2) - 21.2 \quad (5)$$

$$CH_4\ (g/d) = (-100.8 \times \textit{trans}\text{-}16+\textit{cis}\text{-}14\ C18{:}1) + (6.78 \times C16{:}0) + (13.1 \times \text{forage intake}) + 80.1 \quad (6)$$

where individual milk FA are expressed as g/100 g MFA and forage intake as kg DM/d

The work of Chilliard et al. (2009) showed the potential of MFA to predict $CH_4$ output. However, as the authors stated, the validity of the relationships established in their study is

limited to diets varying in linolenic acid supply. Indeed, the diversity in a dataset is an important factor for a model to increase its generalization capacity.

In that regard, Dijkstra et al. (2011) developed a linear model [$R^2$ = 0.73; Eq. (7)] from data of 3 experiments with dairy cattle with a total of 10 dietary treatments and 50 observations:

$$CH_4 \text{ (g/kg DMI)} = 24.6 + (8.74 \times \textit{anteiso} \text{ C17:0}) - (1.97 \times \textit{trans}\text{-10} + \textit{trans}\text{-11 C18:1}) - (9.09 \times \textit{cis}\text{-11 C18:1}) + (5.07 \times \textit{cis}\text{-13 C18:1}) \quad (7)$$

where individual milk FA are expressed as g/100 g MFA

Variation in dietary treatments included supplementation with calcium fumarate, diallyldisulfide, caprylic acid, capric acid, lauric acid, myristic acid, extruded linseed, linseed oil and yucca powder. Dijkstra et al. (2011) found a positive relationship between $CH_4$ (g/kg DMI) and *anteiso* C17:0 and *cis*-13 C18:1; and a negative relationship between $CH_4$ and *trans*-10 + *trans*-11 C18:1 and *cis*-11 C18:1. *Anteiso* C17:0 had a negative relation with dietary crude protein in the diet (Cabrita et al., 2003), and stoichiometrically, fermentation of protein is associated with lower $CH_4$ production (Bannink et al., 2008), which might explain the negative relationship between $CH_4$ and this FA (Dijkstra et al., 2011). *Trans*-10 + *trans*-11 C18:1 is a biohydrogenation intermediate and as in the case of Chilliard et al. (2009) might be related to hydrogenation of linoleic acid. The inclusion of *cis*-13 C18:1 and *cis*-11 C18:1 in the equation are somehow more difficult to explain, even though it is known that a number of unsaturated fatty acids (UFA) originate in the rumen (Dijkstra et al., 2011). However, the microorganisms and enzymes responsible for their production are not yet well characterized or understood (Wallace et al., 2007).

Mohammed et al. (2011) studied the relationships between $CH_4$ (g/d) and MFA from an experiment with 16 lactating dairy cows offered 4 diets in four 28-d periods in a latin square

design. The experimental diets were sunflower seed, linseed, canola seed and a control diet. Different models were developed from only MFA concentrations, MFA combined with intake and production parameters, and MFA combined with intake, production and rumen fermentation parameters. The equation based on MFA profiles only [$R^2 = 0.74$; Eq. (8)] was:

$$CH_4 \text{ (g/d)} = (-486.2 \times \textit{cis}\text{-9 C17:1}) - (122.7 \times \textit{cis}\text{-11 C18:1}) + (2220 \times \text{t,t CLA}) - (11.76 \times \sum \textit{trans}\text{- C18:1}) + (260.1 \times \textit{anteiso}\text{ C15:0}) + 272.4 \quad (8)$$

where individual milk FA are expressed as g/100 g MFA

The authors found positive relationships between $CH_4$ and *trans*, *trans* CLA and *anteiso* C15:0; and negative relationships with *cis*-9 C17:1, *cis*-11 C18:1 and the sum of *trans*- C18:1. *Cis*-9 C17:1 is a desaturation product of C17:0 (Fievez et al., 2003), whose precursor is propionate. Thus, C17:0 is expected to be negatively related to $CH_4$. The inclusion of other variables is less clear.

It is worth to mention that the best equation in the study of Mohammed et al. (2011) was the one including MFA and intake, production and rumen fermentation parameters in the analysis:

$$CH_4 \text{ (g/d)} = (-910.8 \times \textit{cis}\text{-9 C17:1}) + (331.2 \times \textit{iso}\text{ C16:0}) + (0.0001 \times \sum \text{entodionomorphs}) + 242.5 \quad (9)$$

where individual milk FA are expressed as g/100 g MFA and $\sum$ entodionomorphs as $\times 10^5$/ml

This equation included two OBCFA in their final equation (*cis*-9 C17:1, *iso* C16:0) along with total entodiniomorphs [$R^2 = 0.9$; Eq. (9)]. The inclusion of *iso* C16:0 might be related its presence in fibrolytic bacteria, normally linked with higher $CH_4$ production (Fievez et al., 2012). The positive relation between $CH_4$ production and total entodiniomorphs could be explained by the symbiotic association between methanogens and protozoa (Tokura et al., 1997). However, in the experiments from which these equations were developed (Beauchemin

et al., 2009), total protozoa decreased between 23 and 38% compared with the control, along with decreases in $CH_4$. It is likely that protozoa would not be a strong predictor of $CH_4$ for those conditions in which a decrease in $CH_4$ does not come along with a decrease in the number of protozoa.

Furthermore, Mohammed et al. (2011) reported that the accuracy of the predictions increased by developing models specific to individual diets, as compared with the accuracy of the model combining all data, which reflects the limitations of the equations to be applied under other conditions.

Finally, Weill et al. (2009) reported a model Eq. (10) to predict $CH_4$ based on the sum of fatty acids with carbon chains shorter than 16 (FA < C16):

$$CH_4 \text{ (g/L milk)} = (\text{Sum MFA} \leq \text{C16}) \times (11.368) \times [\text{Milk production (L/year)}^{-0.4274}] \quad (10)$$

where individual MFA ≤ C16:0 are expressed as g/100 g MFA

The main advantage of this equation is that the sum of these MFA can be routine analyzed. Ruminants utilize acetate produced in rumen fermentation of carbohydrates as the major carbon source for *de novo* synthesized fatty acids (Bauman and Griinari, 2003). Knowing that acetate has a clear positive relationship with $CH_4$, it could be expected that *de novo* synthesized fatty acids could serve as good predictors for $CH_4$, even though other studies (Chilliard et al, 2009; Dijkstra et al., 2011; Mohammed et al., 2011) have identified other MFA than the ones proposed by Weill et al. (2009) as predictors of rumen $CH_4$.

The main uncertainty about the above-mentioned models is their ability to predict $CH_4$ from animals in conditions different to those from which the models were developed. Obviously, in developing a model, the MFA selected should allow for predictions with the highest generalization capacity.

In the development of a model, it is possible to include only particular independent variables known to have a relationship with the dependent variable. This should be done carefully and only based on expert knowledge. This has been previously done in a study using MFA to identify acidotic cows (Colman et al., submitted) finding that regressions on selected variables performed as good as models from a complete set of variables. This might play an even more important role when having a limited number of observations and a relatively large number of independent variables, which increases the risk of over fitting.

With that in mind, we believe that milk odd- and branched-chained fatty acids (OBCFA) have a high potential to be linked to $CH_4$ emissions. These MFA are of microbial origin in the rumen as constituents of the microbial membrane. Moreover, their profile depends on the presence of specific microbial communities (Keeney et al., 1962) and rumen conditions, which in turn, relate directly to enteric $CH_4$ emissions.

Previous studies have examined the prediction of rumen volatile fatty acid (VFA) proportions from milk OBCFA using various modelling approaches (e.g. Vlaeminck et al., 2006; Craninx et al., 2008; Bhagwat et al., 2012). Furthermore, the stoichiometric relationship between the amount of $CH_4$ produced and formation of acetate, propionate and butyrate in the rumen (Demeyer and Fievez, 2000) might enable prediction of $CH_4$ proportion from milk OBCFA. Vlaeminck and Fievez (2005) used a dataset from 6 experiments (n = 83) and were the first to relate calculated enteric $CH_4$ production to milk OBCFA. Positive relationships were found between calculated $CH_4$ production and *iso* C15:0, while negative relationships were observed between calculated $CH_4$ production and C15:0.

The results of Vlaeminck and Fievez (2005) suggest that there is scope for OBCFA as predictors of $CH_4$, and a larger dataset may increase the accuracy of these predictions. Therefore, in the next section we aim at exploring the relationships between milk OBCFA and

calculated $CH_4$ proportion (mmol/mol VFA) over a wide range of feeding conditions in order to identify the most important milk OBCFA potentially of value to predict $CH_4$ production in lactating dairy cows.

## Relationships between odd- and branched-chain fatty acid profiles in milk and calculated enteric methane proportion for lactating dairy cattle

A dataset, consisting of molar proportions of acetate, propionate, butyrate and $CH_4$ (mmol/mol VFA) as well as milk FA profiles from 13 studies (Table 2.2) resulting in 224 observations (*i.e.*, animal in a diet averages), was used in this study.

Acetate, propionate and butyrate proportions were measured and daily averages were calculated. Rumen $CH_4$ as mmol/mol VFA were calculated according to a stoichiometric relationship based on VFA proportions as mmol/mol VFA as:

$$CH_4 \text{ (mmol/mol VFA)} = 0.450 \times \text{Acetate} - 0.275 \times \text{Propionate} + 0.400 \times \text{Butyrate} \quad (11)$$

which assumes an $H_2$ balance of 0.9 (Demeyer and Fievez, 2000).

### *Variables included*

Seven milk OBCFA (g/100 g milk FA) were considered as input variables. Their mean, standard deviation, minimum and maximum are presented in Table 2.1.

Table 2.1. Descriptive statistics of OBCFA used for model development

| | *iso* C13:0 | *iso* C14:0 | *iso* C15:0 | *anteiso* C15:0 | C15:0 | *iso* C16:0 | C17:0 + *cis*-9 C17:1 |
|---|---|---|---|---|---|---|---|
| Mean | 0.025 | 0.081 | 0.216 | 0.457 | 1.079 | 0.192 | 0.701 |
| Standard deviation | 0.0053 | 0.0197 | 0.0398 | 0.0662 | 0.1703 | 0.0395 | 0.0938 |
| Minimum | 0.007 | 0.032 | 0.116 | 0.309 | 0.700 | 0.119 | 0.507 |
| Maximum | 0.045 | 0.154 | 0.325 | 0.701 | 1.751 | 0.402 | 1.133 |

These FA were determined by gas liquid chromatography as described by Vlaeminck et al. (2005) and Boeckaert et al. (2008).

***Model development***

Three approaches were used to model calculated rumen $CH_4$ proportion based on milk OBCFA [Figure 2.1 (A)]. First, a multiple linear regression (MLR) with a forward variable selection was performed using the PROC GLMSELECT procedure of SAS. The root mean square error (RMSE) was used as selection criterion. Second, partial least squares (PLS) regression was performed with the PROC PLS procedure of SAS, in which the NIPALS algorithm was used to develop the model. The variable selection was based on the VIP criterion (*i.e.*, variable of importance for projection with 0.8 as threshold value; Wold, 1994) and the absolute value of each regression coefficient. The PLS regression aims to extract latent factors (scores) accounting for most of the variation in the response, and predicts responses (Y) based on the covariance of X and Y (Mevik and Wehrens, 2007). Both for MLR and PLS, a 7 fold cross-validation was applied.

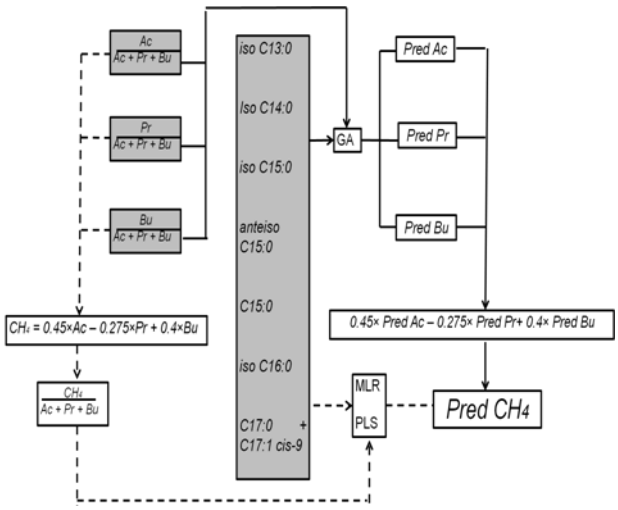
Table 2.2. Summary of the 13 experiments used for model development

| Experiment # | # of data points | Cows | Lactation stage (DIM[a]) | Experiment (days) | Forage | Concentrate (kg/d) | Acetate[b] | Propionate[b] | Butyrate[b] | Calculated $CH_4$[b] | Source |
|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | 21 | 6 | 185 ± 75 | 21 | Grass, clover, alfalfa, mixed silages | 8 | 661.8 ± 13.9 | 209.5 ± 13.6 | 128.6 ± 5.8 | 291.7 ± 9.8 | Dewhurst et al. (2003) |
| 2 | 16 | 4 | 145 ± 37 | 28 | Grass silage | F:C ratios (80/20, 65/35, 50/50 | 663.5 ± 13.4 | 220.7 ± 10.1 | 115.8 ± 8.9 | 284.2 ± 7.4 | Moorby et al. (2006) |
| 3 | 16 | 4 | 130 ± 24 | 28 | Grass silage | 17 | 688.3 ± 15.5 | 196.1 ± 16.1 | 115.6 ± 14.4 | 302.1 ± 11.3 | Hindle et al. (2005) |
| 4 | 16 | 4 | 275 ± 22 | 21 | Grass silage | 4.5 | 710.3 ± 10.3 | 175.4 ± 9.9 | 114.3 ± 5 | 317.1 ± 7.2 | Bruinenberg et al. (2004) |
| 5 + 6 | 50 | 5 | 114 ± 45 (exp 5) 294 ± 33 (exp 6) | 21 | Grass + maize silage | TMR (50/50) | 624.5 ± 27.4 | 233.5 ± 29.7 | 142 ± 10.7 | 273.6 ± 21.4 | Abrahamse et al. 2008 |
| 7 + 8 | 17 | 3 | 308 ± 142 | 13 | Grass + maize silage | 5.1 | 667.1 ± 12.9 | 187. 7± 5.8 | 145 ± 14.7 | 304.3 ± 3.9 | De Brabander et al. (2004) |
| 9 + 10 | 8 | 2 | 284 ± 101 | 13 | Grass + maize silage | 5.1 | 668.8 ± 6.1 | 176.8 ± 3.6 | 154.6 ± 4.4 | 314.1 ± 2.6 | De Brabander et al. (2005) |
| 11[c] | 20 | 4 | 212 ± 35 | 14 | Grass + maize silage + grass seed hay | 4 | 670.7 ± 22.2 | 198.3 ± 21.7 | 130.9 ± 9.3 | 299.6 ± 15.7 | Craninx, M., unpublished |
| 12 | 48 | 12 | 246 ± 103 | 7 | Grass + maize silage | 10 to 16 | 653.5 ± 24.2 | 201.3 ± 21.8 | 145.2 ± 9.8 | 298.8 ± 15.8 | Colman et al. (2010) |
| 13[d] | 12 | 3 | 158 ± 40 | 21 | Grass + maize silage | 8 to 10 | 684.6 ± 28.1 | 192.3 ± 22.4 | 123.1 ± 11.6 | 304.4 ± 16.4 | Tas, B. and van Straalen, W., unpublished |

[a] DIM, days in milk
[b] mmol/mol VFA: mean ± standard deviation
[c] Craninx, M., Laboratory of Animal Nutrition and Animal Product Quality, Ghent University, Ghent, Belgium; unpublished data.
[d] Tas, B.M., W.M. Schothorst Feed Research, Lelystad, the Netherlands; unpublished data

In a third approach, rumen VFA proportions were predicted from milk OBCFA using a rational model structure (Figure 2.1B). These predicted acetate, propionate and butyrate proportions were used to calculate enteric $CH_4$ proportion (Figure 2.1A). Relevant milk OBCFA were selected by a genetic algorithm (GA) (Bhagwat et al. 2012) and the optimal model parameters were estimated with the Nelder-Mead simplex method. In the GA, predictive performance of randomly generated models was evaluated following a cross-validation approach. The best among them constituted the future generation by combining, modifying or copying them. This process was repeated during 80 generations for a population of 40 individual models. The model with the highest predictive performance during the entire search process was retained as the best model. The variance of the prediction error (Var PE) selection criterion was used to quantify the prediction accuracy.

(A)




(B)

$$AC = 1000 \text{ mmol/mol} \quad \frac{\sum_i a_i \times OBCFA_i}{\sum_i a_i \times OBCFA_i + \sum_i p_i \times OBCFA_i + \sum_i b_i \times OBCFA_i}$$

$$PR = 1000 \text{ mmol/mol} \quad \frac{\sum_i p_i \times OBCFA_i}{\sum_i a_i \times OBCFA_i + \sum_i p_i \times OBCFA_i + \sum_i b_i \times OBCFA_i}$$

$$BU = 1000 \text{ mmol/mol} \quad \frac{\sum_i b_i \times OBCFA_i}{\sum_i a_i \times OBCFA_i + \sum_i p_i \times OBCFA_i + \sum_i b_i \times OBCFA_i}$$

Ac, acetate; Pr, propionate; Bu, butyrate; $CH_4$; Pred, predicted

Figure 2.1 (A) Model development for the prediction of calculated $CH_4$ proportion (mmol/mol VFA) based on milk odd- and branched-chain fatty acids (OBCFA), following a multiple linear regression (MLR, ----), partial least squares (PLS, -----) and a rational genetic algorithm (GA, ——) approach. (Model development based on 224 paired observations of proportions of VFA in rumen fluid and OBCFA in milk fat. Measured variables are indicated by grey boxes) (B) Model structure based on rational functions

***Experiment effect***

Due to the different settings in the observed outputs from the various experiments, previous models of linear (Vlaeminck et al. 2006) and rational structure required inclusion of an experiment effect for prediction of VFA proportions. This random effect, which could be considered as accounting for a 'farm factor' in the model, was also included in our study. For both PLS and MLR the experiment effect was included as a dummy variable during the regression process, which allowed for better estimation of the regression coefficients. For the GA approach, the experiment effect was considered after model development by correcting predicted values by the mean error of each experiment. To evaluate the predictive performance of the models, the Var PE was used to express intra-experiment variation, the RMSE to express the average error expected to be associated with future predictions, and $R^2$ to measure the proportion of variance explained by the model. In addition, the concordance correlation coefficient was used to express accuracy and precision (CCC; Lin, 1989). These predictive performance parameters are reported for the cross-validated models.

## RESULTS

The models MLR Eq. (12), PLS Eq. (13) and GA Eq. (14) had a relatively low prediction error (Table 2.2), varying between 12.7 and 13.4 mmol $CH_4$/mol VFA, which represents about 17.5 g $CH_4$/d assuming a VFA production of 80 mol/d for a 650 kg cow in mid-lactation with a DM intake of 12.9 kg/d (Sutton, 2003). Milk OBCFA patterns explained up to 66% of the variance in the dataset of molar $CH_4$ proportions. Because $CH_4$ was calculated in our dataset, it is not surprising that the predictive performances of these models were intermediate compared to the performance of models which predicted acetate, propionate and butyrate proportions from milk OBCFA. In general, both regression techniques resulted in models with comparable predictive performance. The MLR model had a slightly lower intra-experimental variation compared to the GA approach. The PLS model had the largest Var PE**.**

$$CH_4\left(\frac{mmol}{mol\ VFA}\right) = 342.1 + (275.6 \times iso\ \text{C14:0}) - (30.1 \times \text{C15:0}) - [47.6 \times (\text{C17:0} + cis\text{-9 C17:1})] \quad (12)$$

$$CH_4\left(\frac{mmol}{mol\ VFA}\right) = 336.2 + (222.6 \times iso\ \text{C14:0}) + (49.7 \times iso\ \text{C16:0}) - (28.9 \times \text{C15:0}) - [48.0 \times (\text{C17:0} + cis\text{-9 C17:1})] \quad (13)$$

$$CH_4\left(\frac{mmol}{mol\ VFA}\right) = \frac{(0.252 \times iso\ \text{C14:0}) - (0.0012 \times \text{C15:0}) - [0.0018 \times (\text{C17:0} + cis\text{-9 C17:1})]}{(0.757 \times iso\ \text{C14:0}) + (0.0087 \times iso\ \text{C15:0}) - (0.0151 \times iso\ \text{C16:0})} \times 1000 \quad (14)$$

Partial least square regression is known to be a better approach when variables show structural problems related to repeated measurements (e.g. collinearity) and data show structural problems such as skewness (Farahani et al., 2010). However, no improvement occurred using PLS compared with MLR. The GA approach allowed a rational model structure to fit the VFA to better reflect their proportional nature. Nonetheless, the GA resulted in no improvement over the MLR or PLS approaches. In general the three modeling approaches resulted in similar predictions (Table 2.3, Fig 2.2).

Table 2.3. Root mean square error (RMSE), coefficient of determination ($R^2$), concordance correlation coefficient (CCC) and variance of the prediction error (Var PE) of the cross-validated models predicting calculated rumen enteric $CH_4$ proportion with a multiple linear regression model (MLR), a partial least squares model (PLS) and a rational genetic algorithm model (GA)

| | Predicted $CH_4$[a] | RMSE (%) | $R^2$ | CCC | Var PE[b] $(mmol/mol)^2$ |
|---|---|---|---|---|---|
| MLR | 294.1 | 4.2 | 0.66 | 0.79 | 133.5 (34.0) |
| PLS | 294.1 | 4.2 | 0.63 | 0.77 | 154.7 (39.3) |
| GA | 293.9 | 4.1 | 0.66 | 0.82 | 140.9 (35.8) |

[a] mmol/mol VFA. Mean calculated enteric $CH_4$ proportion of the dataset = 294.1 mmol/mol VFA

[b] Data between brackets indicate the proportion, expressed as percentage, of the variance of the prediction error relative to the variance of the $CH_4$ calculated from the measured rumen VFA proportions $(mmol/mol\ VFA)^2$

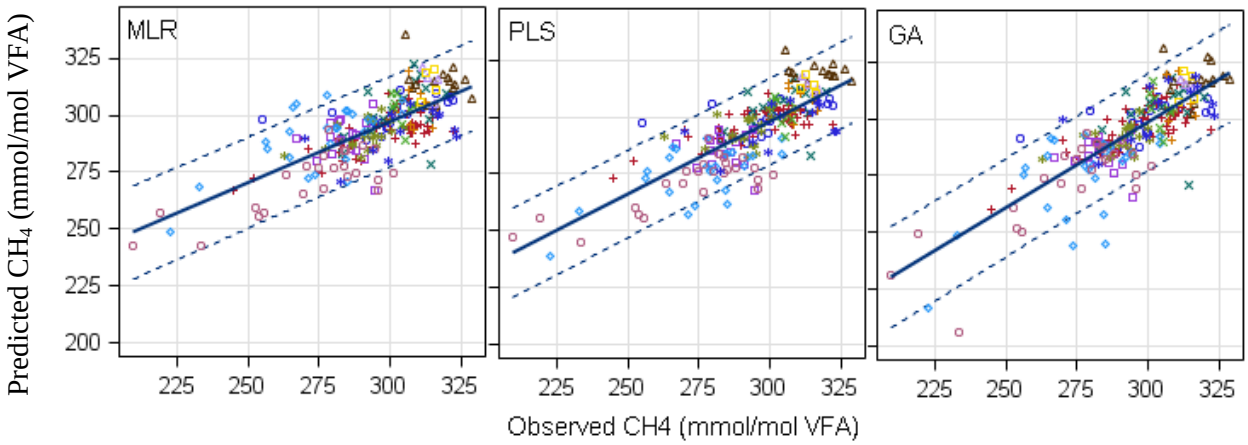



Figure 2.2 Observed vs. predicted ruminal calculated methane proportion (mmol/mol VFA) (n=224) for the cross-validated models developed from milk OBCFA by using MLR, PLS and a rational approach based on GA. Dotted lines indicate 95% confidence interval of calculated methane proportions

Results of the three modeling approaches showed that milk OBCFA most relevant to calculated $CH_4$ proportion are: *iso* C14:0 and *iso* C16:0 which were positively related to $CH_4$ production, and C15:0 and the sum of C17:0 and *cis*-9 C17:1 which were negatively related to $CH_4$ production.

## DISCUSSION

A previous model developed by Vlaeminck and Fievez (2005) indicated only *iso* C15:0 and C15:0 as main predictors. Differences between our study and that of Vlaeminck and Fievez (2005) are likely due to the large differences in size and variability of the datasets. In addition, Vlaeminck and Fievez (2005) used the significance of the *P* value of each regression coefficient as a criterion to select variables included in the model, whereas our selection was based on model performance evaluated by the RMSE of the cross-validation. *Iso*-FA are more abundant in cellulolytic bacteria (Vlaeminck et al. 2006), which in turn are usually related to a higher $CH_4$ production. In contrast, amylolytic bacteria are generally enriched in linear FA (*e.g.*, C15:0 and C17:0; Vlaeminck et al., 2006), and associated with high starch diets which produce less $CH_4$.

In the multivariate analysis of Dijkstra et al. (2011), *anteiso* C17:0 was the only OBCFA selected in their final model Eq. (7). However, by univariate regression the authors found positive relationships between $CH_4$ production (g/kg DM intake) and *iso* C14:0 and *iso* C15:0; and negative relationships between $CH_4$ and *cis-9* C17:1. As mentioned before, the FA *cis-9* 17:1 is derived directly from C17:0 (Fievez et al., 2003) and often has a relatively high correlation with C15:0. Both C15:0 and C17:0 + *cis-9* C17:1 were negatively associated with $CH_4$ estimates in our study. Moreover, the best equation Mohammed et al. (2012) found to predict $CH_4$ (g/d) included both *cis*-9 C17:1 (negatively related) and *iso* C16:0 (positively related). Mohammed et al. (2011) also found a tendency for a negative correlation between $CH_4$ (g/d) and C15:0 (r = –0.3), also in agreement with our findings.

Conversely, Chilliard et al. (2009) found a positive correlation between C15:0 and $CH_4$ (g/d). This contrasting relationship may have been associated with the supplementation of diets with linseed, which may have reduced *de novo* synthesis of microbial FA. Under conditions in which FA are supplemented in the diet, microbes tend to incorporate those FA directly, as opposed to synthesizing them *de novo* (Demeyer et al., 1978). Milk OBCFA were not retained in the predictive equation of Chilliard et al. (2009) Eq. (5), but it has to be kept in mind that their predictions were based on data from specific dietary treatments being sources of UFA. Mohammed et al. (2011) and Dijkstra et al. (2011) considered diverse dietary treatments with a larger number of observations and in their analyses some milk OBCFA show correlations with enteric $CH_4$ production, which might indicate the potential of these MFA to predict rumen methanogenesis in a relatively broader range of diets and conditions.

## Comparison of the OBCFA model with previously reported MFA-based models

The discussion above shows that the variables selected in the models developed from calculated $CH_4$ Eq. (12) – (14) were according to the theoretical expectations, and in some

cases in conformity with studies with *in vivo* $CH_4$ measurements. However, other models have been developed under different conditions, making it difficult to fairly compare our models with those ones. Therefore, in an attempt to assess the reliability of our model in relation to others we used the database from the same studies listed in Table 2.2 including the complete MFA profile along with DMI, forage intake and milk production data to calculate $CH_4$ emissions from previous models and compare the predictions with the predictions of the OBCFA model.

In a second approach calculated $CH_4$ was regressed on the MFA included in each of the five formerly developed models [Eq. (5), (7), (8), (10) and (15) using MLR as described in the previous section. The performances of each model to predict calculated $CH_4$ were compared to the performance of the OBCFA model.

***Methodology***

Five models developed from *in vivo* data were selected for this comparative analysis. The equations are from Chilliard et al. [2009, Eq. (5)], Dijkstra et al. [2011, Eq. (7)], Mohammed et al. [2011, Eq. (8)] and Weill et al. [2009, Eq. (10)]. An additional equation from Mohammed et al. (2011) which takes into account DMI in it was also included in the analysis Eq. (15).

$$CH_4\,(g/d) = (-124.9 \times \textit{cis}\text{-}11\ 18{:}1) + (2115.9 \times t,t\ CLA) - (10.04 \times \sum \textit{trans}\ C18{:}1) + (455.5 \times \textit{anteiso}\ C15{:}0) - (305.9 \times C17{:}0) + (7.83 \times DMI) + 104.8 \qquad (15)$$

The results were compared by a scatter plot against the predictions obtained with our MLR model Eq. (12). Knowing that the three models developed above performed similar, we decided to use the MLR model Eq. (12) for its most straightforward approach which allows an easier interpretation of the results.

Two MFA were not available in our database and a fixed value was taken from previous literature: *Trans*-16 + *cis*-14 C18:1 = 0.518 g/100 g MFA, was taken from the average of Chilliard et al., 2009, Mohammed et al., 2011 and Abecia et al., 2012. *Trans, trans* CLA, which refers to the sum of the *trans, trans* - CLA peaks 7,9; 8,10; 9,11; and 10,12 = 0.0214 g/100 g MFA was taken from the average of 10 treatments in Lerch et al. (2012).

Additionally, for the equation of Weill et al., (2009) a fixed milk yield of 8500 L/year was set for all observations.

## RESULTS

The relationships between $CH_4$ predictions from previous models and the OBCFA model developed in this chapter are low for all the cases (Figure 2.3). The predictions from the model of Chilliard et al. (2009) were best correlated with data predicted from the OBCFA model, but the accordance between both approaches was still limited ($R^2$ = 0.3, Figure 2.3.A), whereas both Mohammed et al. (2011, Figure 2.3.C) and Weill et al. (2009, Figure 2.3.E) had the lowest association to the OBCFA model.

Mohammed et al. (2011) suggested that DMI variables may be more suitable for predicting $CH_4$ compared to MFA variables. In this line, it is interesting to note that between the two equations of Mohammed et al. (2011) the one including DMI Eq. (15) (Fig 2.3.D) related better to $CH_4$ estimated from the OBCFA model. Furthermore, the predictions from Chilliard et al. (2009) also included a DMI variable in the model (forage intake, kg DM/d) and the model of Dijkstra et al. (2011) contained DMI, because their model predicts $CH_4$ as g/kg DMI. The models considering DMI variables related slightly better with the OBCFA model.

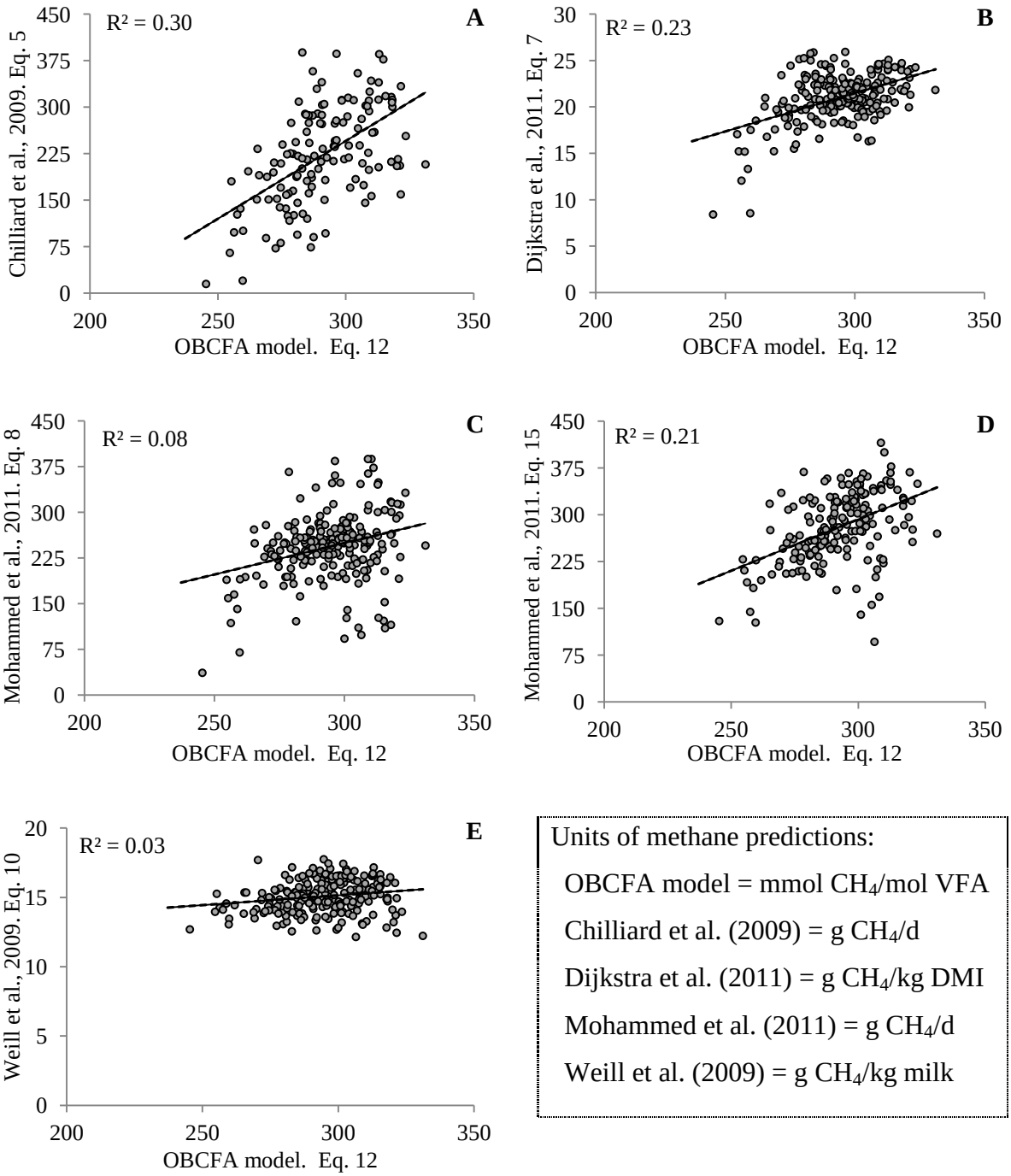



Figure 2.3. Comparison of predicted $CH_4$ from the OBCFA model Eq. (12) with previous models developed from *in vivo* data **A)** Chilliard et al., 2009. Eq. (5) **B)** Dijkstra et al., 2011. Eq. (7) **C)** Mohammed et al. 2011. Eq. (8) **D)** Mohammed et al., 2011. Eq. (15) **E)** Weill et al., 2009. Eq. (10).

The small concordance between the models can be due to factors as the nature of the experiments based on which equations were developed and the units in which $CH_4$ is expressed. To aid to the fair comparison of the OBCFA model in relation to other models the calculated $CH_4$ of the original dataset was regressed on the MFA retained in each model Eq. (5), (7), (8) and (10). For instance for the model of Chilliard et al. [2009; Eq. (5)] $CH_4$ was regressed on C16:0, *trans*-16 + *cis*-14 C18:1, *cis*-9 C14:1 and *n-6* C18:2.

The results of this approach (Table 2.4) show that the model based on OBCFA explained a larger part of the variation in $CH_4$ data ($R^2$) and showed a lower error associated with future predictions (RMSE) than most of the other models except when all MFA ≤ C16 were included in the regression.

Table 2.4. Mean, root mean square error (RMSE) and coefficient of determination ($R^2$) of the regressions of calculated rumen enteric $CH_4$ proportion on MFA included in the respective previous model.

| | Mean (mmol/mol VFA) | RMSE (%) | $R^2$ |
|---|---|---|---|
| OBCFA model | 294.1 | 4.2 | 0.66 |
| Chilliard et al., 2009 | 294.1 | 4.9 | 0.50 |
| Weill et al., 2009 (1)[a] | 293.5 | 5.1 | 0.47 |
| Weill et al., 2009 (2)[a] | 293.5 | 4.1 | 0.65 |
| Dijkstra et al., 2011 | 296.8 | 4.4 | 0.37 |
| Mohammed et al., 2011 | 293.5 | 4.3 | 0.61 |

[a] In Weill et al., 2009 (1) only the Sum of FA ≤ C16 was used for the regression, whereas in Weill et al., 2009 (2) all the individual MFA ≤ C16 were used.

By looking at the MFA kept in each regression (Table 2.5) it is noteworthy that when all individual MFA ≤ C16 were included in the regression, two OBCFA (C15:0 and *iso* C14:0) remained in the final model, which were also present in the original OBCFA model. Moreover, *cis*-9 C17:1, another MFA present in the OBCFA model, was also kept in the model of Mohammed et al (2011), which might indicate that OBCFA could be more relevant

predictors of $CH_4$ output than other MFA. Moreover, the MFA retained from the model of Mohammed et al. (2011) kept their sign respective to the original model Eq. (8). In the model of Chilliard et al. (2009), *cis*-9 C14:1 kept the original sign, but not so *n-6* C18:2. Similarly, *cis*-11 C18:1 kept the sign of the original model of Dijkstra et al. (2011), but not *anteiso* C17:0.

Table 2.5. Milk fatty acids selected in each regression. Fields in gry are MFA negatively related to calculated $CH_4$

| Study | Milk fatty acids | | | |
|---|---|---|---|---|
| OBCFA model | *Iso* C14:0 | C15:0 | C17:0 + *cis*-9 C17:1 | |
| Chilliard et al., 2009 | *Cis*-9 C14:1 | *n-6* C18:2 | | |
| Weill et al., 2009 (1) [a] | Sum FA ≤ C16 | | | |
| Weill et al., 2009 (2) [a] | C12:0 | C15:0 | *Iso* C14:0 | C16:0 |
| Dijkstra et al., 2011 | *Anteiso C17:0* | *Cis*-11 C18:1 | | |
| Mohammed et al., 2011 | Sum *trans* C18:1 | *Cis*-11 C18:1 | *cis*-9 C17:1 | |

[a] In Weill et al., 2009 (1) only the Sum of FA ≤ C16 was used for the regression, whereas in Weill et al., 2009 (2) all the individual MFA ≤ C16 were used.

## CONCLUSIONS

The models help to highlight the most important milk OBCFA related to calculated $CH_4$ (as mmol/mol VFA) in the rumen, *i.e. iso* C14:0, *iso* C15:0, *iso* C16:0, C15:0 and the sum of C17:0 and *cis*-9 C17:1. Overall, linear regressions developed using an MLR or PLS approach, and the rational GA model, had similar predictive performance. Concordance between $CH_4$ estimated from the OBCFA model and $CH_4$ estimated from published models was low. When the calculated $CH_4$ was regressed on individual MFA included in previous models some OBCFA remained in the model, which affirmed the potential of OBCFA to predict $CH_4$ output. Reliability of these MFA as predictors of enteric $CH_4$ production should be confirmed with direct measurements using lactating dairy cows.

# CHAPTER 3. THE POTENTIAL OF MILK FATTY ACIDS AS BIOMARKERS FOR METHANE EMISSIONS: A QUANTITATIVE MULTI-STUDY SURVEY OF LITERATURE DATA

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## ABSTRACT

Both univariate and multivariate analyses were performed to explore the relationships between milk fatty acids (MFA) and methane ($CH_4$) emissions from dairy cattle. Data from a total of 15 studies including 48 treatments were gathered in our database. Methane was expressed as daily emissions (g/d), relative to dry matter intake (g/kg DMI), relative to milk production (g/kg milk) and relative to metabolic body weight (g/kg $BW^{0.75}$/d). The univariate correlations between MFA and $CH_4$ were based on absolute means and on relative changes of each treatment compared with its corresponding control. The univariate analyses suggest that saturated fatty acids, odd- and branched-chain fatty acids and long chain poly-unsaturated fatty acids are positively related to $CH_4$, while mono-unsaturated fatty acids are negatively related to $CH_4$. However, in general, the coefficient of determination ($R^2$) of these univariate regressions ranged from 0.1 to 0.6, indicating that individual MFA are not able to largely explain variation in $CH_4$. Furthermore, four multivariate models were developed linking MFA to $CH_4$ in each of the four units (g/d, g/kg DMI, g/kg milk and g/kg $BW^{0.75}$/d). Milk fatty acids retained in each equation differed, highlighting the importance of the functional unit to express $CH_4$. The present findings contribute to the exploration of the potential of MFA as biomarkers for methane emissions from dairy cattle.

## INTRODUCTION

Milk represents an easy-to-take sample that is frequently used in dairy farms to monitor specific milk traits (*e.g.* fat, acetone) as biomarkers to assess nutritional and health status of dairy cattle. In the context of environmental emissions, biomarkers such as urea in milk not only indicate the excess of protein intake by an animal, but also give an indication of the

nitrogen excretion by production animals (Jonker, Kohn & Erdmann, 1999). Similarly, given the major emphasis on greenhouse gases emissions in recent years, efforts were made towards the development of simple techniques to estimate methane ($CH_4$) emissions under practical conditions, with major attention to milk and its components, specifically milk fatty acids (MFA) (*e.g.* Chilliard et al. 2009; Dijkstra et al., 2011; Mohammed et al., 2011). Although several studies reported changes in MFA along with changes in $CH_4$ production (*e.g.* Sauer et al., 1998; Hristov et al., 2009; Van Zijderveld et al., 2011), variations in MFA profiles and $CH_4$ were not consistent across studies, which makes it difficult to draw conclusions from individual experiments on the relationships between MFA and $CH_4$ and more generally, on the ability of MFA to predict $CH_4$. In the same sense, models aiming at predicting $CH_4$ (Chilliard et al., 2009; Dijkstra et al., 2011, Mohammed et al., 2011) have few MFA in common. This general lack of concurrence may originate mainly, but not only, from the diverse conditions of the studies (e.g. basal diet, type of animal, dietary treatments) which limits their generalization capacity.

Therefore, in this multi-experiment survey we gathered data from multiple studies which reported both $CH_4$ emissions and MFA profiles to identify robust relations between MFA and $CH_4$ emissions in an attempt to highlight individual or groups of MFA predominantly linked with $CH_4$ and to develop a more general predictive equation from a multi-study database.

## MATERIALS AND METHODS

### Database development

Publications were obtained from the ISI Web of Science and from Pubmed databases using "*in vivo*", "methane", and/or "milk fatty acids" as keywords. Table 3.1 presents an overview of published studies reporting both $CH_4$ production and MFA profiles. Fifteen studies comprising 48 treatments (17 controls and 31 supplements) were included.

It is known that the concentration of certain fatty acids in milk will be influenced by the addition of fats in the diet. Therefore, all treatments found in the studies in Table 3.1 were classified in three categories according to the major source and the amount of supplementary fatty acid intake: supplementary medium chain FA (**MCFA**), supplementary unsaturated FA [**UFA,** including mono- and poly-unsaturated fatty acids (MUFA and PUFA)], and no supplementary fatty acids (**Other**). By supplementary intake we refer to the extra amount of fatty acids consumed by the animal within a certain treatment compared with the corresponding control treatment. These amounts (g FA/d and g FA/kg BW) and the category of each treatment are given in appendix (Table A.1). All of the controls were included in the category **Other**, along with those treatments not containing any fatty acid source (*e.g.* monensin, bromochloromethane). The study of Van Zijderveld et al. (2011), supplementing the cows with a mix of dietary additives containing lauric acid, myristic acid, linseed oil and calcium fumarate, was included in the **MCFA** group, because the addition of these fatty acids exceeded that of UFA (254 and 216 g/d of MCFA and UFA, respectively).

Hundred twenty-six MFA were found across the studies, of which 53 were selected for the statistical analysis (with an average concentration higher than 0.02 g/100 g MFA, reported at least in five studies and being represented in each of the three categories previously described) as continuous predictor variables. Summary statistics of the variables used for the analysis are presented in appendix (Table A.2). Methane was expressed as daily emissions (g/d), relative to DMI (g/kg DMI) and relative to milk production (g/kg milk). Furthermore, two of the studies included were performed with goats, therefore, methane relative to metabolic body weight (g/kg $BW^{0.75}$/d) was also included in the analysis.

Table 3.1. Summary of studies reporting methane output and milk fatty acid profiles

| Study (Experiment #) | Supplement and dose | N animals | Basal diet | Measuring technique | $CH_4$ (g/d) | | DMI (kg/d) | | Milk yield (kg/d) | | Fat (g/kg milk) | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | | | | Control | Treatment | Control | Treatment | Control | Treatment | Control | Treatment |
| **Fat supplements. PUFA sources** | | | | | | | | | | | | |
| Sauer et al., 1998 (1) | Soybean oil (10% of DM, *ca.* 600 g/cow/d) | 10 | Corn/alfalfa forage and concentrate | Environmentally controlled barn | 402 | 397 | 19.8 | 17.3* | 24.9 | 24.9 | 36.6 | 37.1 |
| Johnson et al., 2002 (2) | 4 % fat from cottonseeds and canola seed (50:50) | 4 | Alfalfa forage and concentrate | Room; tracer ($SF_6$) | 389 [a] | 394 | 25.2 | 27.2* | 32.3 | 39.3* | 39.0 | 36.3 |
| | 5.6% fat from cottonseeds and canola seed (50:50) | 4 | | | | 456 | | 26.9* | | 39.1* | | 36.1 |
| Chilliard et al., 2009 (3) | Whole crude linseed [b] | 8 | Hay, corn silage, concentrate | $SF_6$ tracer technique | 418 | 369* | 19.8 | 19.5 | 23.0 | 21.5 | 41.1 | 45.4 |
| | Extruded linseed [b] | 8 | | | | 258* | | 16.7* | | 20.8 | | 35.3* |
| | Linseed oil [b] | 8 | | | | 149* | | 14.7* | | 18.9* | | 32.3* |
| Mohammed et al., 2011 (4) | Sunflower seeds [c] | 4 | Barley silage, concentrate (45:55, DM basis) | Environmental chamber | 293 [d] | 264* | 18.1 | 18.2 | 27.0 | 26.1 | 32.0 | 30.0 |
| | Linseeds [c] | 4 | | | | 241* | | 18 | | 25.7 | | 33.0 |
| | Canola seeds [c] | 4 | | | | 265* | | 19.4* | | 26.4 | | 31.0 |
| Moate et al., 2013 (5) | Algal meal (125 g/d) [e] | 8 | Alfalfa hay, concentrate (75:25) | Calorimetric chamber | 543 | 563 | 24.1 | 24.1 | 22.2 | 26 | 49.7 | 37.8* |
| | Algal meal (250 g/d) [e] | 8 | | | | 553 | | 22.7 | | 23.1 | | 37.0* |
| | Algal meal (375 g/d) [e] | 8 | | | | 520 | | 21.5* | | 22.3 | | 38.3* |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **Fat supplements. MCFA sources** | | | | | | | | | | | | |
| Odongo et al., 2007 (6) | Myristic acid (5% DM basis) | 6 | Hay, corn silage, concentrate | Open circuit indirect calorimetry | 399 | 256* | 15.2 | 14.2 | 14.9 | 13.4 | 42.0 | 41.0* |
| Hristov et al., 2009 (7) | Lauric acid (240 g/d) | 6 | TMR[g]. Alflafa + barley forage | $SF_6$ tracer technique | 156 [h] | 170 | 26.5 | 24 | 30.4 | 29.1 | 36.0 | 33.9 |
| | Coconut oil (530 g/d) [f] | 6 | | | | 60* | | 24.5 | | 29 | | 346 |
| van Zijderveld et al., 2011 (8) | Mix of additives [i] | 10 | Grass silage, maize silage, hay, concentrate | Calorimetric chamber | 362 | 325* | 16.4 | 15.9 | 28.9 | 26.1 | 46.3 | 41.0 * |
| Hristov et al., 2011 (9) | Lauric acid (240 g/d) | 6 | TMR. Corn silage + grass hay | $SF_6$ tracer technique | 300 [h] | 290 | 26.9 | 20* | 44.6 | 35.8 | 34.2 | 25.9* |
| | Myristic acid (240 g/d) | 6 | | | | 312 | | 25.7 | | 44.2 | | 31.2 |
| Hollman et al., 2012 (10) | Coconut oil (1.3% DM basis) [j] | 6 | Corn silage, alfalfa silage, grass silage, supplement | Environmentally controlled rooms | | 449* | | 21.3* | | 37.5* | | 36.6* |
| | Coconut oil (2.7% DM basis) [j] | 6 | | | 464 | 291* | 21.1 | 17.4* | 37.1 | 33.7* | 34.7 | 27.9* |
| | Coconut oil (3.3% DM basis) [j] | 6 | | | | 253* | | 16.7* | | 32.4* | | 26.7* |
| **Other supplements** | | | | | | | | | | | | |
| Hamilton et al., 2010 (11) | Monensin 14 d (600 mg/d) | 9 | TMR. Alfalfa hay | Environmental chamber | 270 | 294 | 27.7 | 27.5 | 39.8 | 39.9 | 37.5 | 38.7 |
| | Monensin 60 d (600 mg/d) | 9 | | | 224 | 237 | 28.5 | 25.1 | 40.8 | 40.1 | 39.5 | 38.4 |
| Hristov et al., 2010 (12) | *Saccharomyces cerevisiae* (56 g/d) | 8 | TMR. Corn + alfalfa + grass forage | $SF_6$ tracer technique | 233 | 240 | 27.4 | 27.6 | 46.5 | 46.4 | 34.8 | 32.7 |
| Sauer et al., 1998 (1) | Monensin (24 ppm) | 10 | Corn/alfalfa forage and concentrate | Environmentally controlled barn | 440 | 345* | 16.1 | 14.5* | 27.6 | 31.5* | 36.1 | 27.0* |

| | | | | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Abecia et al., 2012 [k] (13) | Bromochloromethane (0.3 g/100 kg BW) | 9 | Alfalfa hay, concentrate | Open circuit chamber | 14.6 | 9.9* | 0.99 | 1.04 | 0.97 | 1.32* | 54.6 | 49.8 |
| Heidarian Miri et al., 2013 [k] (14) | Cumin seed extract (1.27% DMI) [l] | 6 | Berseem clover hay, concentrate | $SF_6$ tracer technique | 16.5 | 14.5* | 1.12 | 1.17 | 1.36 | 1.53* | 36.3 | 35.4 |
| | Cumin seed extract (2.53% DMI) [l] | 6 | | | | 14.9* | | 1.17 | | 1.34 | | 33.6 |
| Hristov et al. 2013 (15) | Oreganum leaves 250 g/d | 8 | TMR. Grass hay + alfalfa haylage + corn silage | $SF_6$ tracer technique | 500 | 476* | 28.3 | 28.3* | 43.4 | 45.2 | 32.6 | 32.5 |
| | Oreganum leaves 500 g/d | 8 | | | | 321* | | 27.5* | | 44.1 | | 31.1 |
| | Oreganum leaves 750 g/d | 8 | | | | 365* | | 26.6* | | 43.4 | | 35.7 |

[a] Control: 2.3% fat from cottonseeds and canola seeds (50:50)
[b] Proportions of whole crude and extruded linseed and linseed oil were added to achieve a dietary fatty acid level of 5.7% on DM basis
[c] Crushed. Added to the diet to deliver 3.3% of fat on DM basis
[d] Control: calcium salts of long chain fatty acids (palm oil)
[e] Algal meal DM (DHA-Gold) containing 20% *n-3* C22:6
[f] Coconut oil containing 46 g lauric acid/100 g and 20 g myristic acid/100 g
[g] TMR: total mixed ration
[h] Control: Stearic acid (240 g/d)
[i] Lauric acid, myristic acid, linseed oil and calcium fumarate were added at 0.4, 1.2, 1.5 and 0.7%, respectively on DM basis
[j] Coconut oil containing 41 g lauric acid/100 g and 20 g myristic acid/100 g
[k] Experiments with goats
[l] The cumin seed extract supplemented at 1 and 2 g/L rumen volume

## Statistical analysis

Two approaches were used for the univariate analyses. Firstly, absolute means of $CH_4$ production were regressed on absolute means of milk fatty acid proportions (g/100 milk fat) to determine the relationship between individual MFA and $CH_4$ production. In a second approach, relative changes of each variable (changes proportional to the corresponding control) were used for the analysis. Therefore, relative changes in $CH_4$ were regressed on relative changes in MFA proportions. Both approaches were applied for the regression of $CH_4$ in each of the four units of expression on MFA. The analysis of the data was done following a statistical meta-analysis approach (Sauvant, Schmidely, Daudin & St-Pierre, 2008). Using the MIXED procedure of SAS (version 9.3), the following model was applied:

$$Y_{ijk} = \mu + \beta X_{ij} + S_i + \tau_j + \beta_{ij}(X \times \tau) + e_{ijk}$$

where $Y_{ijk}$ = dependent variable; μ = overall intercept across all experiments; $\beta$ = linear regression coefficient of $Y$ on $X$ (fixed effect); $X_{ij}$ = value of the continuous predictor variable (*i.e.* MFA proportion); $S_i$ = the random effect of the $i^{th}$ study; $\tau_j$ = the fixed effect of the $j^{th}$ level of factor $\tau$ (discrete variable, *i.e.* **UFA, MCFA, Other**); $\beta_{ij}$ = the linear regression coefficient of $Y$ on the interaction of $(X$ and $\tau_j)$ and $e_{ijk}$ = the unexplained residual error.

After running the univariate analysis, the interaction effect was not significant for any variable. Hence, this effect was removed from the model, retaining

$$Y_{ijk} = \mu + \beta X_{ij} + S_i + \tau_j + e_{ijk}$$

The Akaike information criterion (AIC) for this model was always lower than that of the previous model including the interaction effect across all variables. The variable 'study' was declared in the CLASS statement as it was considered as a random outcome of a population of studies and even though each study represented a different location and/or research station, the intention of this analysis was to obtain a more generalizable conclusions. Data were

weighed by the inverse of the standard error of the mean (SEM) (Sauvant et al., 2008). In order to maintain the square root of the mean square error in the same scale as the original measurements (*Y*), each weight was divided by the mean of all weights and the resulting values were used as weighing factors for the analysis as suggested by St-Pierre (2001). For a few cases, no measure of dispersion was reported (Hamilton, DePeters, McGarvey, Lathrop & Mitloehner, 2010), or it was not possible to use it to estimate the SEM (Abecia et al., 2012). In those cases the average variance of the specific variable across all the studies was calculated and was used to estimate the SEM, by dividing it by the square root of the respective number of observations.

Not all MFA included in the univariate analysis were reported in every study. Therefore, in order to develop a multivariate model, the MFA missing in the original dataset were estimated using the expectation-maximization (EM) algorithm for missing data with an arbitrary pattern. In this methodology the means and covariances from complete cases, and the means and standard deviations from other available cases are used as the initial estimates for the EM algorithm. This process was repeated ten times. The average estimation across the ten iterations was used to substitute the missing value in the original dataset. This was done with PROC MI of SAS. This new dataset was used for the development of multivariate models using a stepwise variable selection, with $CH_4$ production expressed either in g/d, g/kg DMI, g/kg milk and g/kg $BW^{0.75}$/d as independent variables. The selection of absolute MFA was based on the significance level of the *F* statistic ($P < 0.05$). The model development was set to yield the minimum AIC statistic. A study effect was included as a dummy factor during the regression process, which allowed a better estimation of the regression coefficients. This was done with PROC GLMSELECT of SAS. Four studies (Sauer et al. 1998, Johnson et al., 2002, Hristov et al., 2010 and Hristov et al., 2013) were excluded from the multivariate

analysis as they reported too few MFA, resulting in an excessive increase in the number of missing data.

## RESULTS

### Univariate regression

The linear regressions relating absolute $CH_4$ emissions (g/d, g/kg DMI, g/kg milk, g/kg $BW^{0.75}$/d) to MFA proportions (g/100 g MFA) are presented in appendix (Table A.3). Linear regressions predicting relative changes in $CH_4$ emissions (g/d, g/kg DMI, g/kg milk, g/kg $BW^{0.75}$/d) from relative changes in MFA proportions are presented in appendix (Table A.4). Table 3.2 presents a summary of MFA having an effect on absolute and relative methane emissions. Only those variables with a regression coefficient significantly different ($P < 0.05$) or tending to be different ($P < 0.1$) from 0 are presented.

Milk fatty acid proportions of C8:0, *anteiso* C15:0 (tendency with g/d and g/kg DMI), *iso*-C15:0, C15:0 (tendency with g/kg DMI), and *n-3* C20:5 (tendency with g/d and g/kg $BW^{0.75}$/d) were positively associated with absolute $CH_4$ irrespective of the functional unit for its quantification. Similarly, *cis*-11 C18:1, *cis*-13 C18:1, *trans*-6 + 7 + 8 C18:1, *trans*-12 C18:1, *trans*-13 + 14 C18:1 and total *trans* MUFA had a negative relationship with absolute $CH_4$ expressed in every functional unit. Some of these MFA (i.e. C8:0, *cis*-13 C18:1, *trans*-12 C18:1, *trans*-13 + 14 C18:1) were also related to relative changes in $CH_4$. Additionally, relative changes in individual C6:0 (tendency with g/kg $BW^{0.75}$/d), *n-6* C20:2 (tendencies with g/d, g/kg milk and g/kg $BW^{0.75}$/d) and total SFA (tendencies with g/kg DMI and g/kg milk) were positively related with relative changes in $CH_4$ expressed in all four units and relative changes in *trans*-9 C16:1 (tendencies with g/d and g/kg DMI), and total MUFA (tendency with g/kg milk) were negatively related to relative changes in $CH_4$ for all units of expression.

Table 3.2. Summary of MFA having an effect ($P < 0.10$) on absolute and relative methane emissions expressed in four different units

| | Absolute $CH_4$ | | | | Relative changes in $CH_4$[a] | | | |
|---|---|---|---|---|---|---|---|---|
| Milk fatty acid | g/d | g/kg DMI | g/kg Milk | g/kg $BW^{0.75}$/d | g/d | g/kg DMI | g/kg Milk | g/kg $BW^{0.75}$/d |
| C4:0 | + | + | | | | | | |
| C6:0 | + | | + | + | + | + | + | + |
| C8:0 | + | + | + | + | + | + | + | + |
| C10:0 | + | + | | | + | | + | + |
| C11:0 | + | | + | + | | | | |
| C13:0 | + | | + | + | + | | | |
| *Iso* C14:0 | | | | | + | | | + |
| *Anteiso* C15:0 | + | + | + | + | | | | |
| *Iso* C15:0 | + | + | + | + | + | | | + |
| C15:0 | + | + | + | + | + | | + | + |
| *Trans*- 9 C16:1 | | | | | − | − | − | − |
| *Cis*-9 C17:1 | | − | − | | | − | − | |
| C17:0 | | | + | | + | | + | |
| *Cis*-11 C18:1 | − | − | − | − | − | | − | − |
| *Cis*-13 C18:1 | − | − | − | − | − | − | − | − |
| *Trans*- 6 + 7 + 8 C18:1 | − | − | − | − | − | | | − |
| *Trans*-9 C18:1 | − | | − | − | − | − | | |
| *Trans*-10 C18:1 | − | | | − | − | | | − |
| *Trans*-11 C18:1 | | − | | | − | | | |
| *Trans*-12 C18:1 | − | − | − | − | − | − | − | − |
| *Trans*-13 + 14 C18:1 | − | − | − | − | − | − | − | − |
| C20:0 | | | | | + | | | + |
| *n-6* C20:2 | | | | | + | + | + | + |
| *n-6* C20:3 | | + | + | | + | + | | + |
| *n-6* C20:4 | | + | + | | + | + | | + |
| *n-3* C20:5 (EPA) | + | + | + | + | + | + | | + |
| *n-3* C22:5 | | + | | | + | + | | + |
| FA < C16 | + | | + | + | + | | | + |
| FA > C16 | − | − | | | | | | − |
| Total OBCFA | + | + | + | + | + | | + | + |
| Total SFA | + | | + | | + | + | + | + |
| Total MUFA | − | | − | − | − | − | − | − |
| Total PUFA | − | − | | | | | | |
| Total *cis* C18:1 | − | | | | | | | |
| Total *trans* MUFA | − | − | − | − | − | | | − |
| Total *trans* C18:1 | | | − | − | | | | − |
| Total *n-6* PUFA | + | | + | + | + | + | | + |

[a] Relative changes of each variable (changes proportional to its corresponding control)

Whether or not MFA associate with $CH_4$ emissions partially depends on the functional units used to express these emissions. Absolute daily $CH_4$ and $CH_4$ per kg $BW^{0.75}$/d hardly correlated with individual milk PUFA, whereas $CH_4$ per kg DMI or per kg milk were positively related with these MFA. Conversely, relative changes in daily $CH_4$ emissions and $CH_4$ in g/kg $BW^{0.75}$/d were positively correlated with relative changes in milk PUFA, while relative changes in $CH_4$ per kg milk did not correlate with these MFA. Furthermore, in most cases relative changes in daily $CH_4$ emissions and g $CH_4$/kg $BW^{0.75}$/d were positively associated with relative changes in the OBCFA C13:0, *iso*-C14:0, *iso*-C15:0 and C15:0, and negatively associated with relative changes in *trans*-6 to 11 isomers of C18:1. However, these two clusters of MFA barely associated with relative changes of $CH_4$ expressed per kg of DMI or per kg of milk.

In most cases, MFA associated with absolute $CH_4$ are also associated to relative changes in $CH_4$, with some exceptions (e.g. C4:0, C11:0 and *anteiso* C15:0) being related to absolute $CH_4$ but not to relative changes in $CH_4$. On the other hand, some MFA appeared to better reflect changes in $CH_4$ as compared with a control situation: *iso* C14:0, *cis*-11 C16:1, *trans*-9 C16:1 and *trans*-11 C18:1; two long chain MFA C20:0 and *n-6* C20:2, had a relationship with relative changes in $CH_4$ for most units of expression, but had no relationship with absolute $CH_4$.

Groups of MFA (reported as 'total' in Table A.3) only explain a low to modest amount of the variation of the absolute $CH_4$ emissions ($R^2$ between 0.06 and 0.66). Total OBCFA had the highest positive associations with $CH_4$ across the four functional units, whereas total MUFA had the highest negative association with absolute daily emissions and emissions per kg of milk and metabolic bodyweight. The highest negative association with absolute g $CH_4$/kg DMI was observed with total PUFA. It does not appear that groups of MFA are better

predictors of absolute emissions than individual MFA. This was confirmed for relative changes in $CH_4$ emissions, with $R^2$ ranging from 0.05 to 0.62. Relative changes in total OBCFA and total MUFA in most cases had the highest correlations. Moreover, relative changes in most groups of MFA did not associate with relative changes in $CH_4$ expressed per unit of milk or DMI.

The determination coefficients ($R^2$) of regressions relating changes in $CH_4$ and MFA or relating absolute $CH_4$ emissions to MFA proportions were similar. This indicates that MFA equally perform in predicting changes in $CH_4$ as compared with a standard situation (*e.g.* a diet with an additive vs. diet without an additive), as to predict absolute amounts of $CH_4$ emitted. Milk FA explained 7 to 80% of the variation in absolute daily $CH_4$ emissions; between 7 and 76% of the variation in g $CH_4$/kg DMI; between 6 and 58% of the variation in g $CH_4$/kg milk; and between 12 to 83% of the variation in g $CH_4$/kg $BW^{0.75}$/d, whereas the variation explained by relative changes in MFA ranged between 20 and 80% for relative changes in g $CH_4$/d; between 14 and 79% for relative changes in g $CH_4$/kg DMI; between 5 and 60% for relative changes in g $CH_4$/kg milk; and between 2 and 91% for relative changes in g $CH_4$/kg $BW^{0.75}$/d. The proportion of the variance explained by individual MFA was consistently lower for $CH_4$ in g/kg milk.

In the analysis of absolute means of $CH_4$ emissions, C11:0 and *n-3* C20:5 consistently had the highest $R^2$ for $CH_4$ in g/d, g/kg milk and g/kg $BW^{0.75}$/d, with C13:0 also having high correlation with $CH_4$ in g/kg $BW^{0.75}$/d. C4:0 and *cis*-9 C17:1 had the highest correlation with $CH_4$ in g/kg DMI.

In the analysis of relative changes of $CH_4$ emissions, *n-6* C20:2 was the individual MFA with the highest correlation with $CH_4$ expressed in g/d, in g/kg DMI and in g/kg $BW^{0.75}$/d. *Cis*-9

C17:1 had the highest correlation with $CH_4$ in g/kg milk and also correlated well with $CH_4$ in g/kg DMI.

Furthermore, regression coefficients in the equations correlating relative changes in $CH_4$ emissions to MFA (Table A.4) could give some indication of the sensitivity of MFA to identify variations in $CH_4$ emissions, with *n-6* C20:2, *iso* C15:0, C15:0 and *cis*-9 C17:1 being most responsive. It has to be noted that the intercept (representing the average difference between the control and treatments) never differed from zero.

It is important to note that the majority of the $R^2$ across all MFA in the univariate analysis range from 0.1 to 0.6, which indicates that MFA could explain some variation in $CH_4$ emissions, but relying on a single MFA to predict $CH_4$ would not be appropriate. Furthermore, with the exception of C4:0 and *cis*-9 C17:1, MFA showing high correlations were reported in only 5 to 7 out of the 15 studies included in the database and their average concentration in milk is around 0.1 g/100 g MFA (Table A.2). Hence, the effective potential of these MFA as biomarkers for $CH_4$ emission should be investigated (and confirmed) further. Ideally, a good predictor should be measurable through routine analysis, which is not possible for several minor MFA showing high correlations with $CH_4$.

*3.2 Multivariate regression*

A multiple linear regression analysis was performed to describe $CH_4$ in g/d, g/kg DMI, g/kg milk and g/kg $BW^{0.75}$/d from MFA proportions. The parameters selected for each equation were each significant at $P < 0.05$. The standard error of each regression coefficient is presented between brackets. Adjusted $R^2$ and the root mean square error (RMSE), both in absolute values and expressed relative to the average $CH_4$ emission, are reported to allow evaluation of the model's performance:

$CH_4$ (g/d) = – 36.4 $_{(\pm 8.11)}$ × C12:0 + 143.2 $_{(\pm 33.45)}$ × *cis*-9 C16:1

– 199.4 $_{(\pm 63.86)}$ × *trans*-12 C18:1 + 532.2 $_{(\pm 118.43)}$ × *n-6* C20:2 + 226.1 $_{(\pm 75.30)}$ (16)

Adj $R^2$ = 0.76 and RMSE = 76.4 g/d (27.9%).

$CH_4$ (g/kg DMI) = 14.9 $_{(\pm 2.15)}$ × C15:0 + 0.24 $_{(\pm 0.084)}$ × C16:0 – 4.02 $_{(\pm 2.273)}$ (17)

Adj $R^2$ = 0.87 and RMSE = 2.16 g/kg DMI (14.1%).

$CH_4$ (g/kg milk) = 23.5 $_{(\pm 3.77)}$ × C17:0 – 1.52 $_{(\pm 0.356)}$ × *trans*-13+14 C18:1 + 2.95 $_{(\pm 1.858)}$ (18)

Adj $R^2$ = 0.92 and RMSE = 1.75 g/kg milk (14.5%).

$CH_4$ (g/kg $BW^{0.75}$/d) = 1.22 $_{(\pm 0.356)}$ × C15:0 + 0.66 $_{(\pm 0.149)}$ × *cis*-9 C16:1

– 0.28 $_{(\pm 0.067)}$ × *trans*-13+14 C18:1 + 4.21 $_{(\pm 0.451)}$ × *n*-6 C20:2

+ 0.09 $_{(\pm 0.396)}$ (19)

Adj $R^2$ = 0.94 and RMSE = 0.27 g/kg $BW^{0.75}$/d (12.0%).

Thirty-five observations were used for the models. Individual FA are in g/100g FA. Experiment effects (included as dummy factor) are not shown.

## DISCUSSION

Most of the MFA reported in previous models to predict $CH_4$ [*e.g.* Chilliard et al., 2009 (g $CH_4$/d); Dijkstra et al., 2010 (g $CH_4$/kg DMI), Mohammed et al., 2011 (g $CH_4$/d)] do not coincide across the different equations, and based on the findings of this study some of these differences could be due to differences in the units in which $CH_4$ is expressed.

In general, the results of this study suggest that saturated fatty acids are usually positively related to $CH_4$. The same is valid for OBCFA and long-chained PUFA, while MUFA are negatively related to $CH_4$.

Fatty acids < Cl6 are *de novo* synthesized in the mammary gland from acetate and 3-hydroxy-butyrate (Bernard, Leroux & Chilliard, 2008). Usually a lower production of $CH_4$ is associated with a lower production of acetate in the rumen (Moss, Jouany & Newbold, 2000). Accordingly, lower $CH_4$ emissions would be expected to be accompanied by lower

concentrations of fatty acids with 16 C or less. Similarly, Mohammed et al. (2011) found a positive correlation ($r$ = 0.76) between *de novo* FA and $CH_4$ (g/d). In our study C6:0, C8:0 and C10:0 consistently were positively related to $CH_4$. Particularly, C8:0 was also found to have a positive relationship with $CH_4$ by Mohammed et al. (2012), Chilliard et al. (2010) and Dijkstra et al. (2011). The latter two also found that C6:0 and C10:0 positively related to $CH_4$, which was confirmed when including more experiments in the current study.

Total SFA was positively associated with $CH_4$ agreeing with the findings of Mohammed et al. (2012). Similarly, Weill, Chesneaue, Chilliard, Doreau & Martin (2009) proposed total FA < C16 as predictor of $CH_4$ (g/kg milk), which agrees with our findings where total FA < C16 had a positive relationship with $CH_4$ expressed in g/d, g/kg milk and g/kg $BW^{0.75}$/d. However, even though *de novo* synthesized FA might give an indication of changes in $CH_4$ output, it would not be recommendable to rely only on total FA < C16 to predict $CH_4$, as the $R^2$ of the relations with $CH_4$ were rather low ($R^2$ between 0.07 and 0.48).

Another group of MFA proposed to have potential to predict $CH_4$ emissions are the milk OBCFA (Vlaeminck and Fievez, 2005), some of which were related to $CH_4$ in this study. *Anteiso* C15:0 and *iso* C15:0 were positively associated with $CH_4$ production, which agrees with the assumption that higher numbers of cellulolytic bacteria, enriched in those branched-chain fatty acids (Fievez et al., 2012) are linked with higher $CH_4$ emissions. However, C15:0 is usually associated with amylolytic bacteria and is expected to be negatively related with $CH_4$. The univariate analysis of Dijkstra et al. (2011) found a positive relationship between $CH_4$ and *iso* C15:0 and C15:0, but not with *anteiso* C15:0. Furthermore, Chilliard et al. (2010) found that C15:0 had a positive correlation with $CH_4$ ($r$ = 0.74), whereas Mohammed et al. (2012) found a tendency to a negative correlation ($r$ = –0.3). Neither of them found a relationship between $CH_4$ and *iso* C15:0, whereas the multivariate analysis of Mohammed et al. (2012) selected *anteiso* C15:0 as one of the positive predictors of $CH_4$.

It might be worthwhile to mention that *cis*-9 C17:1, a product of desaturation of C17:0 by the mammary desaturase system (Fievez et al., 2003) was negatively related with $CH_4$ (g/kg DMI and g/kg milk) in our study. This is in agreement with Mohammed et al. (2012), who found that *cis*-9 C17:1 was the major contributor to predict $CH_4$ in the two models they developed relying on MFA and intake variables, and including or not rumen variables. Dijkstra et al. (2011) also found a strong negative relationship between this *cis*-9 C17:1 and $CH_4$.

The feeding of diets rich in unsaturated FA has been accepted as an effective strategy to decrease $CH_4$ emissions (e.g. Patra, 2012), with an associated increase in biohydrogenation intermediates. Thus, in such cases a decrease in $CH_4$ production would be associated with an increase in unsaturated fatty acids in milk. This assumption is supported by our findings, where several individual UFA were found to have a negative relationship with $CH_4$.

Similar to our results, in the (single experiment) papers of Chilliard et al. (2010) and Mohammed et al. (2012), $CH_4$ emissions were negatively associated to UFA. Chilliard et al. (2010) found that the most negative correlations ($r$ = –0.72 to –0.90) with $CH_4$ output were observed for several *cis* and *trans* isomers of C18:1 and C18:2. Likewise, Mohammed et al. (2012) found significant negative correlations between $CH_4$ output and a number of MUFA, including *cis*-9 C17:1, *cis*-11 C18:1, *cis*-13 C18:1, *trans*-6-7-8 C18:1 and isomers of C18:2. All of them, except the isomers of C18:2, were identified as being related to $CH_4$ in our study. One could assume that these associations are related more to the supplementation with dietary UFA sources rather than to a robust prediction of $CH_4$, since both Chilliard et al. (2010) and Mohammed et al. (2012) fed the animals with sources of UFA. However, in the current study 12 out of 31 dietary treatments (excluding control diets) included sources of UFA, whereas a number of C16:1, C17:1 and C18:1 isomers were still negatively related to $CH_4$ emissions. Moreover, Dijkstra et al. (2011), whose analysis considered 50 observations with a minority originating from UFA supplemented cows, also found that milk content of UFA, like *cis*-9

C18:1, *cis*-11 C18:1, *cis*-12 C18:1, *trans*-10 + 11 C18:1, *cis*-14 + *trans*-16 C18:1 and *trans*-11, *cis*-15 C18:2, were negatively associated with $CH_4$ production.

Finally, several long chain PUFA had a positive association with $CH_4$. Previous studies relating MFA and $CH_4$ found no or few correlations between $CH_4$ and individual long chain PUFA. Chilliard et al. (2009) found a positive correlation ($r$ = 0.83) between *n-6* C20:4 and $CH_4$, similar to our findings. Mohammed et al. (2012) found a negative correlation ($r$ = –0.36) of total *n-6* PUFA with $CH_4$, in contrast to our results. These MFA are only present in trace amounts in milk fat and their relevance in connection with enteric $CH_4$ is unknown. However, it should be noted that only in few cases (15 to maximum 21 out of 48 observations) these long chain PUFA were reported. Although MFA only were considered when reported in at least five studies and being represented in each of the three treatment categories, the influence of a single experiment obviously becomes more important when the data set gets smaller. Hence further investigation is required to assess whether the positive correlation between these long chain PUFA and $CH_4$ is confirmed.

The models obtained after the multivariate analysis Eqs. (3) to (6) are able to explain most of the variation in $CH_4$ emissions ($R^2$ between 0.76 and 0.94), the errors associated to their predictions, especially for Eq. (3), are relatively high. Variables selected to predict $CH_4$ emissions by the multivariate analysis of our data showed some coincidences across equations expressing $CH_4$ in different units, especially between the models predicting daily emissions Eq. (16) and $CH_4$ relative to metabolic bodyweight Eq. (19). Furthermore, coincidences also appeared between the models developed in this study and previous models (Chilliard et al., 2009; Dijkstra et al., 2011; Mohammed et al., 2011). *Cis*-9 C16:1 Eq. (16) and (19) had no relationship with $CH_4$ output in previous studies. As expected and confirmed based on MFA data of the current dataset, this desaturase product was highly correlated with *cis*-9 C14:1 and *cis*-9 C17:1 ($r$ = 0.62 and 0.52, respectively). These two desaturase products were retained in

previous models (Chilliard et al., 2009; Mohammed et al., 2011). *Trans*-12 C18:1 Eq. (16) and *trans*-13 + 14 C18:1 Eq. (18) and Eq. (19) are products of the biohydrogenation of PUFA. In our dataset *trans*-12 C18:1 and *trans*-13 + 14 C18:1 were strongly correlated ($r > 0.75$) with other *trans* isomers of C18:1 and with total *trans* C18:1, variables appearing in other models (Mohammed et al., 2011). C16:0 Eq. (17) is related to *de novo* synthesized FA and appears in the equation of Chilliard et al. (2009). Other MFA like C12:0 Eq. (16), C15:0 Eq. (17) and C17:0 Eq. (18) were correlated with $CH_4$ in previous studies without being retained in their final models. However, none of the previous studies identified relationships between $CH_4$ and *n-6* C20:2.

Even though the results of this literature study offer a broader view of the relationships between MFA and $CH_4$ emissions, it has to be noted that the nature of the data was not ideal for the multivariate analysis. The data set had a relatively limited number of observations (35) for a comparably large number of independent variables (53). Carrascal et al. (2009) stated that if a sample size is not large enough compared to the number of predictor variables, the ability of regression analysis to find a significant effect is reduced. Furthermore, within our data set a significant proportion of the dietary strategies or mitigation additives were fat sources (21 out of 48 treatments). It is known that fat supplementation is a major factor influencing changes in the MFA profile (see e.g. Chilliard et al. 2000). Moreover, not all the studies included reported the same amount of individual MFA and usually studies with dietary fat as additives tended to look deeper into the MFA profile, which causes a certain leverage in favor of the tendencies observed in such studies.

## CONCLUSIONS

Previous studies already explored the relationships between MFA and $CH_4$ emissions. This is the first paper quantitatively gathering these literature data. The functional unit to express

$CH_4$ might play an important role in discrepancies between models reported in literature. Moreover, MFA seem suitable to identify both changes in $CH_4$ compared with a standard situation and to predict an absolute amount of $CH_4$ emitted. In general, in this study SFA and PUFA are positively related to $CH_4$ emissions, whereas MUFA are negatively related. It has to be noted that this relationships might have been influenced by the nature of the data set, where many of the studies included dietary treatments of sources of UFA that modified the MFA profile in milk, in general causing an increase in the proportions of milk UFA and a concomitant decrease in the proportions of milk SFA and long chain-PUFA. Knowing that differences and variation in a dataset are key for the generalization capacity of a model, this literature review and both the univariate and the multivariate analyses performed have contributed to explore the potential of MFA to predict $CH_4$.

# CHAPTER 4. DEVELOPING MODELS TO PREDICT METHANE EMISSIONS FROM MILK FATTY ACIDS

## ABSTRACT

Milk fatty acid (MFA) profiles have been previously used to develop models to predict methane ($CH_4$) emissions in dairy cows. However, small data sets and low variability in diets and/or experiments used to develop the models have affected the ability of these models to predict $CH_4$ under other more general. In this study, a data set containing 145 observations from 9 experiments with lactating Holstein cows was used to develop models to predict $CH_4$ emissions expressed in four functional units (g/d, g.kg DMI, g/kg milk and g/kg $BW^{0.75}$/d) and to explore the ability of MFA profiles to differentiate between high and low $CH_4$-emitter animals. A generalized linear mixed model was fitted to the data and marginal ($R^2_{(m)}$, variation explained by fixed effects) and conditional ($R^2_{(m)}$, variation explained by fixed and random effects combined) were calculated to evaluate the models. In general MFA had a modest potential to explain variations in $CH_4$, based on $R^2_{(m)}$ ranging between 0.19 and 0.55. Milk FA explained higher variation in $CH_4$ when this was expressed relative to DMI. Standardized coefficients across the equations showed that C17:0 and its desaturase product *cis*-9 C17:1 is a highly relevant MFA in the prediction of $CH_4$ emissions. Furthermore, a novel approach adopted from recent studies on microbial community organization was used to represent the cumulative distribution of MFA and its relationship with $CH_4$ emissions. Gini coefficients were calculated for different subsets of the data set (based on the quantity of each individual MFA) and for three categories depending on the amount of $CH_4$ produced (HIGH, MEDIUM and LOW $CH_4$ producers). Only Gini coefficients of the subset of MFA including MFA with concentrations lower than 0.625 g/100 g MFA showed relationship with $CH_4$ emissions. Furthremore, Gini coefficients of MFA belonging to the category HIGH, differed from those of categories MEDIUM and LOW. However, the differences observed in Gini

coefficients between groups and categories were minimal and might not aid to the identification of low or high emitters.

## INTRODUCTION

The potential of MFA as predictors of $CH_4$ emissions has been already highlighted in previous studies (e.g. Mohammed et al., 2011). However, the models published so far to predict $CH_4$ from MFA proportions have been developed from a rather limited number of observations or from experiments including treatments directly influencing the MFA profile regardless of their effects in $CH_4$ emissions. Hence, there is still the need to develop a model from a larger data set obtained under more general conditions.

Furthermore, MFA have been associated to $CH_4$ emissions by traditional correlation and/or linear regression analysis. However, it is not only of interest to predict an actual amount of $CH_4$ produced by a cow, but also, in the frame of genetic selection for animals with a natural tendency to emit high or low quantities of $CH_4$, the profile of MFA and possible changes occurring in it might aid to the identification of these animals.

In that regard, the objectives of this study were to develop $CH_4$ prediction models, for $CH_4$ expressed as daily emissions and relative to dry matter intake (DMI), milk yield and metabolic bodyweight ($BW^{0.75}$/d) from a larger number of observations and experiments, as well as identification of the most important MFA related to $CH_4$. Moreover, a novel approach to relate MFA profile to changes in $CH_4$ was also tested aiming at differentiating high from low $CH_4$-producing animals, based on the cumulative proportions of MFA in samples across our dataset.

## MATERIALS AND METHODS

### Data development

Data from 9 experiments, with a total of 145 observations from 41 Holstein-Friesian lactating dairy cows were used (Table 4.1). Eight of the experiments were completed at the Institute for Agricultural and Fisheries Research (ILVO, Belgium). The experimental procedures with animals were approved by the ethical commission of ILVO (EC 2011, 154). The set-up of these experiments was similar to that described for the *in vivo* experiments in Chapter 1B. Briefly, cows adapted to an experimental diet during 4 weeks. From two weeks prior to the first $CH_4$ measurements onwards dry matter intake (DMI) was restricted to 95% of the *ad libitum* intake. The trial lasted six weeks, and enteric methane was measured in weeks 0, 2, 4 and 6 by keeping the animals in individual open circuit chambers (De Campeneere and Peiren, 2012). Cows were fed, cleaned and milked twice daily (0700h and 1700h).

A ninth experiment was conducted at the ruminant metabolism research unit of Trawsgoed IBERS Aberystwyth University farm in Trawscoed (United Kingdom). All procedures used in this experiment were licensed and regulated by the United Kingdom Home Office under the Animals (Scientific Procedures) Act of 1986. The experiment was a complete 3 x 3 Latin Square with two replicate blocks of 3 cows, with 32 day periods starting 7 days apart. Enteric $CH_4$ measurements were done in a larger version of the small ruminant respiration chambers at Aberystwyth University described in detail by Hart et al. (2012). A total mixed ration (TMR) based diet was offered for *ad libitum* intake once a day (0800h), with refusals removed just before feeding.

Table 4.1. Overview of experiments included in the data set

| Exp # | n | Basal diet (proportion in parenthesis, g/kg DM) | Dietary treatment | CH4 (g/d) | | | Period |
|---|---|---|---|---|---|---|---|
| | | | | Avg. | Min | Max | |
| 1 | 12 | Grass silage (460), Maize silage (370), Concentrate (120), bypass soybean meal (50) | Essential oils | 259 | 198 | 319 | Oct-Dec/2011 |
| 2 | 12 | Grass silage (460), Maize silage (370), Concentrate (120), bypass soybean meal (50) | Organosul-phurous compound | 268 | 245 | 285 | Oct-Dec/2011 |
| 3 | 12 | Grass silage (200), Maize silage (490), Concentrate (130), bypass soybean meal (50), soybean meal + rapeseed cake (50), sugar beet pulp (80) | Essential oils | 323 | 281 | 360 | Jan-Mar/2012 |
| 4 | 12 | Grass silage (390), Maize silage (310), Concentrate (290), bypass soybean meal (10) | Organosul-phurous compound | 340 | 287 | 418 | Jan-Mar/2012 |
| 5 | 16 | Grass silage (380), Maize silage (320), Concentrate (210), bypass soybean meal (50), soybean meal + rapeseed cake (40) | None | 372 | 329 | 416 | Mar-May/2012 |
| 6 | 16 | Grass silage (310), Maize silage (460), Concentrate (170), bypass soybean meal (20), soybean meal + rapeseed cake (40) | Saponins | 381 | 299 | 504 | Oct-Dec/2012 |
| 7 | 16 | Grass silage (290), Maize silage (430), Concentrate (200), bypass soybean meal (40), soybean meal + rapeseed cake (40) | Essential oils | 330 | 263 | 392 | Oct-Dec/2012 |
| 8 | 31 | Grass silage (230), Maize silage (230), concentrate (300), soybean meal + rapeseed cake (240) | Medium chain fatty acids | 440 | 345 | 547 | Feb-Abr/2013 |
| 9 | 18 | Grass silage (300), Maize silage (300), Concentrate (400) | Nitrate and linseed | 395 | 289 | 487 | Aug-Dec/2012 |

For all experiments, water was available *ad libitum* throughout the entire experiment. Dry matter intakes and milk production (a.m. and p.m.) were recorded daily throughout the duration of the experiments, however only those recordings during the presence of the cows in

the open circuit chambers were considered for this study (3 days). A milk sample was obtained at each milking, evening and morning samples were pooled and weighted by production to one sample for analyses and were stored at –20 °C until being analyzed for FA composition. These analyses were done at the facilities of the Laboratory of Animal Nutrition and Animal Product Quality (LANUPRO, Belgium) as described by Colman et al. (2013).

Milk FA were quantified by gas chromatography (GC) after extraction (Chouinard et al., 1997) and methylation (Stefanov et al., 2010) and were expressed as grams per 100 g of MFA methyl esters. Tridecanoic acid (C13:0, as triacylglyceride; Sigma, Bornem, Belgium) was added as internal standard to assess the accuracy of the chromatograms.

Analysis of the FA was done by a GC (HP 7890A; Agilent Technologies Belgium NV, Diegem, Belgium) equipped with a 75-m SP-2560 capillary column (i.d.: 0.18 mm; film thickness: 0.14 μm; Supelco Analytical, Bellefonte, PA) and a flame ionization detector. A combination of 2 oven temperature programs was used in this study to achieve separation of most *cis* and *trans* C16:1 and C18:1 *iso*mers according to the method of Kramer et al. (2008) with modifications (Stefanov et al., 2010). A first temperature program was as follows: at the time of sample injection, the column temperature was 70 °C for 2 min, which was then increased at 15 °C/min to 150°C, followed by a second increase of 1 °C/min to 165 °C, which was maintained for 12 min, followed by a third increase at 2 °C/min to 170 °C, which was maintained for 5 min, and a final increase at 5 °C/min to 215 °C, which was maintained for 10 min. A second temperature program was used to separate most of the coeluting isomers: at the time of sample injection, the column temperature was 70 °C, which was then increased at 50 °C/min to 175 °C and maintained isothermal for 13 min, followed by a second increase at 5 °C/min to 215 °C, which was maintained for 10 min. For both programs, inlet and detector temperatures were 250 and 255 °C, respectively. The split ratio was 100:1. The flow rate for the hydrogen carrier gas was 1 mL/min. Most FA peaks were identified using quantitative

mixtures of methyl ester standards (BR2 and BR3, Larodan Fine Chemicals AB, Malmo, Sweden; Supelco 37, Supelco Analytical; PUFA-3, Matreya LLC, Pleasant Gap, PA). Fatty acids for which no standards were available commercially were identified by order of elution according to Precht et al. (2001) and Kramer et al. (2008).

## Statistical Analysis

Summary statistics of the variables included in the analysis are presented in appendix (Table A.5). Milk FA ≤ 0.02 g/100 g fat (*trans*-5 C18:1, *trans*-10, *cis*-12 CLA, C22:0, *n-6* C22:4 and C24:0) were not included in the statistical analyses. Furthermore, one peak was find to be the result of coelution of *trans*-14 C18:1 and *cis*-9 C18:1, however it is known that the concentration of *trans*-14 C18:1 is minimal compared with the amount of *cis*-9 C18:1 and therefore, we refer to this peak only as *cis*-9 C18:1.

### Multiple Linear Regression

Regression analysis was done using the GLIMMIX procedure of SAS. Daily methane emissions (g/d) and emissions relative to DMI (g/kg, DMI), milk production (g/kg milk) and metabolic body weight (g/kg $BW^{0.75}$/d) were used as the dependent variable. Individual MFA were used as independent variables and were included in the model as fixed effects. Experiment and cow were included as random effects. A forward variable selection was used having the F statistic (the higher the better), the *P* value ($P < 0.1$) and the standardized regression coefficients (absolute higher value) as selection criteria. Only those variables with *P* value < 0.1 were retained in the final model. The first variable included in the model was that with the highest F statistic from the univariate analysis (PROC GLIMMIX). Variables highly correlated ($r > 0.6$) with a variable which was already retained in the model were excluded as the risk for colinearity increases. Pearson correlations among all variables were determined using the PROC CORR procedure of SAS. Conditional ($R^2_{(c)}$) and marginal ($R^2_{(m)}$) coefficients of determination were calculated according to Nakagawa and Schielzeth

(2013) using the function *lmer* in the R package lme4 (version 0.999375-42; Bates et al., 2011).

**Cumulative abundance of MFA**

The Lorenz curve and the Gini coefficient (Morgan, 1962) were used to estimate the cumulative abundance of MFA acids. Knowing that milk fat is mainly dominated by a small number of MFA and a larger number of MFA have a relative low concentration, Gini coefficients were calculated for the total set of MFA (**Group 100**) and for subsets of MFA according to their mean cumulative proportions in milk fat across the complete data set (Figure 4.1).

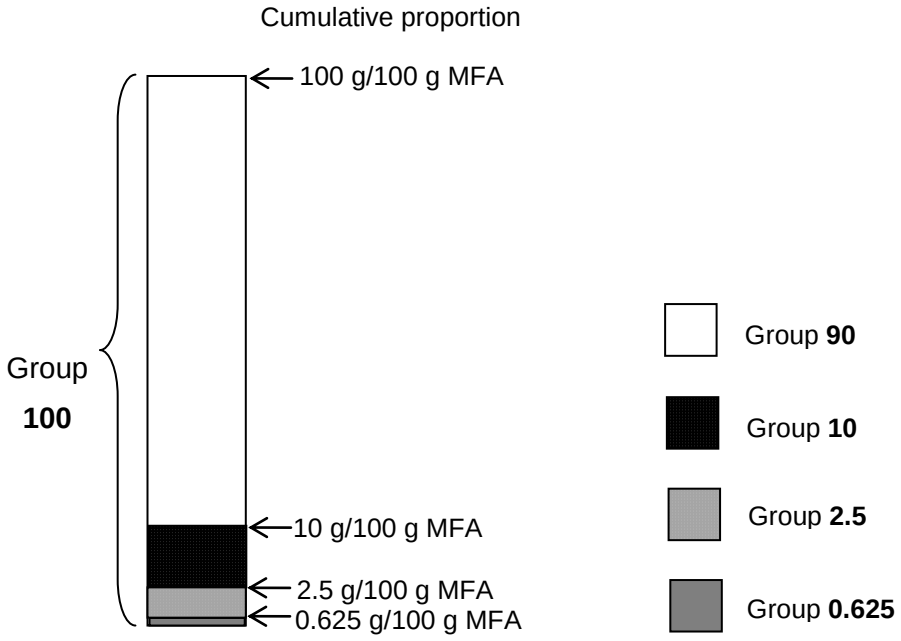


Figure 4.1. Partitioning of MFA by their cumulative proportions

To define these subsets, MFA were organized from large to small according to their proportion in milk fat. Starting from the less abundant to the most abundant, the first group included the less abundant MFA by calculating the cumulative proportion of MFA until summing up 0.625 g/100 g MFA (*trans*-9 C16:1, *trans*-10 C16:1, *iso* C18:0, *n-3* C20:5, *trans*-11 + 12 C16:1, C11:0, *n-6* C22:5, *n-3* C22:6, *cis*-11 C16:1, *cis*-9 C20:1, *trans*-14 C16:1, *n-6*

C20:2, C9:0, C5:0, C7:0; **Group 0.625)**. The next gorup included MFA with cumulative concentrations between 0.625 and 2.5 g/100 g MFA (*iso* C15:0, *cis*-7 C16:1, *iso* C16:0, *cis*-13 C16:1, *cis*-9 C17:1, *trans*-9 C18:1, *n-3* C20:3, *trans*-11, *cis*-15 C18:2, *trans*-6 + 7 + 8 C16:1, *n-6* C18:3 + C20:0, *iso* C14:0, C12:1, *n-3* C22:5, *n-6* C20:3, *cis*-13 C18:1; **Group 2.5).** A third group included MFA with a cumulative proportion between 2.5 g/100 g MFA and 10 g/100 g MFA (C15:0,*trans*-11 C18:1, C17:0, *trans*-13 C18:1, *cis*-9, *trans*-11 CLA, *cis*-14 + *trans*-16 C18:1, *anteiso* C17:0, *cis*-11 C18:1, *n-3* C18:3, a*nteiso* C15:0, *Trans*-10 C18:1, C10:1, *trans*-12 C18:1, *iso* C17:0, *trans*-15 C18:1, *cis*-12 C18:1, *n-6* C20:4, *trans*-6 + 7 + 8 C18:1, *cis*-15 C18:1, **Group 10**); and a final group included the most abundant MFA with cumulative proportions between 10 and 100 g/100 g MFA (C16:0, *cis*-9 C18:1, C14:0, C18:0, C4:0, C12:0, C10:0, C6:0, *cis*-9 C16:1, *n-6* C18:2, C8:0, C14:1; **Group 90**). The Gini coefficient is defined as a ratio of the areas on the Lorenz curve diagram, and was calculated according to the formula of Gastwirth (1972):

$$G = \frac{1}{\mu} - \int_0^{y^*} (1 - F(y))^2 \, dy$$

Where

F(y) is the cumulative probability distribution of MFA abundance, μ is its mean, assumed finite, and y* is its upper limit, which may be infinite.

Methane emissions (g/d, g/kg DMI, g/kg milk and g/kg $BW^{0.75}$/d) were regressed on each group of the Gini coefficient using the GLIMMIX procedure of SAS including the Gini coefficient as fixed variable and cow and experiment as random variable.

Furthermore, observations were classified into three categories according to their daily $CH_4$ production (g/d) (Figure 4.2) and according to $CH_4$ in g/kg DMI: High $CH_4$ observations ($CH_4$ production within the 75% quantile, **HIGH**), middle producing observations ($CH_4$ production between the 25 and 75% quantile, **MEDIUM**) and low producing observations

($CH_4$ production within the 25% quantile, **LOW**). An analysis of variance (PROC MIXED, SAS) was performed using categories as an independent variable (fixed effect) and Gini coefficients as dependent variable. Experiment and cow were included as random variables. This analysis was done separately for each of the five MFA groups from which Gini coefficients were calculated.

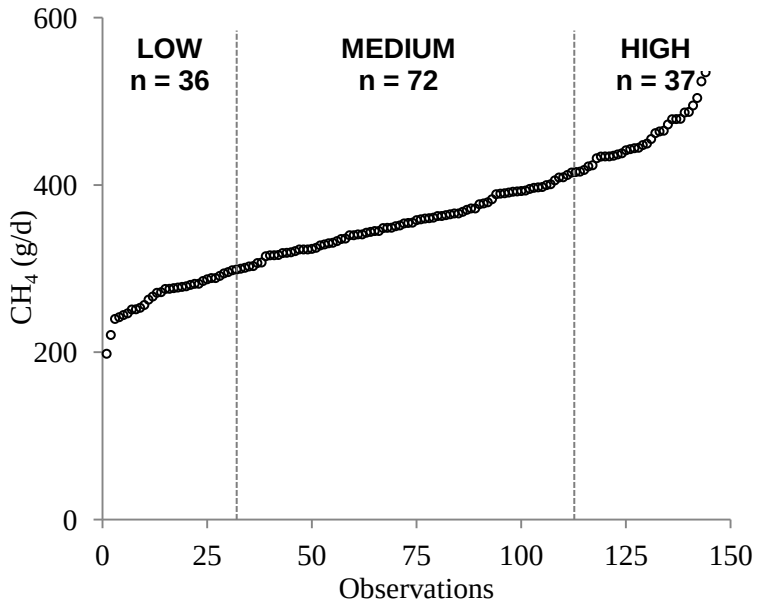


Figure 4.2. Categories of LOW, MEDIUM and HIGH daily $CH_4$ (g/d) producing observations

## RESULTS

### Relationships of MFA with methane emissions

Correlations between $CH_4$ emissions and individual MFA were modest with absolute values ranging from 0.1 to 0.6. Highest positive and negative correlations between MFA and $CH_4$ are presented in Table 4.3. All the correlations between individual MFA and methane are presented in Appendix A.6

A multiple linear regression analysis was performed to describe $CH_4$ in g/d, g/kg DMI, g/kg milk and g/kg $BW^{0.75}$/d from MFA proportions. The parameters selected for each equation

were each significant at $P < 0.10$. The standard error of each regression coefficient is presented between brackets. Marginal ($R^2_{(m)}$) and conditional ($R^2_{(m)}$) determination coefficients are provided for each equation. Standardized regression coefficients are presented in Table 4.4.

$$CH_4 \text{ (g/d)} = -323.8_{(\pm 97.62)} \times \text{C17:0} + 267.7_{(\pm 107.82)} \times \textit{anteiso}\text{ C17:0} + 9.1_{(\pm 3.12)} \times \text{C18:0} - 36.6_{(\pm 21.15)} \times \textit{cis}\text{-11 C18:1} + 351.4_{(\pm 50.07)} \quad (20)$$

$$R^2_{(m)} = 0.33 \qquad R^2_{(c)} = 0.91$$

$$CH_4 \text{ (g/kg DMI)} = -0.63_{(\pm 0.196)} \times \text{C14:0} - 10.1_{(\pm 2.87)} \times \text{C17:0} - 16.2_{(\pm 4.58)} \times \textit{cis}\text{-9 C17:1} + 0.23_{(\pm 0.069)} \times \textit{cis}\text{-9 C18:1} + 30.2_{(\pm 0.2.55)} \quad (21)$$

$$R^2_{(m)} = 0.55 \qquad R^2_{(c)} = 0.80$$

$$CH_4 \text{ (g/kg milk)} = -3.41_{(\pm 1.193)} \times \text{C4:0} - 69.5_{(\pm 34.67)} \times \text{C7:0} - 1.20_{(\pm 0.338)} \times \text{C14:0} - 73.8_{(\pm 19.21)} \times \textit{trans}\text{-9 C16:1} + 28.4_{(\pm 8.93)} \times \textit{iso}\text{ C16:0} - 10.1_{(\pm 4.80)} \times \text{C17:0} - 32.2_{(\pm 7.40)} \times \textit{Cis}\text{-9 C17:1} + 0.42_{(\pm 0.116)}\ \textit{cis}\text{-9 C18:1} - 39.6_{(\pm 13.31)} \times \textit{n-6}\text{ C22:5} + 44.2_{(\pm 6.32)} \quad (22)$$

$$R^2_{(m)} = 0.38 \qquad R^2_{(c)} = 0.89$$

$$CH_4 \text{ (g/kg BW}^{0.75}\text{/d)} = 1.41_{(\pm 0.405)} \times \textit{trans}\text{-9 C18:1} - 1.91_{(\pm 0.648)} \times \textit{trans}\text{-12 C18:1} + 3.63_{(\pm 1.938)} \times \textit{n-3}\text{ C20:5} + 2.88 \quad (23)$$

$$R^2_{(m)} = 0.19 \qquad R^2_{(c)} = 0.80$$

One hundred forty-five observations were used for the models. Individual FA are in g/100g FA.

Table 4.3. Pearson correlations between methane emissions and proportions of individual milk fatty acids (g/100 g MFA)

| Positive | | Negative | |
|---|---|---|---|
| **$CH_4$ (g/d)** | | | |
| *Trans*-15 C18:1 | (*r* = 0.35) | *Cis*-9 C17:1 | (*r* = –0.60) |
| C12:1 | (*r* = 0.32) | *Trans*-12 C18:1 | (*r* = –0.52) |
| *n-3* C22:5 | (*r* = 0.30) | *Trans*-13 C18:1 | (*r* = –0.50) |
| **$CH_4$ (g/kg DMI)** | | | |
| *Trans*-14 C16:1 | (*r* = 0.51) | C17:0 | (*r* = –0.67) |
| *Cis*-15 C18:1 | (*r* = 0.46) | C14:0 | (*r* = –0.61) |
| *Cis*-9 C16:1 | (*r* = 0.36) | *n-6* C18:3 + C20:0 | (*r* = –0.60) |
| **$CH_4$ (g/kg milk)** | | | |
| *Trans*-14 C16:1 | (*r* = 0.45) | C6:0 | (*r* = –0.44) |
| *Trans*-10 C18:1 | (*r* = 0.33) | *n-6* C18:3 + C20:0 | (*r* = –0.42) |
| *Trans*-15 C18:1 | (*r* = 0.31) | C14:0 | (*r* = –0.39) |
| **$CH_4$ (g/kg $BW^{0.75}$/d)** | | | |
| *Trans*-15 C18:1 | (*r* = 0.34) | *Cis*-9 C17:1 | (*r* = –0.58) |
| *Cis*-15 C18:1 | (*r* = 0.27) | *Trans*-12 C18:1 | (*r* = –0.54) |
| C16:0 | (*r* = 0.26) | *Trans*-13 C18:1 | (*r* = –0.48) |

From the standardized coefficients, C17:0 was identified as the most influencing MFA for $CH_4$ in g/d and g/kg DMI, whereas *cis*-9 C17:1, a desaturase product of C17:0, was the most determining predictor for $CH_4$ in g/kg milk. A polyunsaturated MFA, *n-3* C20:5, was the most influencing predictor for $CH_4$ in g/kg $BW^{0.75}$/d.

Table 4.4. Standardized coefficients of the modeling of the effects of individual MFA (g/100 g MFA) on $CH_4$ emissions expressed in g/d, g/kg DMI, g/kg milk and g/kg $BW^{0.75}$/d

| $CH_4$ (g/d) | | $CH_4$ (g/kg DMI) | | $CH_4$ (g/kg milk) | | $CH_4$ (g/kg $BW^{0.75}$/d) | |
|---|---|---|---|---|---|---|---|
| C17:0 | –486 | C17:0 | –14.6 | *Cis*-9 C17:1 | –27.7 | *n-3* C20:5 | +3.63 |
| *Anteiso* C17:0 | +186 | *Cis*-9 C17:1 | –13.9 | C14:0 | –24.6 | *Trans*-12 C18:1 | –1.91 |
| C18:0 | +166 | C14:0 | –12.9 | *Trans*-9 C16:1 | –15.7 | *Trans*-9 C18:1 | +1.41 |
| *Cis*-11 C18:1 | –95.2 | *Cis*-9 C18:1 | +8.21 | *Cis*-9 C18:1 | +14.9 | | |
| | | | | *n-6* C22:5 | –14.8 | | |
| | | | | C17:0 | –14.5 | | |
| | | | | *Iso C16:0* | +12.0 | | |
| | | | | C4:0 | –10.9 | | |
| | | | | C7:0 | –7.42 | | |

**Cumulative distribution of MFA.**

Unevenness (higher Gini coefficients) in the distribution of MFA proportions was higher for both Group ALL and **Group 90** and changed towards a more balanced distribution (smaller Gini coefficients) in groups of less abundant MFA (Figure 4.3).

In general, correlations between Gini coefficients and $CH_4$ emissions were weak, with **Group 0.625** having somewhat stronger correlations with $CH_4$ relative to DMI, milk production and $BW^{0.75}$/d (Table 4.5). This was confirmed by regressing $CH_4$ emissions on Gini coefficients, which showed significant relationships between the group of less abundant MFA (**Group 0.625**) and $CH_4$ expressed relative to DMI, milk production and $BW^{0.75}$/d.

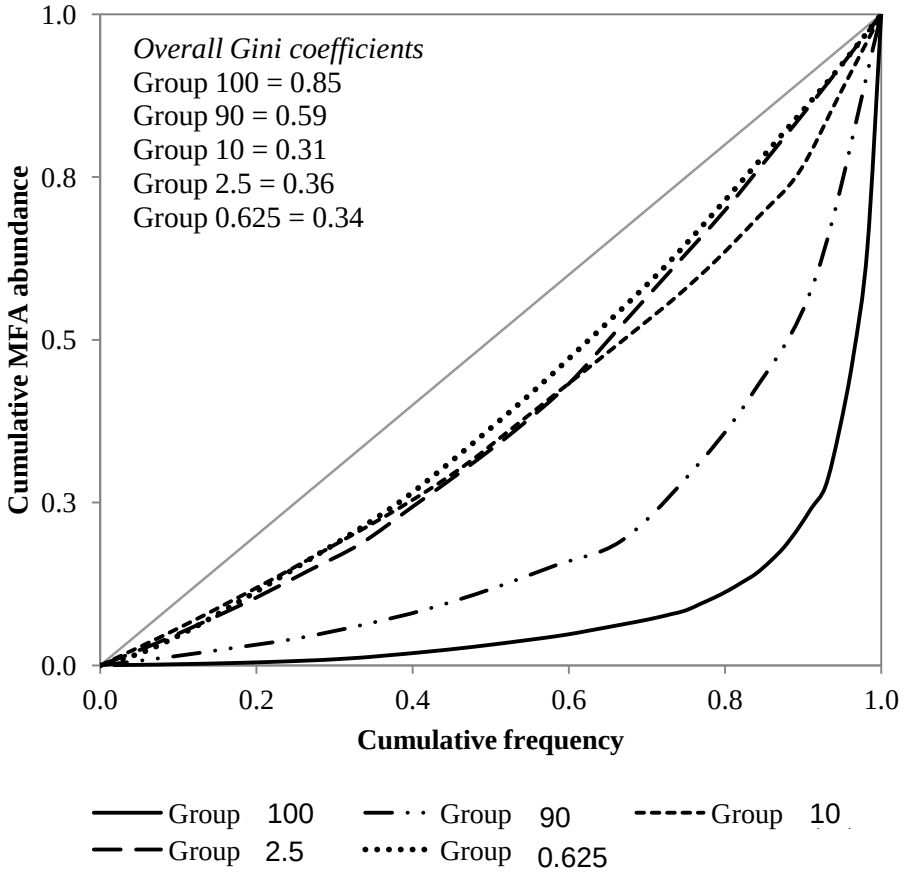


Figure 4.3. Gini coefficients and Lorenz curves for the total MFA and the subsets of MFA

Table 4.5. Correlation coefficients between Gini coefficients and $CH_4$ emissions and *P* values of the regression of methane emissions on Gini coefficients

| | CH4 | | | |
|---|---|---|---|---|
| | g/d | g/kg DMI | g/kg milk | g/kg $BW^{0.75}$/d |
| *Correlations* | | | | |
| Group 100 | 0.24 | 0.28 | 0.10 | 0.25 |
| Group 90 | 0.07 | 0.55 | 0.34 | 0.21 |
| Group 10 | 0.39 | 0.32 | 0.10 | 0.44 |
| Group 2.5 | 0.04 | 0.47 | 0.35 | 0.17 |
| Group 0.625 | 0.36 | 0.69 | 0.49 | 0.49 |
| *P values for the univariate analysis* | | | | |
| Group 100 | 0.40 | 0.89 | 0.39 | 0.43 |
| Group 90 | 0.32 | 0.74 | 0.85 | 0.45 |
| Group 10 | 0.37 | 0.73 | 0.91 | 0.21 |
| Group 2.5 | 0.40 | 0.58 | 0.60 | 0.22 |
| Group 0.625 | 0.13 | 0.06 | 0.02 | 0.08 |

Furthermore, Gini coefficients of MFA profiles belonging to observations in the category HIGH were different from those of categories MEDIUM and LOW for **Group 100**, **Group 10** and **Group 0.625** (Table 4.6). The differences between the observations in category HIGH and the rest were clearer when observations were ordered according to g $CH_4$/kg DMI, in which category HIGH had a higher Gini coefficient for every group (Table 4.7). This would mean that observations of HIGH $CH_4$ emissions were associated with a lower number of MFA which were relatively more abundant.

Table 4.6. Results of analysis of variance of Gini coefficients by category according to daily $CH_4$ emissions (g/d) and MFA group

| | Category | | | P-value |
|---|---|---|---|---|
| | HIGH | MEDIUM | LOW | |
| Group 100 | 0.859[a] | 0.851[b] | 0.850[b] | 0.02 |
| Group 90 | 0.592 | 0.584 | 0.588 | 0.40 |
| Group 10 | 0.337[a] | 0.306[b] | 0.295[b] | <0.001 |
| Group 2.5 | 0.374 | 0.352 | 0.366 | 0.29 |
| Group 0.625 | 0.391[a] | 0.336[b] | 0.308[b] | <0.001 |

Table 4.7. Results of analysis of variance of Gini coefficients by category according to $CH_4$ emissions relative to DMI (g/kg DMI) and MFA group

| | Category | | | P-value |
|---|---|---|---|---|
| | HIGH | MEDIUM | LOW | |
| Group 100 | 0.860 [a] | 0.851[b] | 0.849 [b] | <0.001 |
| Group 90 | 0.611 [a] | 0.579 [b] | 0.581 [b] | <0.001 |
| Group 10 | 0.333 [a] | 0.306 [b] | 0.299 [b] | <0.001 |
| Group 2.5 | 0.410 [a] | 0.349 [b] | 0.335 [b] | <0.001 |
| Group 0.625 | 0.436[a] | 0.318 [b] | 0.300 [b] | <0.001 |

## DISCUSSION

Correlations between $CH_4$ and individual MFA were generally weak across the complete data set, although in other studies high correlations between some individual MFA and $CH_4$ were reported, but this was with data sets from a single experiment with relatively limited dietary diversity (Chilliard et al., 2009; Mohhammed et al., 2011). When observations were collected from multiple experiments (Dijkstra et al., 2011) or when combining diverse diets (Mohammed et al., 2011), correlation coefficients were lower and, similar to our study, were not higher than 0.6 in most cases.

The multivariate analysis was done by fitting a generalized linear mixed model to our data. Generalized linear mixed models have the advantage of dealing with data showing correlations among some or all observations. Such correlations can arise from repeated observations of the same sampling units and/or shared random effects in an experimental design (Pinheiro and Baetes, 200), like the case with our dataset.

The proportion of the variance explained by MFA depended on the functional units to express $CH_4$. Two different $R^2$ were calculated for each model, a marginal ($R^2_{(m)}$) which represents the variance explained by fixed effects (*i.e.* MFA) in our models and a conditional ($R^2_{(c)}$) which accounts for the variance explained by fixed effects and random effects combined. Milk FA had a greater ability to express variation in $CH_4$ emissions when this was expressed relative to DMI Eq. (21), and lowest when $CH_4$ was expressed relative to metabolic bodyweight. Moreover, the model to predict $CH_4$ in g/kg DMI is of particular interest given the potential for C14:0 and *cis*-9 C18:1 concentrations in milk to be determined by spectrofotometrically (Soyeurt et al., 2011). However, the determination of C17:0 in milk still remains out of the scope of routine analysis.

Some common MFA occurred in the models developed to predict $CH_4$ emissions expressed in different units, particularly C17:0 was kept in Eqs. (20), (21) and (22). It is worth noticing

that the model to predict $CH_4$ relative to milk Eq. (22) contained all the predictors of Eq. (21) (g $CH_4$/kg DMI) plus additional MFA, which confirmed the similarities in the MFA related to both methane relative to DMI and methane relative to milk, which was found from statistical analysis previously performed on literature data (Chapter 3).

The models developed in Chapter 4 confirmed the findings of Chapter 3 regarding the similarities between the MFA related to $CH_4$ in g/kg DMI and $CH_4$ in g/kg milk. Indeed, all of the variables included in the former model were included in the latter. On the other hand, there were no coincidences within the MFA related to daily emissions (g/d) or emissions relative to metabolic bodyweight (g/kg $BW^{0.75}$/d), in contrast to the findings in Chapter 3. In Chapter 3 $CH_4$ in g/kg $BW^{0.75}$/d was reported as such only in one study (Van Zijdereveld et al., 2011), in seven studies body weight by treatment was reported, whereas for five studies only the average bodyweight of all the animals used in the experiment was reported. This caused an increase in the similarity between $CH_4$ expressed as daily emissions or relative to metabolic body weight.

In three of the four models, C17:0 or its desaturated derivate *cis*-9 C17:1 was the predominant MFA contributing to the prediction of $CH_4$, based on standardized regression coefficients, which agrees with findings of Mohammed et al. (2011). Linear odd-chain MFA, like C17:0, are *de novo* synthesized by rumen microbes or endogenously produced when propionyl-CoA, a derivative of propionic acid, is used as primer instead of acetyl-CoA (Fulco, 1983; Kaneda, 1991). Propionate, in turn, has a negative relationship with $CH_4$ production in the rumen (*e.g.* Demeyer and Van Nevel, 1975). Moreover, C15:0 is the most abundant odd-chain MFA and, similar to C17:0, its origin is from microbial *de novo* synthesis or endogenous synthesis using propionyl-CoA as primer (Vlaeminck et al., 2006). However, C15:0 was not kept as predictor of $CH_4$ in any model in this or in previous studies, and the reason why C17:0 seem to be a

superior predictor than other MFA, especially than C15:0, with which holds the same origin is unknown and is worth to be further investigated.

*Cis*-11 C18:1 Eq. (20) appeared in models previously reported by Dijkstra et al. (2011) and (Mohammed et al. (2011), which highlights its possible relevance as predictor of $CH_4$. C*is*-11 C18:1 is believed to have its origin in the isomerisation of *trans*-11 C18:1 (vaccenic acid). Vaccenic acid, in turn, is related to higher ruminal concentrations when diets rich in PUFA are fed (Chilliard et al., 2000) and when rumen pH decreases (Martin and Jenkins, 2002), both factors negatively related to $CH_4$ production. Moreover, *trans*-9 C16:1 Eq. (22), a product of partial oxidation of vaccenic acid, and *trans*-9 C18:1 Eq. (23) were strongly correlated with *trans*-11 C18:1 ($r$ =0.7 and $r$ = 0.92, respectively), which might explain their inclusion in our models.

Furthermore, *cis*-9 C18:1 Eq. (21) and (22) was correlated with *cis*-13 C18:1 ($r$ = 0.65) a MFA reported in the model of Dijkstra et al. (2011). Whereas *iso* C16:0 was kept in the best model of Mohammed et al. (2011) and was retained in our model to predict $CH_4$ relative to milk yield Eq. (22).

*Anteiso* C17:0 Eq. (20) was also reported in the model of Dijkstra et al. (2011), whereas Mohammed et al. (2011) reported *anteiso* C15:0, a MFA highly correlated with *anteiso* C17:0 in our dataset ($r$ = 0.86).

Other MFA kept in our models were not previously reported in models to predict $CH_4$ (C18:0, C14:0, *n-6* C22:5, *n-3* C20:5, C4:0, C7:0 and *trans*-12 C18:1).

Milk FA appear to have a modest potential to predict amounts of $CH_4$ emitted by the cow. However, MFA might hold potential to identify high and low $CH_4$ emitting animals, which could be of interest for researchers dedicated to selection of livestock as a means to reduce ruminant $CH_4$ emissions (e.g. Hegarty et al. 2007). To study this possibility, we applied the

principles of the Lorenz curve and Gini coefficients to find the cumulative distribution of MFA across our data set.

Even though the Lorenz curve and Gini coefficient approach has its origin in economics, recently, they have been applied in microbiology to represent graphically and numerically the species evenness within an ecosystem, based on the DGGE profiles (e.g. Mertens et al., 2005; Marzorati et al., 2008). They have been also used to describe the community organization of rumen archaea in relation to treatments intended to decrease $CH_4$ emissions (Patra et al., 2012; Panyakaew et al., 2014).

In this study the Lorenz curve and the Gini coefficient constituted a convenient representation of the cumulative distribution of MFA, which we considered might be meaningful given the existing link between MFA profiles, VFA proportions and rumen fermentation and potentially, rumen microbioal community. However, characteristics describing the cumulative distribution of MFA did not seem very powerful in reflecting variations in $CH_4$ emissions, and, based on correlations and univariate regression (Table 4.5), only the group including the minor MFA, summing up 0.625 g/100 g MFA (**Group 0.625**), was related to daily $CH_4$ emissions. This group included mainly isomers of C16:1, long chain PUFA and linear odd-chain MFA like C5:0, C7:0 and C9:0. When comparing the MFA cumulative distribution of observations in the HIGH, MEDIUM and LOW daily $CH_4$ categories, those observations in category HIGH had a higher Gini coefficient than MEDIUM and LOW for **Group 100**, **Group 10** and **Group 0.625**. However, the differences in means were minimal and might not represent a practical way to identify high from medium and/or low producers. Nevertheless, when the categories were separated based on $CH_4$ emissions relative to DMI, observations in the category HIGH had consistently showed higher Gini coefficients than both MEDIUM and LOW for all groups. Remarkably, such differences were greater in this case as compared with classification based on daily emissions. Furthermore, the smaller the group of MFA, the more

obvious the differences between categories. This means that those observations belonging to the lower quartile of $CH_4$ in g/kg DMI had a more even distribution of MFA, and in particular, those MFA present in smaller quantities, were more evenly distributed within the MFA group. These results show some scope for this approach to discriminate animals producing higher $CH_4$ emissions from those producing less within a population.

## CONCLUSIONS

Milk fatty acids hold a modest potential to predict $CH_4$ emissions. The most important MFA seem to be C17:0, C14:0 along with isomers of C18:1. Milk FA explained the greatest part of the variation in $CH_4$ when this was expressed relative to DMI. On the other hand, MFA only poorly explained variation in $CH_4$ relative to metabolic bodyweight. Some of the MFA selected in the different models can be determined spectrofotometrically, which shows scope for routine quantification f $CH_4$ emissions. A novel approach based on recent developments in describing microbial community organization and which was used here to describe the cumulative distribution of MFA in relation with $CH_4$, did not seem to aid further on the identification of MFA linked to $CH_4$.

# CHAPTER 5. GENERAL DISCUSSION AND FUTURE PERSPECTIVES

**CHAPTER 5**

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# GENERAL DISCUSSION

## Introduction

Practical estimation of $CH_4$ emitted by cattle has become an important issue, as a mean to obtain inventories of emissions in a farm, and due to the need to test the effectiveness of mitigation strategies. Currently, equipment and/or facilities to do this are not only expensive, but also improbable to apply under practical conditions. Researchers have directed some of their efforts to develop models to estimate $CH_4$ emissions. Empirical models based on dry matter intake and chemical dietary characteristics are available as well as mechanistic models based on the biochemistry of the fermentation. More recently, empirical models based on milk fatty acids (MFA) have been developed to predict $CH_4$. Milk is easy to be sampled and holds a large amount of metabolites, including fatty acids, containing information on rumen metabolism.

On the other hand, historically *in vitro* systems have been used to assess the potential of an additive to decrease $CH_4$ and they have been regarded as a good tool to screen large amounts of samples and treatments.

This PhD dissertation focuses on 1) *in vitro* techniques and their perspective to be used for routine assessment of $CH_4$ mitigation, and 2) the potential of MFA to predict $CH_4$.

## *In vitro* techniques for routine assessment of $CH_4$ mitigation

Flachowsky and Lebzien (2009) highlighted the large volume of work done *in vitro* dealing with substances to decrease $CH_4$ emissions from ruminants, but also noticed inconsistent results and challenges for extrapolating of *in vitro* measurements to *in vivo* situations.

Therefore, *in vitro* systems might need to adapted in order to better reflect *in vivo* conditions. The main objective of Chapters 1A was to study the interaction effects between additives and basal substrate *in vitro* as a potential source of limited correlation between *in vitro* and *in vivo* results. The main objective of Chapter 1B was to explore the potential that specific modifications in the *in vitro* set-up had to improve the assessment of the inhibitory effect of an additive (*i.e.* a blend of essential oils) *in vivo*.

***In vitro vs. in vivo results***

A number of articles have reviewed the effects of diverse additives on $CH_4$ emissions *in vitro* (e.g. Cottle et al. 2011; Klevenhusen et al., 2012; Patra, 2012), and *in vivo* (e.g. Cottle et al., 2011; Eckardt et al., 2010; Patra, 2012). These reviews show that in general it is more usual to observe effects *in vitro* than *in vivo*, this is likely related to the fact that inhibitory effects on $CH_4$ are strongly dependent on the dose applied, and *in vitro* systems allow for the testing of large concentrations of a compound. This can not be done *in vivo*, where the dosage of a compound has to be done with much more care.

Even though many compounds have been tested both *in vitro* and *in vivo*, few studies have done this within the same experiment and/or ensuring that the same product is used in both systems. In this regard, Table 5.1 summarizes studies that fed animals with the same product incubated *in vitro* either within the same or in a previous study. The main difference between both systems is the concentration needed to observe inhibitory effects. But the relation of *in vitro* to *in vivo* doses varies largely across studies and depending on the product tested, with some compounds requiring 10 to 20 times greater concentrations (*m/v*) *in vivo* (hoseradish oil, coconut oil) than *in vitro* to see similar effects (Table 5.1); whereas yucca saponins required applied at lower doses (*m/v*) *in vivo* found comparable effects *in vitro* (Table 5.1) On the other hand, quillaja saponins, did not have any effect on methane inhibition *in vitro* eventhough

doses used (*m/v*) were higher than dose showing an effect *in vivo* (Table 5.1). The blend of essential oils tested in Chapter 2 needed to be included at a higher concentration (*m/v*) *in vitro* (10-20 times higher) to see similar effects to those observed *in vivo*. It is clear that the differences between *in vitro* and *in vivo* are not uniform across compounds. Therefore, in order to optimize *in vitro* systems towards monitoring *in vivo* inhibitory effects of dietary additives, more studies should be performed comparing the effects of the same compound both *in vitro* and *in vivo*, giving especial attention to the possible sources of discrepancies between both systems.

Table 5.1 Comparison of *in vitro* and *in vivo* with testing the same additive to mitigate $CH_4$. Calculations of concentrations (*m/v*) assuming rumen voulmes of 30 L for steers, 10 L for wethers and 100 L for dairy and beef cattle.

| Additive | *In vitro* | | | | *In vivo* | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | Substrate | Concentration | | $CH_4$ inhibition (%) | Animal | Diet | Concentration | | $CH_4$ inhibition (%) |
| | | ppm (*m/v*) | ppm (*m/m*) | | | | ppm (*m/v*) | ppm (*m/m*) | |
| Horseradish oil (Batch system) [a] | Corn starch | 5.95 | 893 | 18 | 6-7 months old steers | Sudangrass + concentrate (50:50) | 91 | 700 | 19 |
| | | 29.8 | 4463 | 59 | | | | | |
| | | 59.5 | 8930 | 90 | | | | | |
| Yucca saponins (continuous culture) [b] | Concentrate + oat (50:50) | 210 | 16.8 | 17 | Wethers | Italian ryegrass + concentrate (60:40) | 148 | 1244 | 12 |
| | | 420 | 33.7 | 29 | | | | | |
| | | 630 | 50.5 | 42 | | | | | |
| Quillaja saponins (continuous culture) [b] | Concentrate + oat (50:50) | 138 | 11.1 | 0 | Wethers | Italian ryegrass + concentrate (60:40) | 97 | 841 | 17 |
| | | 276 | 22.1 | 0 | | | | | |
| | | 415 | 33.2 | 0 | | | | | |
| Coconut oil (RUSITEC) [c] | Maize silage + hay + concentrate (69:8:23) | 360 | 26000 | 43 | Wethers | Hay + concentrate (71:29) | 3797 | 35000 | 28 |
| | Maize silage + hay + concentrate (49:5:46) | 720 | 53000 | 72 | Wethers | Hay + concentrate (45:55) | 6293 | 70000 | 73 |
| Blend of essential oils (Batch system) [d] | Grass silage + maize silage + concentrate (35:35:30) | 5 – 40 | 500 – 4000 | 0 | Dairy cows | Grass silage + maize silage + concentrate (46:37:12) | 2 | 13 | 15 |
| | | 45 – 50 | 4500 – 5000 | 15 | Beef cattle | Maize silage + concentrate (50:50) | 4 | 11 | 10 |

[a] (Mohammed et al., 2004).
[b] *In vitro* (Pen et al., 2006); *in vivo* (Pen et al., 2007)
[c] *In vitro* (Machmüller et al., 1998); *in vivo* (Machmüller et al., 1999)
[d] (Chapter 4)

***Importance of substrate × additive interaction***

One of the features in which *in vitro* systems clearly differ from *in vivo* systems is the choice of substrate. *In vitro* experiments usually rely on the combination of an additive with a single substrate (e.g. hay, mix of concentrate and grass). However, as outlined by Flachowsky and Lebzien (2012), additives to inhibit $CH_4$ should ideally be tested in combination with different feedstuffs. Moreover, we consider that these substrates should directly reflect diets used in feeding target animals (e.g. dairy cattle are rarely fed solely on hay). Our results in Chapter 1A suggest that there are additive × substrate interaction on the inhibitory effects on $CH_4$, in agreement with other studies with e.g. MCFA (Machmüller et al, 2006) or monensin (Wischer et al., 2013). However, these effects were not common for all the additives tested, indicating that additive × substrate interaction depends on the type of additive and it should be individually studied for each supplement.

Even though results among studies are inconsistent, based on literature and our findings in Chapter 1A, we attempt to indicate which additives show interaction effects with substrates, and the type of substrate (using NDF (g/kg DM) as an indicator of the proportion of fiber in the substrate) with which a stronger $CH_4$ inhibition might occurred.

There were three additives for which we did not find interaction effects: cinnamaldehyde, fumaric acid and garlic oil. In our study, cinnamaldehyde strongly inhibited $CH_4$ and the complete fermentation. Therefore, it was not possible to test for interaction effects. However, Pellikaan et al. (2011) found a complete inhibition of $CH_4$ when cinnemaldehyde was added to maize (NDF = 82 g/kg), but only a 65% inhibition in combination with soybean hulls (NDF = 648 g/kg). Similarly, Mateos et al. (2013) showed that cinnamaldehyde had a stronger inhibitory effect in combination with a high concentrate substrate rather than with a medium concentrate one (77% and 32% inhibition, respectively).

Furthermore, in agreement with our findings in Chapter 1A, the meta-analysis by Ungerfeld et al. (2007) showed that there was no interaction between the ratio of concentrate to roughage in the diet and fumaric acid on $CH_4$ production. However, the results of different studies (Figure 5.1) suggest, that *in vitro* fumaric acid might had a higher inhibition effect when combined with a more fibrous diet.

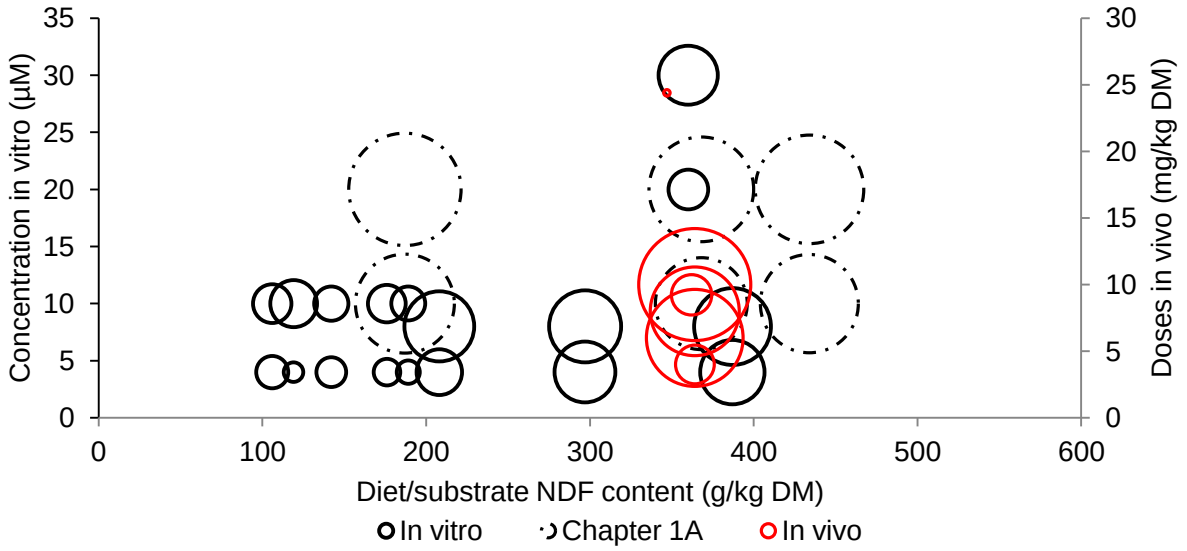


Figure 5.1. *In vitro* and *in vivo* inhibition of $CH_4$ production by fumaric acid in relation to NDF content of the substrate/diet and doses. Biggest circle = 42% inhibition.

Adapted from Asanuma et al. (1999), Beauchemin and McGinn (2006), Caro and Ranilla (2003), García-Martínez et al. (2005), McGinn et al. (2004) and Molano et al. (2008).
Circles with dashed lines represent findings in our study in Chapter 1A.

Moreover, a recent study with garlic oil (Mateos et al., 2013) reported an interaction effect of this compound with the substrate (either high or medium concentrate diet). However, $CH_4$ inhibitions reported were similar for both substrates when garlic oil was applied at 15 and 54 mg/ml, doses comparable to the ones we used (15 and 30 mg/ml), which helps to confirm our findings in Chapter 1A, but also gives an indication of a possible effect of doses on the substrate × additive interaction.

On the other hand, interactions with substrates were observed for quillaja saponins, monensin, MCFA and fish oil. In our study we found greater $CH_4$ inhibition when quillaja saponins were

combined either with grass or maize silage than with concentrate although differences are minor as compared with differences between studies (Figure 5.2). Most previous studies were performed within a limited range of NDF content in the diet (Figure 5.2). However, despite this limitation, Figure 5.2 clearly illustrates that saponins, substrate × interaction is not a predominant factor determining $CH_4$ inhibition. It has to be noted that the sources of these compounds vary from study to study (i.e. saponins extract, purified saponins, plant parts) which obviously is another factor that has to be considered when screening compounds, as outlined by Flachowsky and Lebzien (2012).

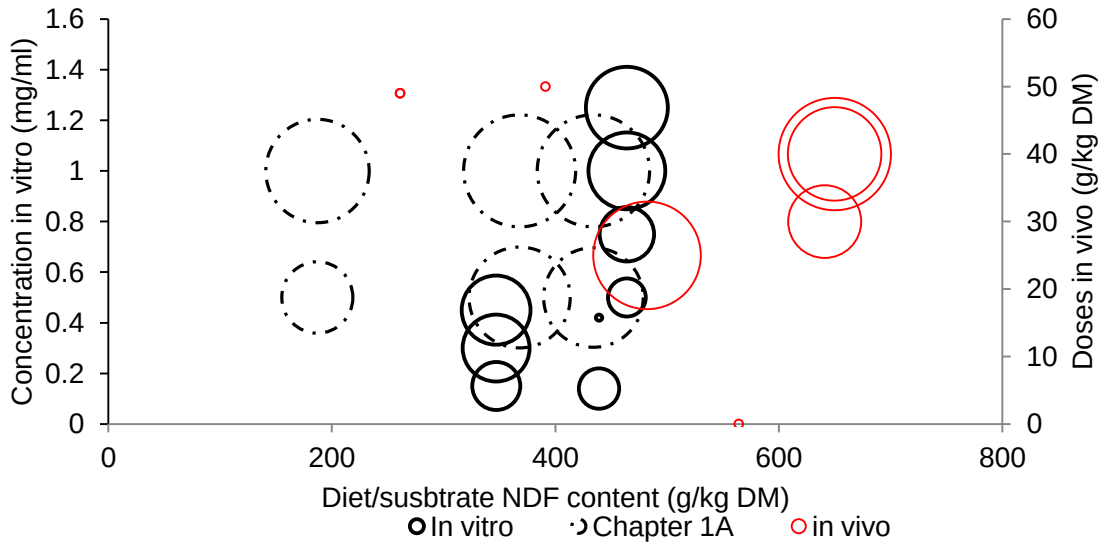


Figure 5.2. *In vitro* and *in vivo* inhibition of $CH_4$ production by saponins sources in relation to NDF content of the substrate/diet and doses. Biggest circle = 32% inhibition.

Adapted from Castro-Montoya et al. (2011), Holtshausen et al. (2009) and Pen et al. (2006).
Circles with dashed lines represent findings in our study in Chapter 1A.

Furthermore, more information from different studies is available on the effects of monensin on $CH_4$ (Figure 5.3), which suggests that stronger effects of monensin might occur for diets with less fiber. Conversely, studies by Wischer et al. (2013) and Pellikaan et al. (2012) with monensin tested with different substrates confirm our findings that monensin has a greater effect on $CH_4$ inhibition when combined with fiber rich materials than in combination with starch rich ones.

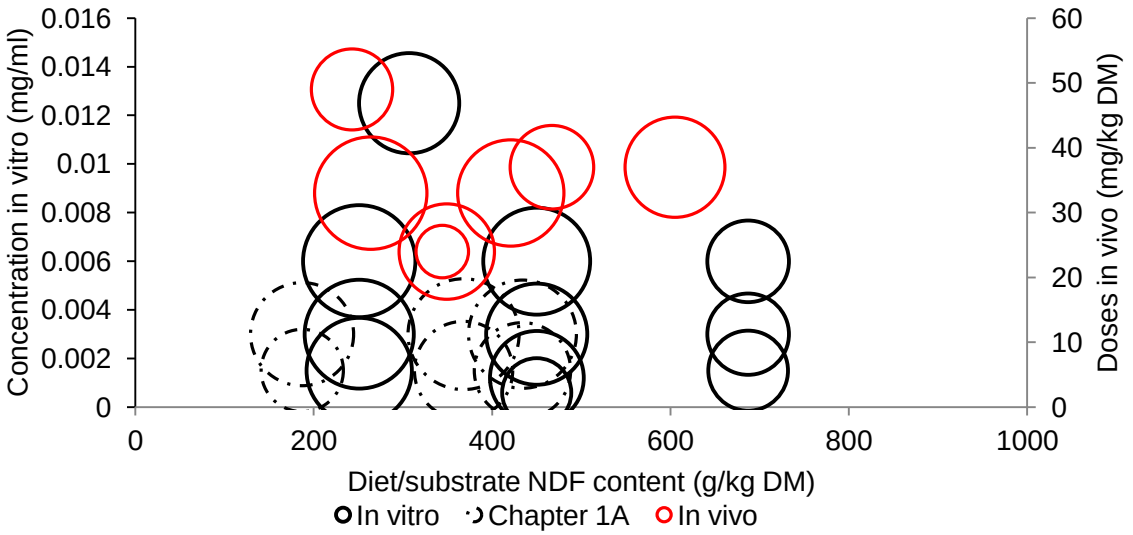


Figure 5.3. *In vitro* and *in vivo* inhibition of $CH_4$ production by monensin in relation to NDF content of the substrate/diet and doses. Biggest circle = 52% inhibition.

Adapted from Callaway and Martin. (1996), Russel and Strobell (1988), Sauer et al. (1998), Odongo et al. (2007), Guan et al. (2006), Thornton and Owens (1981).
Circles with dashed lines represent findings in our study in Chapter 1A.

Moreover, in Chapter 1A it was found that MCFA decrease $CH_4$ to a larger extent in combination with maize silage, while the lowest inhibition occurred in combination with concentrate. In contrast, Machmüller et al. (2006) in their review of MCFA to decrease $CH_4$ stated that MCFA would have a stronger inhibitory effect on $CH_4$ combined with concentrate-based diets, which would be confirmed by the observations in Figure 5.4, where greater inhibition can be seen related with less fiber content of the diet.

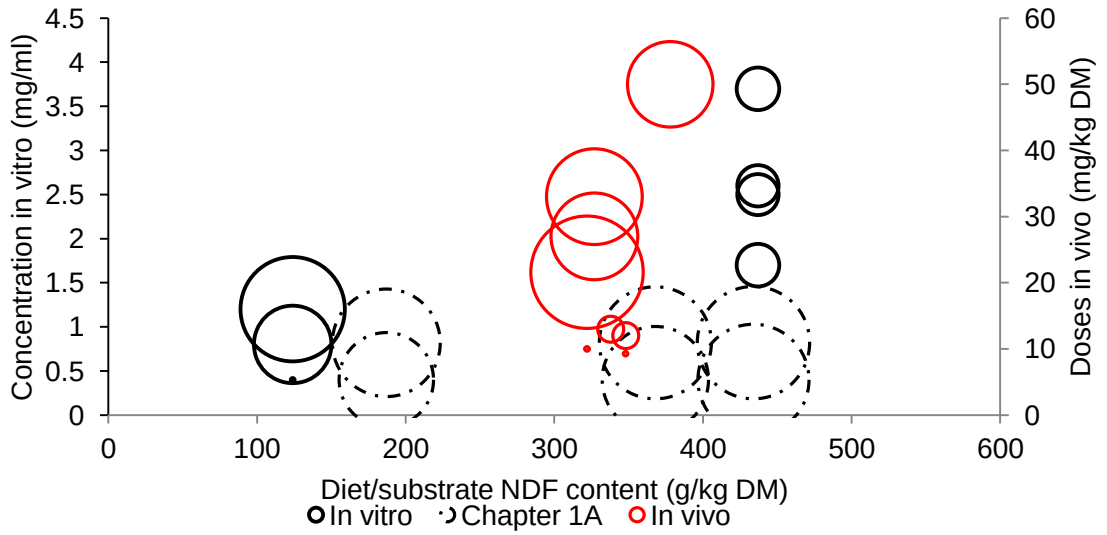


Figure 5.4. *In vitro* and *in vivo* inhibition of $CH_4$ production by sources of MCFA in relation to NDF content of the substrate/diet and doses. Biggest circle = 94% inhibition.

Adapted from Goel et al. (2009), Panyakaeuw et al. (2013) Hristov et al. (2009), Hristov et al. (2011), Odongo et al. (2005), Hollman et al. (2012).
Circles with dashed lines represent findings in our study in Chapter 1A.

Harder to compare are our results from fish oil, given that few studies have studied the effects of this compound, and none has tested it in combination with different substrates. We found that fish oil has a larger potential to inhibit $CH_4$ when combined with grass silage and concentrate. On the other hand, one *in vivo* study by Lee et al. (2008) did not find interactions between fish oil and diet on acetate or propionate proportions when fish oil was supplemented to cattle fed either grass or red clover, suggesting no interaction effects between this additive and the diet on VFA proportions, which are normally linked to $CH_4$ emissions.

It is clear that more research should be done for some compounds and that the effects we observed in the experiment of Chapter 1A should still be tested *in vivo* in order to validate these results.

Additive × substrate interactions likely play a role in the differences between *in vitro* and *in vivo* results. However, these effects are minor as compared with differences between studies,

and they are certainly not the only factor influencing mismatches between *in vitro* and *in vivo* systems.

***Modifications to the batch in vitro set up***

As outlined before, besides possible interaction effects between additives and substrates, there are other factors that might play a role in the differences between *in vitro* and *in vivo* results. Therefore, in Chapter 1B we modified some features (Table 5.2) of routine *in vitro* incubations in an attempt to better reflect effects of additives, for this particular case a blend of essential oils, *in vivo*.

Table 5.2. Factors modified in Chapter 1B to the batch *in vitro* set up to better reflect *in vivo* responses

| Experiment and factor addressed | *In vitro* (modification) | Outcome |
|---|---|---|
| **Exp. 2**: Time of addition | Addition *ca.* 16 h prior the start of the incubation | No effect |
| **Exp. 3**: Longer incubation time (72 h) (GPT) | Longer incubation time (72 h) by the GPT system | Inhibition at 30 ppm after 30 h |
| **Exp. 4 (series 1)**: Regular supply of substrate and essential oils over 96 h incubation | Transfers of inoculums from 24 h incubation adding both substrate and additive at each transfer | No effect of daily supply. Fermentation seemed to be impaired |
| **Exp. 4 (series 2)**: Longer incubation time (14 d) + regular supply of substrate and essential oils | Transfers of inoculums from 48 h incubation adding both substrate and additive at each transfer | No effect of longer incubation time and regular supply of essential oils |

Addition of the additive the day before feeding (experiment 1) attempted to reflect the *in vivo* practice of mixing the product with the feed hours (or days) before feeding. A longer incubation time (experiment 2) was meant to allow sensitization of microorganisms to the essential oils, what might occur *in vivo* after days of supplementation. Ensuring constant

supply of buffer, substrate and additive (experiments 3 and 4) over longer incubation times was an attempt to further reflect *in vivo* practices were additives are fed daily. However, increasing the exposure time of the feedstuff to the additive, longer incubation time and ensuring a regular supply of buffer, substrate and additive failed to cancel the large differences observed in the doses needed *in vitro* and *in vivo* to observe $CH_4$ inhibition. Other factors not tested in our study and that potentially play a role, and should be kept in mind in order to optimize *in vitro* systems are the ratio of buffer to substrate to rumen fluid to and the diet of the donor animal. The ratio of substrate amount to buffer to rumen fluid, can affect fermentation if the buffer is depleted, which can provoke a drop in pH impairing the fermentation, especially in incubation runs lasting more than 24 h. However, it was not possible to confirm this by measuring the pH in the incubation medium. Furthermore, the diet of the donor animal is another factor to be considered, as some authors argued that the *in vitro* fermentation varies depending on the diet of the donor animal (e.g. Cherney et al., 1993; Cone et al., 1996; Khiaosa-ard et al., 2011)

Additionally, another factor we consider important and might deserve a larger discussion is the length of the incubation time. Even though an extension of the incubation time did not help to observe inhibition of $CH_4$ *in vitro*, the technique seems to have potential to reflect rumen fermentation even for a long period of time (14 days), and still meets the routine principle of batch systems. It has to be noted that the modifications we have done to the *in vitro* batch system did not aid on the better reflection of *in vivo* results for this particular compound, and extrapolation of these results to other compounds should not be done. Indeed, different results were observed by Morgavi et al. (2013) when testing the $CH_4$ inhibition potential of *monascus*-fermented rice and of *monascus* extract using a consecutive batch culture (transfers every 24 h). Neither the fermented rice nor the *monascus* extract showed effects on $CH_4$ inhibition after 24 h (first batch) and 48 h (second batch, one transfer).

However, after 72 h (third batch, second transfer) fermented rice and *monascus* extract decreased $CH_4$ production by 60% and 86%, respectively, as compared with their corresponding controls. These results were later validated *in vivo* but the set up of the experiments did not allow for a comparison of the concentrations of the active compound in both *in vitro* and *in vivo*. It is noteworthy that for the latter compound, the consecutive batch incubation system was more appropriate to better simulate inhibition of $CH_4$ *in vivo* than for the blend of essential oils we used, hence the potential of this technique to be used as a model of rumen fermentation should be further studied for other additives.

***Potential to quantitatively assess in vivo $CH_4$ mitigation from batch in vitro set ups***

In the *in vitro* experiments of Chapter 1B, $CH_4$ inhibition was observed only at a much higher concentrations as compared with *in vivo* (50 ppm *vs.* 2-4 ppm, for *in vitro* and *in vivo*, respectively). Other *in vitro* studies with essential oils have reported inhibition of $CH_4$ but at concentrations even higher than those in our study: Evans and Martin (2000) supplemented thymol at 400 ppm, Busquet et al. (2005a) found decreases with garlic oil and diallyl disulfide at 300 ppm. Moreover, the meta-analysis of Klevenhusen et al. (2012) showed that essential oils are added *in vitro* at an average concentration of 45.6 mg/g DM, with minimum of 0.03 mg/g DM and maximum of 500 mg/g DM (under the conditions of our experiments these amounts would be equivalent to 456, 0.3 and 5000 ppm, for the average, minimum and maximum, respectively). These high concentrations of essential oils reflect the amounts needed to observe effects on *in vitro* fermentation, including $CH_4$ production. In contrast, most of the *in vivo* studies with essential oils fed the animals with lower doses, ranging from 0.182 to 5 g per kg DMI, equivalent to 10 and 90 ppm (*m/v*) (Beauchemin and McGinn, 2006; Mohammed et al., 2004, Klevenhusen et al., 2011; Wang et al., 2009). Furthermore, no effect on $CH_4$ was observed when 1 g/d of a mix of essential oils and spices was fed to beef steers (Beauchemin and McGinn, 2006). Interestingly, using the same extract, McIntosh et al.

(2003) reported that the growth of the methanogen *Methanobrevibacter smithii* was inhibited only when a concentration of at least 1000 ppm was added. The maximum concentration used in the study of Beauchemin and McGinn (2006) was estimated to be 33 ppm, according to the authors, which is well below McIntosh et al.'s doses, and may account for the lack of effect on $CH_4$ emissions.

No clear explanation was found for the need of much larger concentrations *in vitro* to observe similar decreases in $CH_4$ production with those *in vivo*. One possibility is that *in vivo* decreases in rumen $CH_4$ production trough essential oils are indirect through a host × additive interaction. Studies with monogastrics have observed these type of effects from dietary additives. For example, antibiotics have been widely used as growth promoter in poultry, and their beneficial effects have been related to their direct impact on intestinal bacteria (see review of e.g. Dibner and Richards, 2005). However, studies with germ-free animals have also found benefits of antibiotics feeding (Dibner and Richards, 2005), and one hypothesis for this is that absorption of nutrients may improve because of a thinning of the small intestine barrier (Forsgren et al., 1980). Indeed both regular animal and germ-free animals fed antibiotics were found to show a reduction in gut size, including thinner intestinal villi and total gut wall (Coates et al., 1952). Such host × additive interactions have not been studied in ruminants, but if they exist it is unlikely that they will be replicated by an *in vitro* system.

*In vitro* approaches still represent an important tool to screen possible dietary additives. However, an ideal *in vitro* technique should correlate well with actually measured *in vivo* parameters. For the specific case of the blend of essential oils tested in Chapter 1B, under the current set-up of routine *in vitro* batch systems, these are not able to effectively asses the inhibition caused by an additive if an equivalent *in vivo* doses is applied *in vitro*.

## The potential of MFA to predict $CH_4$ emissions from dairy cattle

As previously outlined, MFA hold potential to reflect changes in rumen fermentation. However, it is not clear which MFA might have the greatest potential as biomarkers for $CH_4$, and how well MFA predict $CH_4$ emissions compared with feed-based models or models based on rumen biochemistry. Therefore, in this PhD dissertation we aimed at 1) identifying the most relevant MFA as biomarkers for $CH_4$ production. 2) exploring the potential of multivariate MFA-based models to predict $CH_4$ emissions.

### *Individual MFA linked to $CH_4$ emissions.*

So far, diverse studies have explored the relationships between MFA and $CH_4$ emissions, either by a qualitative description of changes in individual MFA when a $CH_4$ mitigation agent was supplemented (e.g. Odongo et al., 2007; Sauer et al., 1998) or by finding associations between MFA and $CH_4$ emissions based on pearson correlation and/or linear regression (Chilliard et al., 2009; Mohammed et al., 2011; Dijkstra et al., 2011).

Most of the data in these previous studies were obtained from a single experiment; hence, they might not represent more general conditions. Nevertheless, from their results and results from Chapter 3 and Chapter 4, C14:0, C17:0, *cis*-11 C18:1, *cis*-13 C18:1 and *trans* isomers of C18:1 were found to be the most relevant MFA linked with $CH_4$. In this regard, C17:0 and its desaturase product (*cis*-9 C17:1) were found to be related with $CH_4$, either by pearson correlations, simple linear regression or multiple linear regression in Chapter 3 and Chapter 4 and in every study linking MFA to $CH_4$, with the exception of Chilliard et al. (2009). Similarly, *cis*-11 C18:1 was among the highest correlated MFA with $CH_4$ in the studies of Dijkstra et al. (2011) and Mohammed et al; (2011) and it was also retained in their equations. *Cis*-13 C18:1 was correlated with $CH_4$ in the studies of Chilliard et al. (2009) and Mohammed

et al. (2011) and it was selected in the model of Dijkstra et al. (2011). Furthermore, *trans* isomers of C18:1 also appear to hold relationships with $CH_4$: *trans*-10 C18:1 (Dijkstra et al., 2011; Chapter 4), *trans*-11 C18:1 (Chilliard et al., 2009; Dijkstra et al., 2011) and *trans*-12 C18:1 and *trans*-13 C18:1 (Chilliard et al., 2009; Chapter 4) were all selected in models or were among the MFA having highest correlation with $CH_4$.

Some MFA showing a negative relationship with methane in Chapter 4 (e.g. C17:0) were found to have a positive relationship with methane in Chapter 3. These differences are likely related to the nature of the dataset used for the meta-analysis in Chapter 3, where important changes in both the MFA profiles as well as $CH_4$ were caused by addition of dietary sources of UFA, and a general decrase in individual SFA and PUFA, while increasing individual MUFA.

It has to be noted, however, that all the correlations reported between individual MFA and $CH_4$ are weak (highest correlation coefficients ranging between 0.3 and 0.6), with the exception of the ones reported by Chilliard et al. (2009). Therefore, even though there are clear links between individual MFA and $CH_4$ emissions, no single MFA can be considered as an appropriate predictor of $CH_4$. Hence, a mulivariate model might improve the prediction of $CH_4$ by combining the information from individual MFA.

Moreover, there is a debate on whether $CH_4$ emissions should be expressed as an absolute daily amount emitted per cow (g/d), relative to the unit of production (e.g. g/kg milk) or even relative to DMI (g/kg DMI). Interestingly, when $CH_4$ was expressed in different units (*i.e.* g/d, g/kg DMI, g/kg milk, g/kg $BW^{0.75}$/d) different MFA were associated with $CH_4$ emissions, highlighting the need to standardized the units in which $CH_4$ is expressed when comparing or reporting models to predict such emissions. In this regard, multivariate models in Chapter 3

and Chapter 4 were developed for $CH_4$ expressed in four functional units (*i.e.* g/d, g/kg DMI, g/kg milk, g/kg $BW^{0.75}$/d).

***Prediction of $CH_4$ emissions through MFA: Modeling techniques***

Multiple regression analysis is a widespread technique with the main purposes of predicting the outcome of an event by finding the linear combination of a set of predictors that provides the best estimates of the dependent variable across a set of observations (Steel and Torrie, 1980). Its use is fostered by its applicability to varied types of data and by its ease of interpretation (Mason and Perreault, 1991). Multiple linear regression analysis has been the preferential choice for the development of models in many areas in biology, including prediction of $CH_4$ emissions. Indeed, the three previous studies predicting $CH_4$ used linear regression to develop their models (Chilliard et al., 2009, Dijkstra et al., 2011; Mohammed et al., 2011). However, most experiments in animal science include repeated measurements from the same individual and/or when data from different trials is combined, which increases the risk of non-desired effects in the data set, such as noise (unexplained variation in a data set) and collinearity between predictors. The presence of such effects is not an ideal condition for the fitting of a linear model across the observations.

Therefore, we tested two other techniques (Partial least squares regression (PLS) and a rational approach with a genetic algorithm selection (GA)) to see if these modeling techniques improve predictions of calculated $CH_4$ (mmol/mol VFA). Partial least squares regression, is a statistical technique particularly well suited to analyzing a large array of related predictor variables (i.e. not truly independent), especially when the number of observations is small compared with the number of independent variables (Carrascal et al., 2009). The rational approach with selection based on a genetic algorithm was used by Bhagwat et al. (2012) to predict VFA from MFA, finding an improvement in the predictions of VFA by using this approach, compared with model developed using linear regression.

In Chapter 2, we found that models from MLR and the GA approach performing slightly better (RMSE = 4.0, $R^2$ = 0.66 for both) than the model developed by PLS (RMSE = 4.1, $R^2$ = 0.63). Based on these results, for the subsequent models developed in Chapter 3 and Chapter 4, with data sets similar to the one used in Chapter 2, mainly in terms of number of observation and source of data, linear regression was the technique of choice, given the simplicity of both the development and the interpretation of the models, as outlined above.

Multivariate models were developed from treatment means gathered from literature (Chapter 3) and from individual observations obtained from 9 experiments *in vivo* with measurements of $CH_4$ emissions paired with MFA profiles (Chapter 4). The models of Chapter 3 were developed by fitting a general linear model, following similar approaches from the previous studies reporting MFA-based models to predict $CH_4$. However, this approach might not have been the most ideal to develop the models, given to the presence of a random categorical variable 'Study', which represented the study from which the data was obtained. If a generalized linear model is to be fitted, this type of variables has to be included either as a class variable or as an interval (e.g. dummy variable) in either case, many degrees of freedom are used and consequently, the risk of type II errors increases. Taking this into account, for the development of the models in Chapter 4, a generalized linear mixed model was fitted across the data. This type of models is an extension of generalized linear models to include both fixed (*i.e.* MFA) and random effects (*i.e.* experiment, cow). Additionally, they are better fitted for variables that may be correlated, and the risk of such correlation increased for the data set used in Chapter 4 due to repeated measurements obtained from the same cow. Additionally, a conditional ($R^2_{(c)}$) and a marginal ($R^2_{(m)}$) determination coefficient were calculated to estimate the variance explained by the complete model (including both random and fixed effects) and the variance explained solely by MFA (fixed effects).

***Performance of $CH_4$ prediction models***

In terms of performance, the models developed in Chapter 3 and Chapter 4 explained a similar degree of variation in $CH_4$ data (including the random effects). But only in Chapter 4 the exclusive contribution of MFA in the explanation of variance was estimated (based on $R^2_{(m)}$). This was low for the prediction of $CH_4$ as daily emissions or expressed relative to either milk or metabolic bodyweight ($R^2_{(m)}$ = 0.33, 0.38 and 0.19, respectively), and modest for the prediction of $CH_4$ relative to DMI ($R^2_{(m)}$ = 0.55). For further discussion we will refer to the marginal coefficient of determination ($R^2_{(m)}$) as this is the parameter indicating the influence of the variables of interest on $CH_4$ production.

The model predicting $CH_4$ relative to DMI performed best, although this would require on farm recording of DMI if an inventory of absolute emissions is to be estimated. Others (e.g. Dijkstra et al., 2011) argued that evaluation of dietary mitigation strategies preferentially should be based on $CH_4$ expressed as a function of DMI because it avoids confounding effects of the latter on emissions when dietary strategies to mitigate $CH_4$ are applied. Furthermore, from a sustainability point of view, $CH_4$ relative to DMI is more relevant as these would allow identifying cows using resources more efficiently. Along the same line, expressing $CH_4$ relative to non-human-edible DM intake would be worth to be studied.

Aside from MFA-based models, there are other models attempting to predict $CH_4$ emissions: 1) Empirical models directly relating nutrient intake to $CH_4$ emissions, and 2) Dynamic models that try to simulate $CH_4$ from ruminal fermentation biochemistry (Kebreab et al., 2009). There are different advantages and disadvantages in each of these types of models, but discussing them is not the aim of this section and it has been previously addressed in deep by other authors (see e.g. Kebreab et al., 2009).

When comparing empirical models found in literature with the MFA-based models developed in Chapter 4, we found that most empirical models reported higher explanation of variance by intake and diet composition variables (see summary in Table 5.3) than the variance explained by MFA in our models.

Table 5.3. Overview of empirical models to predict methane

| Reference | Variables included | $R^2$ | N |
|---|---|---|---|
| Moe and Tyrrel (1979) | Intake of hemicellulose, cellulose, soluble residue (kg/d) | 0.67 | 404 |
| Yan et al. (2000) | Digestible energy intake (MJ/d), proportion of silage ADF intake | 0.89 | 322 |
| Yan et al. (2000) | Digestible energy intake (Mj/d), proportion of silage DMI intake | 0.89 | 322 |
| Mills et al. (2003) | DMI (kg/d) | 0.60 | 159 |
| Mills et al. (2003) | Metabolizable energy intake (MJ/d) | 0.55 | 159 |
| Mills et al. (2003) | Nitrogen, ADF and starch intake (kg/d) | 0.57 | 159 |
| Mills et al. (2003) | Forage proportion, DMI (kg/d) | 0.61 | 64 |
| Ellis et al. (2007) | Forage proportion | 0.56 | 89 |
| Ellis et al. (2007) | DMI (kg/d) | 0.68 | 89 |
| Ellis et al. (2007) | DMI, ADF, NDF (kg/d) | 0.67 | 89 |

On the other hand, a study by Kebreab et al. (2008) found that the mechanistic models COWPOLL (Dijkstra et al. 1992) and MOLLY (Baldwin et al., 1995) performed better that the empirical model of Moe and Tyrrel (1979) when estimating $CH_4$ emissions from feedlot and dairy cattle, confirming the assumption that mechanistic models are better suited to estimate $CH_4$ than empirical ones. However, other parameters different to $R^2$ were reported to evaluate their performance, which do not allow for a comparison with our model. One study of Alemu et al. (2011) compared the ability of four mechanistic models to predict $CH_4$ from their estimation of VFA proportions and found $R^2$ ranging from 0.19 and 0.51, which are

comparable values with those we obtained with our models in Chapter 4. It has to be noted that the study of Alemu et al. (2011) was done by fitting the models on an independent set of observations, which has not been done with the models in Chapter 4.

It is, indeed, a challenging task to calculate $CH_4$ emissions from published data using equations based on MFA, not only for the limited number of studies reporting both MFA and $CH_4$, but mainly because not every MFA included in our models is reported in such studies. However, in an attempt to validate our models, we calculated the emissions based on MFA available from the studies considered in Chapter 3. However, such calculations were possible only for the model of Dijkstra et al. (2011) ($CH_4$ in g/kg DMI). Which also highlights that some of the MFA retained by some models (*i.e.* Eq. (23) Chapter 4; Mohammed et al., 2011; Chilliard et al., 2009) required more detailed analysis to be determined.

For the studies of Chilliard et al. (2009) and Mohammed et al. (2011), there is a clear negative relationship between reported and calculated $CH_4$ from our equation, caused by the presence of C18:0 in our model (positively related), which increases on addition of sources of UFA. This explains also the negative relationship between reported and calculated $CH_4$ relative to DMI for the MFA of Chilliard et al. (2009), where *cis*-9 C18:1 (included in our model) also increases on addition of linseed sources. Better predictions were obtained for $CH_4$ in g/kg DMI for the study of Van Zijderveld et al. (2011), where changes in MFA were not so drastic. The model of Dijkstra et al. (2011) was better to predict $CH_4$ in g/kg DMI from MFA of Chilliard et al. (2009), which was related to the presence of *cis*-11 C18:1 and *cis*-13 C18:1 (negatively related) in their model.

Furthermore, when using our equation to predict $CH_4$ in g/kg $BW^{0.75}$/d from the MFA of Chilliard et al. (2009), better predictions were achieved, caused by the *trans*-9 and *trans*-12 isomers of C18:1, which were negatively related in our model. Moreover, when $CH_4$ in g/d

and $CH_4$ in g/kg $BW^{0.75}$/d were estimated for the MFA reported by Hristov et al. (2009), emissions were overestimated, especially for the coconut oil treatment that strongly decreased $CH_4$ emissions. When predicting $CH_4$ in g/kg $BW^{0.75}$/d from the MFA of Hamilton et al. (2010) emissions were well predicted for the first set of treatments, but not for the second.

Table 5.4. Methane emissions calculated from models based on MFA. No study reported all MFA needed to calculate $CH_4$ in g/kg milk

| Study | Treatment | $CH_4$ (g/d) | | $CH_4$ (g/kg DMI) | | | $CH_4$ (g/kg milk) | |
|---|---|---|---|---|---|---|---|---|
| | | Rep[a] | Calc[a] | Rep | Calc | Dijkstra et al. 2011 | Rep | Calc. |
| Chilliard et al., 2009 | Control | 418 | 329 | 21.1 | 16.4 | 24.1 | 3.17 | 2.72 |
| | Whole crude linseed | 369 | 384 | 18.9 | 20.3 | 23.6 | 2.80 | 2.72 |
| | Extruded linseed | 258 | 387 | 15.5 | 21.7 | 17.2 | 1.96 | 1.97 |
| | Linseed oil | 149 | 361 | 10.1 | 24.2 | 15.9 | 1.13 | 1.44 |
| Van Zijderveld et al., 2011 | Control | | | 22.1 | 22.1 | | | |
| | Mix of additives[b] | | | 20.5 | 17.6 | | | |
| Odongo et al., 2007 | Control | | | 26.2 | | 17.3 | 2.90 | 2.73 |
| | Myristic acid | | | 18.0 | | 17.9 | 1.86 | 2.79 |
| Hristov et al., 2009 | Control | 156 | 230 | 5.9 | | 18.7 | 1.17 | 3.33 |
| | Lauric acid | 170 | 227 | 7.1 | | 17.6 | 1.28 | 3.15 |
| | Coconut oil | 60 | 248 | 2.5 | | 17.1 | 0.45 | 3.18 |
| Mohammed et al. 2011 | Control | 293 | 386 | 16.3 | | 19.7 | | |
| | Sunflower seeds | 264 | 413 | 14.6 | | 17.1 | | |
| | Linseeds | 241 | 408 | 13.4 | | 19.3 | | |
| | Canola seeds | 265 | 408 | 13.7 | | 18.2 | | |
| Hamilton et al. 2010 | Control | | | | | | 2.36 | 2.36 |
| | Monensin 14 d[c] | | | | | | 2.57 | 2.50 |
| | Control | | | | | | 1.95 | 2.36 |
| | Monensin 60 d[c] | | | | | | 2.07 | 2.70 |
| **Pearson correlation** | | | **0.56** | | **–0.69** | **0.40** | | **–0.13** |

[a] Rep = reported $CH_4$ emissions; Calc = calculated $CH_4$ from models in Chapter 4

[b] Lauric acid, myristic acid, linseed oil and calcium fumarate were added at 0.4, 1.2, 1.5 and 0.7%, respectively on DM basis

[c] Monensin after 14 and 60 days of supplementation

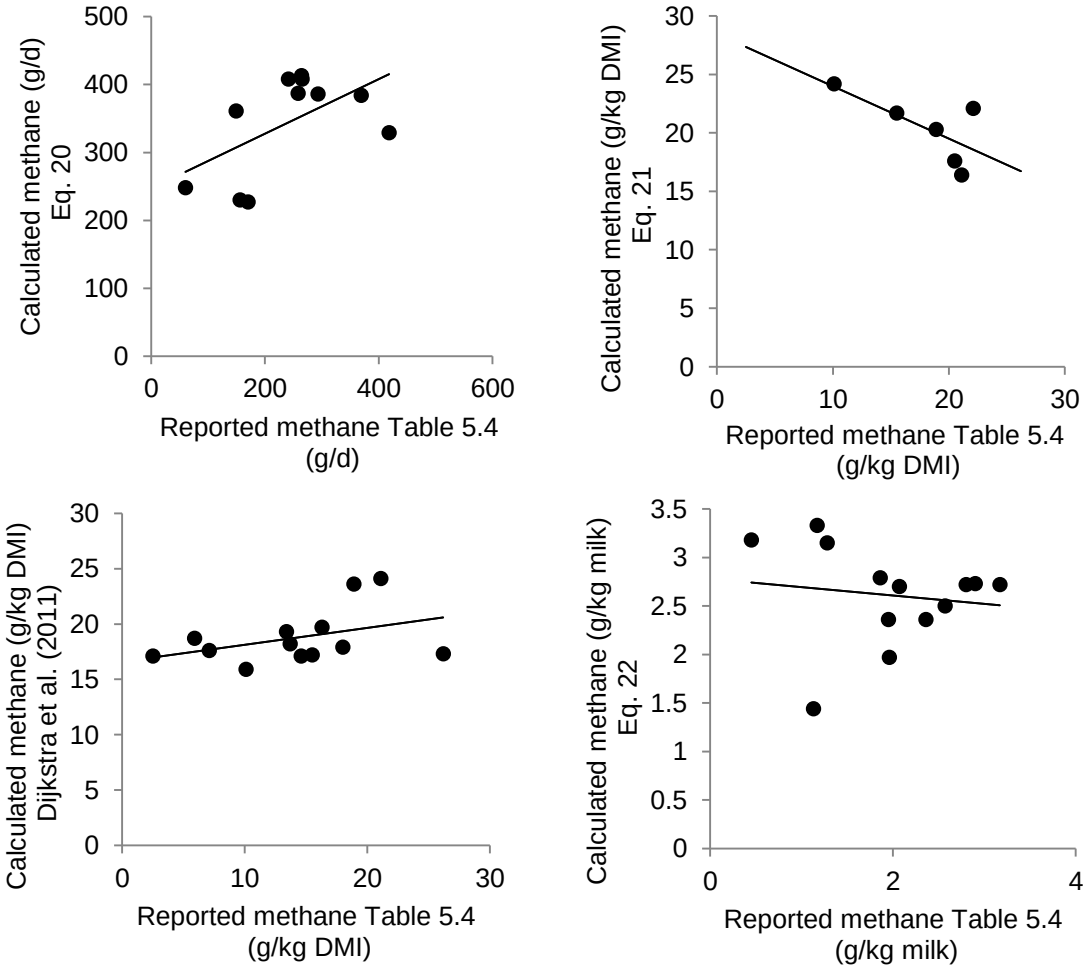


Figure 5.5. Scatter plots of reported and calculated methane emissions from models based on MFA as presented in Table 5.4

Table 5.5. Methane emissions calculated from models based on intake variables. Observed $CH_4$ (Mj/d) was calculated from daily emissions reported (g/d)

| Study | Dietary treatment | Observed $CH_4$ (MJ/d) | Empirical models | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Chilliard et al., 2009 | Control | 23.2 | 24.1 | 24.7 | 19.2 | 17.2 | 16.9 | 14.3 | 20.0 |
| | Whole crude linseed | 20.5 | 23.9 | 24.4 | 19.0 | 17.2 | 16.3 | 13.9 | 19.6 |
| | Extruded linseed | 14.3 | 21.3 | 22.0 | 16.7 | 17.2 | 14.0 | 12.6 | 16.7 |
| | Linseed oil | 8.3 | 19.5 | 20.3 | 15.1 | 17.2 | 12.9 | 11.8 | 15.2 |
| Van Zijderveld et al., 2011 | Control | 20.1 | 21.0 | 20.7 | 16.5 | 15.9 | 15.2 | --- | --- |
| | Mix of additives | 18.1 | 20.6 | 20.3 | 16.1 | 15.9 | 14.5 | --- | --- |
| Sauer et al., 1998 | Control | 22.0 | 24.1 | 25.0 | 19.2 | 17.6 | --- | 17.1 | --- |
| | Soybean oil | 21.6 | 21.8 | 22.8 | 17.2 | 17.6 | --- | 15.7 | --- |
| | Control | 24.0 | 20.7 | 21.7 | 16.3 | 17.6 | --- | 15.6 | --- |
| | Monensin | 18.8 | 19.3 | 20.4 | 15.0 | 17.6 | --- | 14.6 | --- |
| Odongo et al., 2007 | Control | 22.2 | 19.9 | 20.4 | 15.5 | 16.9 | 16.2 | 15.6 | 16.4 |
| | Myristic acid | 14.2 | 19.0 | 19.6 | 14.7 | 16.9 | 14.5 | 14.5 | 14.9 |
| Johnson et al. 2002 | Control | 21.6 | 29.1 | 28.3 | 23.6 | 15.7 | 16.6 | 16.1 | 21.5 |
| | Cottonseed + canola seed fat | 21.9 | 31.0 | 30.0 | 25.2 | 15.7 | 18.9 | 18.0 | 23.5 |
| | Cottonseed + canola seed fat | 25.4 | 30.7 | 29.7 | 25.0 | 15.7 | 18.3 | 18.9 | 22.3 |
| Hristov et al., 2009 | Control | 8.7 | 30.3 | 29.6 | 24.7 | 15.9 | 21.1 | 21.8 | 23.1 |
| | Lauric acid | 9.5 | 28.0 | 27.4 | 22.6 | 15.9 | 19.4 | 20.3 | 21.1 |
| | Coconut oil | 3.3 | 28.5 | 27.8 | 23.1 | 15.9 | 19.8 | 20.6 | 21.5 |
| Hristov et al., 2011 | Control | 16.7 | 30.7 | 30.4 | 25.0 | 16.6 | 22.9 | 20.0 | 26.0 |
| | Lauric acid | 16.1 | 24.3 | 24.4 | 19.4 | 16.6 | 17.8 | 16.4 | 19.9 |
| | Myristic acid | 17.3 | 29.6 | 29.4 | 24.0 | 16.6 | 22.0 | 19.4 | 24.9 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Mohammed et al., 2011 | Control | 16.3 | 22.6 | 21.4 | 17.9 | 14.8 | 14.1 | 13.9 | 16.8 |
| | Sunflower seeds | 14.7 | 22.7 | 21.5 | 18.0 | 14.8 | 14.9 | 14.5 | 17.3 |
| | Linseeds | 16.2 | 22.5 | 21.3 | 17.8 | 14.8 | 14.1 | 13.9 | 16.7 |
| | Canola seeds | 14.7 | 23.8 | 22.6 | 18.9 | 14.8 | 15.4 | 15.1 | 18.0 |
| Hamilton et al., 2010 | Control | 15.0 | 31.4 | 32.8 | 25.6 | 18.8 | 20.1 | 20.4 | 23.5 |
| | Monensin 14 d[c] | 16.3 | 31.2 | 32.6 | 25.5 | 18.8 | 20.0 | 20.3 | 23.3 |
| | Control | 12.5 | 32.2 | 33.5 | 26.3 | 18.8 | 20.6 | 20.8 | 24.1 |
| | Monensin 60 d[c] | 13.2 | 29.0 | 30.5 | 23.5 | 18.8 | 18.5 | 19.0 | 21.5 |
| Hollman et al., 2012 | Control | 25.8 | 27.0 | 26.1 | 21.8 | 15.5 | 19.8 | 27.0 | 26.1 |
| | Coconut oil | 25.0 | 25.6 | 24.8 | 20.5 | 15.5 | 18.4 | 25.6 | 24.8 |
| | Coconut oil | 16.2 | 22.4 | 21.8 | 17.7 | 15.5 | 15.5 | 22.4 | 21.8 |
| | Coconut oil | 14.1 | 20.8 | 20.3 | 16.3 | 15.5 | 14.2 | 20.8 | 20.3 |
| Moate et al., 2013 | Control | 30.2 | 28.1 | 29.7 | 22.7 | 19.0 | 27.3 | 30.2 | 23.0 |
| | Algal meal (125 g/d) | 31.3 | 28.1 | 29.7 | 22.7 | 19.0 | 27.3 | 30.2 | 23.0 |
| | Algal meal (250 g/d) | 30.7 | 26.8 | 28.4 | 21.6 | 18.8 | 26.0 | 26.5 | 23.2 |
| | Algal meal (375 g/d) | 28.9 | 25.7 | 27.3 | 20.6 | 18.7 | 25.0 | 25.3 | 22.3 |
| **Pearson correlation** | | | **0.03** | **0.10** | **0.03** | **0.32** | **0.50** | **0.47** | **0.33** |

1 $CH_4$ (MJ/d) = 5.93 + 0.92 × DMI (kg/d) (Mills et al., 2003)
2 $CH_4$ (MJ/d) = 1.06 + 10.27 × Dietary forage proportion + 0.87 × DMI (kg/d) (Mills et al., 2003)
3 $CH_4$ (MJ/d) = 3.23 + 0.809 × DMI (kg/d) (Ellis et al., 2008)
4 $CH_4$ (MJ/d) = 8.56 + 0.139 × forage (%) (Ellis et al., 2008)
5 $CH_4$ (MJ/d) = 3.14 + 2.11 × NDF (kg/d) (Ellis et al., 2008)
6 $CH_4$ (MJ/d) = 5.87 + 2.43 × ADF (kg/d) (Ellis et al., 2008)
7 $CH_4$ (MJ/d) = 2.16 + 0.493 × DMI (kg/d) - 1.36 × ADF (kg/d) + 1.97 × NDF (kg/d) (Ellis et al., 2008)

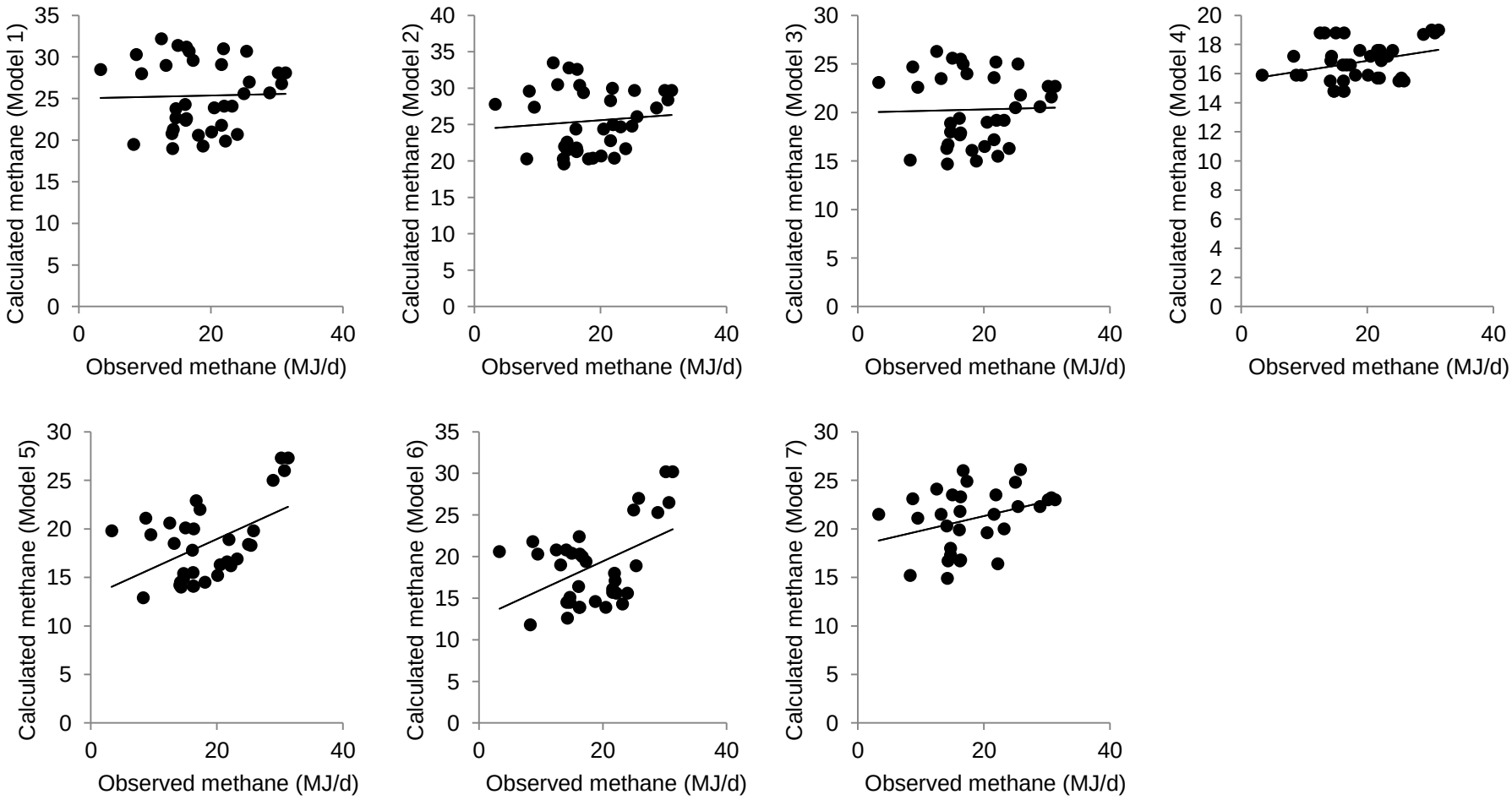


Figure 5.6. Scatter plots of reported and calculated methane emissions from models based on intake variables as presented in Table 5.5

Even though, a modest correlation between calculated and reported daily $CH_4$ was observed, in general, the potential of our models to predict $CH_4$ from literature data is limited. It has to be noted that all of the data available were derived from studies supplementing additives to decrease $CH_4$. Indeed in our data set the majority of the additives to decrease $CH_4$ did not succeed in this and most of the variation in our data set was due to natural variations between cows. Along the same line, in a further attempt to compare our model with other models found in literature (Table 5.3) we calculated $CH_4$ emissions (MJ/d) (Table 5.5).

Pearson correlation between calculated and observed methane from empirical models in Table 5.5 were similar to those with the MFA based models in Table 5.4 (with the exception of g/kg DMI). However, some of these models (e.g. Model 5, 6 and 7) seem to a slightly better ability to predict emissions than MFA-based models (Figure 5.5, Figure 5.6). In general these models are able to predict emissions when a decrease in DMI occurs along $CH_4$ inhibition, but they are not able to predict the proportion of such $CH_4$ inhibition. Relying solely or mainly on the intake of dry matter to predict $CH_4$ emissions might not be suitable for mitigation strategies with small amounts of an additive supplemented decreasing $CH_4$ without affecting the DMI. Yet, DMI is an important factor determining $CH_4$ emissions. This is also highlighted by results in Chapter 4, where the model to predict $CH_4$ relative to DMI had a higher marginal $R^2$ than models predicting daily emissions and $CH_4$ relative both to milk and metabolic bodyweight. To test the influence of DMI in the models, in a brief exercise we included DMI as another predictor variable along with MFA to develop another model to predict daily $CH_4$ emissions:

$$CH_4\ (g/d) = 17.2 \times DMI - 273.2 \times C17{:}0 + 6.5 \times C18{:}0 + 108.7 \quad (24)$$

$$R^2_{(m)} = 0.44;\ R^2_{(c)} = 0.81$$

Indeed, DMI was selected as the most important predictor (based on standardized coefficients) in the model and the variance explained by the independent variables increased from 33% in the model developed only from MFA (Chapter 4) to 44% with the inclusion of DMI. There might be a scope to integrate DMI and other diet-related variables with MFA data to improve the predictions of $CH_4$ emissions. This was done within the study of Mohammed et al. (2011) who found a higher $R^2$ in a model developed from MFA together with intake and production data ($R^2 = 0.83$) than in a model only from MFA ($R^2 = 0.74$). The increase in the additional variance explained when including DMI in the study of Mohammed et al. (2011) is comparable to the increase found by us.

Finally, Chorafas (1965) stated that ideally models should have the following features: 1) simplicity for manipulation and understanding by those applying the model. 2) Representative enough to allow generalization to a wide range of situations. 3) Sufficient complexity to accurately represent the system under study. Moreover, another feature of models regarded by us as important was the simplicity to obtain the parameters included in the model. This constitutes the main criticism given to mechanistic models and even to empirical models. Milk FA profiling requires an extensive analysis, but some MFA have potential to be routinely analyzed. In this regard, from the models developed in Chapter 4, the one for the prediction of $CH_4$ relative to DMI might be of most interest. In that model C14:0 and *cis*-9 C18:1 hold potential to be analyzed spectrofotometrically (Soyeurt et. al., 2011). However, C17:0 and *cis*-9 C17:1 can not be routine analyzed so far. On the other hand, C15:0, another odd-chain FA holding several similarities in their origin and nature which C17:0, has the potential to be analyzed by spectrofotometry (Stefanov et al., 2012) and hold a modest correlation with C17:0 ($r = 0.55$) in our data set. However, when both C17:0 and *cis*-9 C17:1 were substituted by C15:0 in the model to predict $CH_4$ in g/kg DMI, $R^2_{(m)}$ decreased drastically to 0.05, which rules out the possibility of using substituting C17:0 and its

desaturase product with C15:0 to obtain a similar prediction from MFA with the potential to be routine analyzed.

There seem to be concordances across studies and the results of this PhD dissertation on the most important individual MFA related to $CH_4$, as outlined in this discussion. However, no single MFA can be used as a biomarker for emissions. However, multivariate MFA-based models explain a low to modest amount of the variation in $CH_4$, highlighting their limited potential to predict these emissions. This was confirmed with a small set of data from literature.

## FUTURE RESEARCH PERSPECTIVE

As outlined before, there are still other factors not studied within Chapters 1A and 1B that could play a role in the differences between *in vitro* and *in vivo* results, and it might be worthwhile to look at them in future research. Especial attention should be given to the fact that the differences between *in vitro* and *in vivo* results will vary depending on the additive studied. Furthermore, most of the additives inhibiting methanogenesis are regarded as having either a direct effect on microorganisms or as decreasing the availability of hydrogen for $CH_4$ formation. However, the effects of some additives might be beyond their direct detrimental effects on microorganisms, and could be related to a direct effect on the host and this should be studied in the future.

Most of the empirical models to predict $CH_4$ have been already validated by using independent data sets either collected from literature or from own experiments. We were not able to validate our observations with data from other experiments, and the information

available in literature (based on treatment means) is still very limited to allow for a meaningful validation of our models.

Along the same line, it is frequently argued that models are applicable only within the context from which they have been developed. Particularly regarding the prediction of $CH_4$, models derived from measurements in chambers would not be applicable to grazing animals (Storm et al., 2012). We acknowledge that our models respond to specific conditions, and diets could have been more variable to allow for a model with a greater generalization capacity. The fact that most of our observations derived from the same farm, compromises further the generalization capacity of our models. On the other hand, the conditions of the majority of our *in vivo* experiments are in general representative of a typical dairy farm in Belgium and The Netherlands, therefore, our models could be deemed specific for management conditions of dairy cattle in this region.

The prediction of $CH_4$ is still a challenge that will probably keep researchers looking for answers for years to come, and until more practical and more accessible techniques to measure $CH_4$ emissions are available for farmers, we have to rely mainly on models to estimate inventories of $CH_4$. The results of this PhD dissertation have certainly contributed to increase the knowledge on the potential of MFA as predictors of methanogenesis. Even though, their potential seems limited, still some important insights on rumen physiology and, therefore, rumen methanogenesis, can be acquired by them. Hence, the research on MFA should not be abandoned. It would be of great benefit gathering and developing models from already existing data sets containing individual $CH_4$-MFA information from previous studies (e.g. those included in the literature review of Chapter 3), which would account for approximately 276 extra observations.

Most models developed from diets not including fat dietary sources won't be able to reliably predict $CH_4$ emissions from animals fed sources of fat to mitigate $CH_4$. Eventually, specific models for this type of diets should be developed.

It is possible that MFA based models to predict $CH_4$ emissions won't perform as good as empirical and mechanistic models. Still, the potential of MFA to qualitatively explain variations in $CH_4$ (e.g. differentiate groups of high and low emitters) could be explore by using other statistical tools (e.g. linear discriminant analysis, logistic regression) can be used to assess the potential of MFA to identify animals with a natural tendency to be high or low $CH_4$ emitters.

The combination of intake variables and MFA could improve the ability of models to predict $CH_4$ emissions. As mentioned above, this has been already done by Mohammed et al. (2011) with positive effects on $R^2$. A larger data set, like the one we have collected, could provide a more general insight on a combined model.

The importance of certain MFA, particularly, C17:0 has been highlighted with the results of this PhD, however, so far it is not possible to determine concentrations of this MFA in milk on a routine basis. The PhD dissertation of Stefanov (2012) explored the possibility of estimating C17:0 concentrations spectrofotometrically without success. Further research on the estimation of C17:0 in milk might be worthwhile. It is not clear the unique physiological relationship that C17:0 and no other MFA (e.g. C15:0) holds with $CH_4$ emissions. Therefore, it might be worthwhile to take a deeper look at the origin of C17:0 and its possible presence in microorganisms closely linked with methanogenesis (e.g. those competing with methanogens). The fatty acids profile of bacteria in the rumen has been already studied for diverse species, but that of Archaea species remained mainly unstudied and it might give important hints on this topic.

The estimation of the amount of $CH_4$ produced by ruminants is of paramount importance in order to effectively apply mitigation strategies in dairy farms. In this PhD dissertation two approaches have been tested for their ability to quantify those emissions.

First, a number of *in vitro* experiments was performed in order to evaluate their ability to reflect the effects that dietary additive cause *in vivo.* A first series of *in vitro* incubations tested the interaction between seven feed additives [*i.e.,* quillaja saponins, fumaric acid, garlic oil, fish oil, cinnamaldehyde, monensin, medium chain fatty acids (MCFA)], having different modes of action to inhibit $CH_4$, and feeds regularly used in dairy cattle feeding [concentrate (CON), grass silage (GS), maize silage (MS) and a mixture of CON+GS+MS (300:350:350 (dry matter, DM), MIX)]. These incubations were done in a batch system containing buffered rumen fluid for 24 h. No additive × substrate interactions were observed for cinnamaldehyde, garlic oil and fumaric on $CH_4$ (µmol/flask) inhibition. Whereas, fish oil, quillaja saponins, monensin and MCFA had additive × substrate interactions for inhibition of $CH_4$. Monensin was more effective in combination with GS, and quillaja saponins were more effective when combined with MS. Results clearly indicate *in vitro* interactions between additives and substrates. Cinnamaldehyde and MCFA strongly inhibited fermentation which impaired appropriate evaluation of the most promising combination. Further, average calculated $CH_4$ production (based on incuabtions with individual substrates and their proportions within MIX) was compared with the actual $CH_4$ production measured in incubations with MIX. Regression of calculated on measured $CH_4$ indicated no synergism between additive and substrate combinations seemed to exist.

However, it is unlikely that these interactions are the main reason for variable results among *in vitro* studies or the differences observed between *in vitro* and *in vivo* studies. Therefore, other factors that might influence these discrepancies were studied by comparing the results of

both *in vivo* and *in vitro* experiments performed with the same product. Most *in vitro vs. in vivo* comparisons indicated stronger *in vitro* effects. However, in the current PhD dissertation *in vitro* and *in vivo* results were compared for a blend of essential oils which provoked similar $CH_4$ inhibitory effects (–10 to –15%) as compared with a non-supplemented control, but at a much lower dose *in vivo* compared to *in vitro*. A blend of essential oils was supplemented to beef and dairy cattle in two separated *in vivo* experiments during six weeks, and the same product was incubated in four *in vitro* experiments. In both *in vivo* experiments animals were supplemented with 0.2 g/d of the essential oils (*ca.* 2 ppm *m/v*) finding decreases in daily $CH_4$ emissions ranging between 12 and 15%. The *in vitro* experiments attempted to replicate the results observed *in vivo.* A decrease in CH production of 15% was observed in 24 h batch incubations but only at a much higher concentration (50 ppm, *m/v*). Methane was inhibited to a similar extent (17%) when somewhat lower amounts of essential oils (30 ppm) were applied in a gas production technique (GPT) system. Nevertheless, the concentrations needed *in vitro* are still considerably higher than those applied *in vivo*. A longer exposure time of the feedstuff to the essential oils (essential oils added *ca*. 16 h prior the start of the incubation) did not elicit any effect on $CH_4$ production and was not different from addition immediately prior to the start of the incubation. Similarly, a longer incubation time and regular supply of both substrate and additive in a consecutive batch incubation system with transfers every 24 h for 96 h or transfers every 48 h for 14 d did not induce $CH_4$ inhibition at lower doses of essential oils (5, 15 and 30 ppm, *m/v*). In this comparative study modifications to the *in vitro* set-up did not allow to cancel out large differences in the essential oils doses needed *in vitro* to provoke similar $CH_4$ inhibitory effects as observed *in vivo*. For this particular case, these results suggest that essential oils might have a different mode of action *in vitro* and *in vivo*, which merits attention for future research. Essentially, current *in vitro* set-ups only allow to asses antimicrobial effects. It has to be noted that these experiments were performed with essential

oils, and different results might be observed with other dietary additives. Nevertheless, this study serves to highlight the gap, still to be fulfilled, between *in vitro* and *in vivo* systems. *In vitro* systems still need to be improved to better reflect $CH_4$ inhibition *in vivo*, and the modifications done in these experiments were not able to cancel such differences. Therefore, the second part of this PhD dissertation was dedicated to explore another approached, based on the relationships between milk fatty acids (MFA) and $CH_4$ production.

In a first study, a particular group of MFA, odd- and branched-chain fatty acids (OBCFA) were used to predict calculated $CH_4$ proportions (mmol/mol VFA). Three techniques were used for the development of the models: multiple linear regression (MLR), partial least squares regression (PLS) and a genetic algorithm approach (GA). Two-hundred twenty-four observations from 13 experiments were used for this study. Methane proportion (mmol/mol VFA) was calculated from acetate, propionate and butyrate and expressed relative to the sum of these volatile fatty acids (VFA). Calculated $CH_4$ proportion was related to milk OBCFA using MLR and PLS, resulting in a linear prediction model. The GA approach resulted in a model that predicted rumen VFA proportions of total VFA from milk OBCFA. Methane proportion was calculated from predicted acetate, propionate and butyrate proportions in total VFA based on rumen stoichiometry and compared with $CH_4$ proportion calculated from measured VFA proportions. The prediction error was low (*i.e.*, root mean square error < 5%), and the models captured up to 66% of the variance of calculated $CH_4$. However, the variance of the prediction error was less than 40% of the variance of the calculated $CH_4$ proportion. Seven milk OBCFA were initially considered as predictors, from which branched-fatty acids (*iso* C14:0, *iso* C15:0, *iso* C16:0) were positively related to calculated enteric $CH_4$ production and odd-fatty acids (*i.e.,* C15:0 and sum of C17:0 and C17:1 *cis*-9) were negatively related to $CH_4$. Moreover, there were no differences in the performance of the models developed by the three different techniques. Relationships in this large data set allowed to identify the most

relevant OBCFA in milk as potential predictors of rumen methanogenesis. However, further exploration showed evidence that other MFA also might hold potential as predictors of $CH_4$ emissions. Diverse studies have reported changes in MFA profiles when $CH_4$ mitigation additives have been fed to the animals. Nevertheless, it is difficult to draw conclusions from single experiments. Hence, in an attempt to get a broader exploration of the relationships between MFA and $CH_4$, both univariate and multivariate analyses were performed using data from 15 studies including 48 dietary treatments to inhibit $CH_4$. Methane was expressed as daily emissions (g/d), relative to dry matter intake (g/kg DMI), relative to milk production (g/kg milk) and relative to metabolic body weight (g/kg $BW^{0.75}$/d). The univariate correlations between MFA and $CH_4$ were based on absolute means and on relative changes of each treatment compared with its corresponding control. The univariate analyses suggested that SFA, OBCFA and long chain PUFA were positively related to $CH_4$, while MUFA negatively. In general, the coefficient of determination ($R^2$) of the univariate regressions ranged from 0.1 to 0.6, indicating that individual MFA are not able to largely explain variation in $CH_4$. Furthermore, four multivariate models were developed linking MFA to $CH_4$ in each of the four units (g/d, g/kg DMI, g/kg milk and g/kg $BW^{0.75}$/d). Milk fatty acids retained in each equation differed, highlighting the importance of the functional unit to express $CH_4$. Similarities were observed in the MFA related to $CH_4$ in g/d and in g/kg $BW^{0.75}$/d. On the other hand, similarities also appeared in MFA related to $CH_4$ in g/kg DMI and in g/kg milk.

Nonetheless, it is necessary to mention the data set gathered from literature had some limitations especially related with the different methodologies to determine MFA in each study and the type of additives fed to the animals in such experiments. Therefore, even though the findings of this literature review certainly contribute to the exploration of the potential of MFA as biomarkers for $CH_4$ emissions, still these results should be validated by a complete and uniform data set containing, if possible containing a larger amount of observations.

In this regard, eight *in vivo* experiments were conducted in the experimental farm of ILVO, Belgium, measuring $CH_4$ emissions and collecting milk samples for FA profiling. An additional experiment conducted at IBERS, Wales was added to complete a data set containing 145 observations. Models to predict $CH_4$ emissions expressed in four functional units (g/d, g.kg DMI, g/kg milk and g/kg $BW^{0.75}$/d) were developed by multiple linear regression. Marginal ($R^2_{(m)}$, variation explained by fixed effects) and conditional ($R^2_{(c)}$, variation explained by fixed and random effects combined) were calculated to evaluate the models. In general MFA had a modest potential to explain variations in $CH_4$: $R^2_{(m)}$ ranged between 0.19 (g $CH_4$/kg $BW^{0.75}$/d) and 0.55 (g $CH_4$/kg DMI). Milk FA explained higher variation in $CH_4$ when the latter was expressed relative to DMI. Standardized coefficients across the equations showed that C17:0 and its desaturase product *cis*-9 C17:1 is a highly relevant MFA in the prediction of $CH_4$ emissions. Furthermore, a novel approach adopted from recent studies on microbial community organization was used to present the cumulative distribution of MFA and its relationship with $CH_4$ emissions. Gini coefficients were calculated for different subsets of the data set (based on the quantity of MFA) and for three categories depending on the amount of $CH_4$ produced (HIGH, MEDIUM and LOW $CH_4$ producers). Gini coefficients of the subset of MFA including those with concentrations lower than 0.625 g/100 g MFA showed relationship with $CH_4$ emissions. Furthremore, Gini coefficients of MFA belonging to the category HIGH, differed from those of categories MEDIUM and LOW. However, the differences observed in Gini coefficients between groups and categories were minimal and might not aid to the identification of low or high emitters.

There are clear relationships between MFA and $CH_4$. However, from previous studies and from our own results, robustness of the models to predict $CH_4$ seems limited and does not to improve predictions compared with other models (*i.e.* feed based models). Nevertheless, MFA are still able to give important information on the rumen Metabolism, which merits further

research. Milk FA data collected through various experiment by several research centers could be merged within an international consortium to assess the ultimate potential of this approach.

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Table A.1. Amount and source of fatty acids added to the diet from the respective control for each treatment (ordered by the relative change in daily $CH_4$ production) and its classification into one of the categories MCFA, UFA and Other

| # | Treatment | Change in $CH_4$ (%) [a] | g FA/d (g FA/kg BW) | | | | Category |
|---|---|---|---|---|---|---|---|
| | | | MCFA | MUFA | PUFA | Total UFA | |
| *Significant decreasing effect on $CH_4$ (g/d)* | | | | | | | |
| 1 | Linseed oil (Exp 3) | -64.3 | 0 | 91.2 (0.131) | 566 (0.815) | 657 (0.946) | UFA |
| 2 | Coconut oil (Exp 7) | -61.5 | 346 (0.723) | 0 | 0 | 0 | MCFA |
| 3 | Coconut oil 3.3% (Exp 10) | -45.5 | 407 (n.d.) | 36.9 (n.d.) | 9.09 (n.d.) | 46.0 (n.d.) | MCFA |
| 4 | Extruded linseed (Exp 3) | -38.3 | 0 (0) | 58.6 (0.084) | 410 (0.589) | 468 (0.674) | UFA |
| 5 | Coconut oil 2.7% (Exp 10) | -37.3 | 368 (n.d.) | 33.4 (n.d.) | 8.22 (n.d.) | 41.6 (n.d.) | MCFA |
| 6 | Oreganum leaves 750 g/d (Exp 15) | -35.8 | 0 | 0 | 0 | 0 | Other |
| 7 | Myristic acid (Exp 6) | -35.8 | 710 (1.42) | 0 | 0 | 0 | MCFA |
| 8 | Bromochloromethane (Exp 13) | -32.0 | 0 | 0 | 0 | 0 | Other |
| 9 | Oreganum leaves 500 g/d (Exp 15) | -27.0 | 0 | 0 | 0 | 0 | Other |
| 10 | Monensin (Exp 1) | -21.6 | 0 | 0 | 0 | 0 | Other |
| 11 | Linseeds (Exp 4) | -17.8 | 0 | 0 | 506 (1.99) | 506 (1.99) | UFA |
| 12 | Cumin seed extract 2.53% (Exp 14) | -11.8 | 0 | 0 | 0 | 0 | Other |
| 13 | Crude linseed (Exp 3) | -11.7 | 0 | 78.9 (0.113) | 427 (0.615) | 506 (0.728) | UFA |
| 14 | Mix of dietary additives (Exp 8) | -10.2 | 254 (0.328) | 45.3 (0.058) | 170 (0.219) | 215 (0.278) | MCFA |
| 15 | Sunflower seed (Exp 4) | -9.9 | 0 | 0 | 429 (1.68) | 429 (1.68) | UFA |
| 16 | Canola seeds (Exp 4) | -9.6 | 0 | 341 (1.34) | 342 (1.35) | 683 (2.69) | UFA |
| 17 | Cumin seed extract 1.27% (Exp 14) | -9.5 | 0 | 0 | 0 | 0 | Other |
| 18 | Oreganum leaves 250 g/d (Exp 15) | -4.8 | 0 | 0 | 0 | 0 | Other |
| 19 | Coconut oil 1.3% (Exp 10) | -3.2 | 212 (n.d.) | 19.2 (n.d.) | 4.73 (n.d.) | 23.9 (n.d.) | MCFA |

| | | | | | | | |
|---|---|---|---|---|---|---|---|
| *No significant effect on $CH_4$ (g/d)* | | | | | | | |
| 20 | Algal meal 375 g/d (Exp 5) | -4.2 | 0 | 0 | 25 (0.042) | 25 (0.042) | UFA |
| 21 | Lauric acid (Exp 9) | -3.2 | 240 (0.511) | 0 | 0 | 0 | MCFA |
| 22 | Soybean oil (Exp 1) | -1.2 | 0 | 138 (0.165) | 370 (0.443) | 508 (0.608) | UFA |
| 23 | Cottonseed and ground canola oilseeds 4% fat (Exp 2) | +1.2 | 3.1 (n.d.) | 167 (n.d.) | 223 (n.d.) | 390 (n.d.) | UFA |
| 24 | Algal meal 250 g/d (Exp 5) | +1.8 | 0 | 0 | 50 (0.084) | 50 (0.084) | UFA |
| 25 | *Saccharomyces cerevisiae* (Exp 12) | +3.1 | 0 | 0 | 0 | 0 | Other |
| 26 | Algal meal 125 g/d (Exp 5) | +3.7 | 0 | 0 | 75 (0.127) | 75 (0.127) | UFA |
| 27 | Myristic acid (Exp 9) | +4.0 | 240 (0.514) | 0 | 0 | 0 | MCFA |
| 28 | Monensin 60 d (Exp 11) | +5.8 | 0 | 0 | 0 | 0 | Other |
| 29 | Monensin 14 d (Exp 11) | +9.1 | 0 | 0 | 0 | 0 | Other |
| 30 | Lauric acid (Exp 7) | +9.2 | 240 (0.498) | 0 | 0 | 0 | MCFA |
| 31 | Cottonseed and ground canola oilseeds 5.6% fat (Exp. 2) | +17.3 | 4.8 (n.d.) | 355 (n.d.) | 376 (n.d.) | 731 (n.d.) | UFA |

[a] Change relative to the corresponding control
n.d. not determined by lack of information on BW

Table A.2. Summary statistics of the variables selected for the analysis

| Variable | n | avg | stdev | max | min |
|---|---|---|---|---|---|
| *Dependent variables (Methane)* | | | | | |
| $CH_4$ (g/kg DM) | 48 | 15.4 | 8.04 | 35.9 | 1.97 |
| $CH_4$ (g/kg milk) | 48 | 11.5 | 6.77 | 26.7 | 1.66 |
| $CH_4$ (g/d) | 43 | 328 | 154 | 603 | 48.2 |
| $CH_4$ (g/kg $BW^{0.75}$/d) | 41 | 2.40 | 1.38 | 4.90 | 0.35 |
| *Predictor variables (MFA, mg/100 mg milk fat)* | | | | | |
| C4:0 | 29 | 3.2 | 1.77 | 6.7 | 0.26 |
| C6:0 | 38 | 2.4 | 1.46 | 6.0 | 0.53 |
| C8:0 | 38 | 1.4 | 0.85 | 4.5 | 0.41 |
| C10:0 | 38 | 3.5 | 3.21 | 15.7 | 0.84 |
| C11:0 | 23 | 0.14 | 0.07 | 0.3 | 0.01 |
| C12:0 | 42 | 3.5 | 1.58 | 7.4 | 1.12 |
| C13:0 | 22 | 0.13 | 0.04 | 0.2 | 0.04 |
| *Iso* 14:0 | 17 | 0.22 | 0.26 | 0.7 | 0.05 |
| C14:0 | 42 | 10.7 | 4.15 | 24.8 | 4.64 |
| *Cis*-9 C14:1 | 31 | 1.0 | 0.59 | 2.5 | 0.16 |
| *Anteiso* C15:0 | 17 | 0.36 | 0.17 | 0.7 | 0.20 |
| *Iso* C15:0 | 17 | 0.21 | 0.09 | 0.4 | 0.06 |
| C15:0 | 32 | 1.0 | 0.31 | 1.6 | 0.47 |
| C16:0 | 42 | 26.8 | 8.83 | 47.7 | 12.1 |
| *Cis*-9 C16:1 | 24 | 1.5 | 0.62 | 2.8 | 0.58 |
| *Trans*-9 C16:1 | 15 | 0.42 | 0.36 | 1.2 | 0.03 |
| *Anteiso* C17:0 | 17 | 0.34 | 0.16 | 0.6 | 0.12 |
| C17:0 | 34 | 0.48 | 0.21 | 1.0 | 0.18 |
| *Cis*-9 C17:1 | 18 | 0.22 | 0.10 | 0.4 | 0.07 |
| C18:0 | 42 | 9.0 | 4.19 | 17.3 | 1.50 |
| *Cis*-9 C18:1 | 30 | 20.0 | 4.47 | 28.8 | 13.2 |
| *Cis*-11 C18:1 | 19 | 0.51 | 0.19 | 1.0 | 0.35 |
| *Cis*-12 C18:1 | 19 | 0.28 | 0.19 | 0.7 | 0.10 |
| *Cis*-13 C18:1 | 19 | 0.14 | 0.06 | 0.3 | 0.02 |
| *Trans*-6+7+8 C18:1 | 22 | 0.44 | 0.26 | 1.1 | 0.08 |
| *Trans*-9 C18:1 | 22 | 0.33 | 0.14 | 0.7 | 0.17 |
| *Trans*-10 C18:1 | 22 | 1.0 | 1.09 | 4.5 | 0.26 |
| *Trans*-11 C18:1 | 29 | 1.1 | 0.61 | 3.1 | 0.45 |
| *Trans*-12 C18:1 | 22 | 0.46 | 0.28 | 1.2 | 0.17 |
| *Trans*-13+14 C18:1 | 17 | 1.1 | 1.21 | 5.2 | 0.21 |
| *Cis*-9, *trans*-11 CLA | 34 | 0.74 | 0.41 | 2.0 | 0.28 |
| *Cis*-9, *cis*-12 C18:2 | 24 | 2.3 | 1.19 | 5.5 | 0.03 |
| *n-3* C18:3 | 20 | 0.59 | 0.26 | 1.3 | 0.21 |

| | | | | | |
|---|---|---|---|---|---|
| C20:0 | 31 | 0.12 | 0.05 | 0.2 | 0.05 |
| *n-6* C20:2 | 15 | 0.18 | 0.23 | 0.6 | 0.01 |
| *n-6* C20:3 | 15 | 0.08 | 0.03 | 0.1 | 0.02 |
| *n-6* C20:4 | 19 | 0.07 | 0.07 | 0.4 | 0.04 |
| *n-3* C20:5 (EPA) | 19 | 0.09 | 0.04 | 0.2 | 0.02 |
| *n-3* C22:5 (DPA) | 21 | 0.08 | 0.14 | 0.7 | 0.02 |
| Total CLA | 33 | 0.71 | 0.51 | 2.3 | 0.30 |
| Total MUFA | 27 | 24.9 | 9.06 | 55.4 | 14.2 |
| Total SFA | 42 | 62.1 | 21.2 | 114 | 27.7 |
| Total PUFA | 35 | 4.2 | 2.49 | 11.2 | 1.45 |
| Total OBCFA | 25 | 2.7 | 0.85 | 4.8 | 1.36 |
| Total FA < C16 | 41 | 26.5 | 9.85 | 53.7 | 9.73 |
| Total C16 | 42 | 29.0 | 5.22 | 17.8 | 38.7 |
| Total FA > C16 | 25 | 40.0 | 14.01 | 71.2 | 20.9 |
| Total *cis* C18:1 | 23 | 19.6 | 6.87 | 39.8 | 12.0 |
| Total *trans* C18:1 | 31 | 3.8 | 2.32 | 11.0 | 1.62 |
| Total *Cis*-MUFA | 20 | 22.1 | 6.24 | 36.5 | 15.0 |
| Total *Trans*-MUFA | 22 | 4.9 | 3.87 | 17.8 | 2.02 |
| Total *n-3* PUFA | 24 | 1.1 | 0.89 | 2.8 | 0.14 |
| Total *n-6* PUFA | 32 | 2.7 | 1.52 | 6.3 | 0.61 |

Table A.3. Equations linearly relating milk fatty acid proportions (g/100 g milk fat) to methane emissions (g/d, g/kg DMI, g/kg milk, g/kg $BW^{0.75}$/d). Regression coefficients ± standard error. $R^2$ presented in brackets and in bold at the end of each equation

| Parameter | g/d | g/kg DMI | g/kg milk | g/kg $BW^{0.75}$/d |
|---|---|---|---|---|
| C4:0 | $-111_{(\pm 165.1)} + 298x_{(\pm 70.48)}$ **( 0.57)** | $8.87_{(\pm 6.44)} + 3.41x_{(\pm 1.58)}$ **(0.72)** | | |
| C6:0 | $274_{(\pm 124.8)} + 56.5x_{(\pm 26.22)}$ **(0.45)** | | $5.84_{(\pm 4.04)} + 2.64x_{(\pm 0.75)}$ **(0.36)** | $1.50_{(\pm 1.04)} + 0.47x_{(\pm 0.2)}$ **(0.57)** |
| C8:0 | $142_{(\pm 146.3)} + 210x_{(\pm 57.36)}$ **(0.47)** | $7.83_{(\pm 5.04)} + 6.3x_{(\pm 2.16)}$ **(0.33)** | $2.2_{(\pm 4.71)} + 7.44x_{(\pm 1.77)}$ **(0.36)** | $0.50_{(\pm 1.22)} + 1.58x_{(\pm 0.46)}$ **(0.58)** |
| C10:0 | $210_{(\pm 118.0)} + 85.7x_{(\pm 28.29)}$ **(0.46)** | $11.9_{(\pm 4.36)} + 1.02x_{(\pm 0.51)}$ **(0.32)** | | |
| C11:0 | $163_{(\pm 127.8)} + 38.6 \times 10^2 x_{(\pm 1689)}$ **(0.80)** | | $5.49_{(\pm 4.12)} + 146.6x_{(\pm 53.1)}$ **(0.57)** | $1.07_{(\pm 0.97)} + 33.4x_{(\pm 13.5)}$ **(0.83)** |
| C13:0 | $99.7_{(\pm 136.2)} + 24.5 \times 10^2 x_{(\pm 993.8)}$ **(0.56)** | | $3.65_{(\pm 4.83)} + 90.5x_{(\pm 31.5)}$ **(0.55)** | $0.92_{(\pm 1.4)} + 19.9x_{(\pm 9.69)}$ **(0.75)** |
| *Anteiso* C15:0 | $-15.9_{(\pm 219.8)} + 925x_{(\pm 399.9)}$ **(0.42)** | $0.82_{(\pm 6.84)} + 29.2x_{(\pm 12.9)}$ **(0.34)** | $-1.75_{(\pm 6.43)} + 36.5x_{(\pm 12.4)}$ **(0.07)** | $-0.61_{(\pm 1.65)} + 7.92x_{(\pm 3.16)}$**(0.62)** |
| *Iso* C15:0 | $-89.5_{(\pm 122.7)} + 23.8 \times 10^2 x_{(\pm 485.9)}$ **(0.41)** | $-1.73_{(\pm 4.86)} + 77.7x_{(\pm 19.1)}$ **(0.34)** | $-3.26_{(\pm 3.69)} + 88.6x_{(\pm 15.1)}$ **(0.06)** | $-1.19_{(\pm 0.96)} + 20.9x_{(\pm 3.97)}$**(0.61)** |
| C15:0 | $-110_{(\pm 163.6)} + 503x_{(\pm 132.4)}$ **(0.42)** | $6.06_{(\pm 7.36)} + 12.5x_{(\pm 6.4)}$ **(0.58)** | $-3.01_{(\pm 5.22)} + 17.0x_{(\pm 4.29)}$ **(0.39)** | $-1.29_{(\pm 1.34)} + 4.5x_{(\pm 1.07)}$ **(0.63)** |
| *Cis*-9 C17:1 | | $45_{(\pm 8.15)} - 83.5x_{(\pm 32.5)}$ **(0.76)** | $31.4_{(\pm 7.78)} - 64.0x_{(\pm 29.9)}$ **(0.48)** | |
| C17:0 | | | $9.31_{(\pm 4.22)} + 6.28x_{(\pm 3.41)}$ **(0.52)** | |
| *Cis*-11 C18:1 | $661_{(\pm 183.6)} - 677x_{(\pm 182.1)}$ **(0.49)** | $20.3_{(\pm 6.48)} - 20.6x_{(\pm 6.39)}$ **(0.25)** | $20.7_{(\pm 6.23)} - 21.0x_{(\pm 6.54)}$ **(0.22)** | $4.64_{(\pm 1.46)} - 5.28x_{(\pm 1.49)}$ **(0.53)** |
| *Cis*-13 C18:1 | $322_{(\pm 98.6)} - 19.1 \times 10^2 x_{(\pm 243.9)}$ **(0.57)** | $11.2_{(\pm 4.07)} - 59.3x_{(\pm 10.3)}$ **(0.28)** | $11.8_{(\pm 3.22)} - 65.2x_{(\pm 9.7)}$ **(0.25)** | $2.33_{(\pm 0.78)} - 15.7x_{(\pm 2.13)}$ **(0.59)** |
| *Trans*-6 + 7 + 8 C18:1 | $338_{(\pm 96.77)} - 453x_{(\pm 121.9)}$ **(0.39)** | $11.4_{(\pm 3.88)} - 12.6x_{(\pm 4.8)}$ **(0.22)** | $12.6_{(\pm 3.34)} - 14.3x_{(\pm 4.69)}$ **(0.18)** | $2.49_{(\pm 0.78)} - 3.67x_{(\pm 1.0)}$ **(0.45)** |
| *Trans*-9 C18:1 | $388_{(\pm 113.1)} - 465x_{(\pm 201.8)}$ **(0.34)** | | $13.2_{(\pm 4.01)} - 13.2x_{(\pm 7.19)}$ **(0.16)** | $2.63_{(\pm 0.94)} - 3.3x_{(\pm 1.64)}$ **(0.40)** |
| *Trans*-10 C18:1 | $278_{(\pm 95.4)} - 97.9x_{(\pm 45.29)}$ **(0.35)** | | | $2.04_{(\pm 0.77)} - 0.79x_{(\pm 0.36)}$ **(0.41)** |
| *Trans*-11 C18:1 | | $5.35_{(\pm 3.44)} + 3.69x_{(\pm 1.57)}$ **(0.07)** | | |
| *Trans*-12 C18:1 | $417_{(\pm 86.9)} - 392x_{(\pm 82.19)}$ **(0.42)** | $13.8_{(\pm 3.74)} - 11.9x_{(\pm 3.1)}$ **(0.25)** | $15.1_{(\pm 3.17)} - 13.3x_{(\pm 2.92)}$ **(0.17)** | $2.99_{(\pm 0.76)} - 3.12x_{(\pm 0.67)}$ **(0.47)** |
| *Trans*-13 + 14 C18:1 | $444_{(\pm 91)} - 348x_{(\pm 82.58)}$ **(0.50)** | $16.8_{(\pm 4.07)} - 12.0x_{(\pm 2.79)}$ **(0.26)** | $17.3_{(\pm 3.47)} - 12.3x_{(\pm 2.83)}$ **(0.29)** | $3.76_{(\pm 0.73)} - 2.92x_{(\pm 0.68)}$ **(0.47)** |
| *n-6* C20:3 | | $4.13_{(\pm 8.05)} + 183x_{(\pm 64.27)}$ **(0.49)** | $2.90_{(\pm 6.51)} + 149x_{(\pm 65.2)}$ **(0.50)** | |
| *n-6* C20:4 | | $10.1_{(\pm 6.2)} + 46.3x_{(\pm 16.8)}$ **(0.49)** | $8.38_{(\pm 4.62)} + 35.1x_{(\pm 17.8)}$ **(0.49)** | |
| *n-3* C20:5 (EPA) | $166_{(\pm 152.2)} + 29.6 \times 10^2 x_{(\pm 1451)}$ **(0.79)** | $6.86_{(\pm 6.24)} + 140x_{(\pm 41.9)}$ **(0.55)** | $5.00_{(\pm 4.96)} + 110x_{(\pm 44.3)}$ **(0.58)** | $1.00_{(\pm 1.16)} + 25.3x_{(\pm 11.6)}$ **(0.82)** |
| *n-3* C22:5 | | $11.3_{(\pm 6.26)} + 38.2x_{(\pm 13.4)}$ **(0.53)** | | |
| Total FA < C16 | $180_{(\pm 121.1)} + 4.3x_{(\pm 2.23)}$ **(0.25)** | | $6.37_{(\pm 3.66)} + 0.15x_{(\pm 0.07)}$ **(0.07)** | $0.79_{(\pm 0.96)} + 0.05x_{(\pm 0.02)}$ **(0.48)** |
| Total FA > C16 | $401_{(\pm 207.1)} - 4.8x_{(\pm 2.51)}$ **(0.06)** | $15.6_{(\pm 6.55)} - 0.16x_{(\pm 0.09)}$ **(0.17)** | | |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Total MUFA | $866_{(\pm 164.8)} - 43.2x_{(\pm 7.84)}$ | **(0.54)** | | | $17.9_{(\pm 5.41)} - 0.25x_{(\pm 0.13)}$ | **(0.31)** | $4.07_{(\pm 1.35)} - 0.07x_{(\pm 0.03)}$ | **(0.66)** |
| Total SFA | $262_{(\pm 120.3)} + 2.2x_{(\pm 1.28)}$ | **(0.37)** | | | $6.32_{(\pm 4.1)} + 0.08x_{(\pm 0.03)}$ | **(0.36)** | | |
| Total PUFA | $601_{(\pm 156.9)} - 72.3x_{(\pm 34.25)}$ | **(0.34)** | $25_{(\pm 5.74)} - 1.41x_{(\pm 0.77)}$ | **(0.29)** | | | | |
| Total OBCFA | $-182_{(\pm 205.8)} + 218x_{(\pm 56.76)}$ | **(0.58)** | $-0.35_{(\pm 8.2)} + 6.24x_{(\pm 2.35)}$ | **(0.58)** | $-6.02_{(\pm 6.63)} + 7.36x_{(\pm 1.86)}$ | **(0.39)** | $-1.84_{(\pm 1.61)} + 1.83x_{(\pm 0.46)}$ | **(0.65)** |
| Total *cis* C18:1 | $852_{(\pm 297.9)} - 57.8x_{(\pm 26.48)}$ | **(0.39)** | | | | | | |
| Total *trans* C18:1 | | | | | $12.5_{(\pm 2.84)} - 1.33x_{(\pm 0.55)}$ | **(0.14)** | $2.39_{(\pm 0.68)} - 0.43x_{(\pm 0.13)}$ | **(0.12)** |
| Total *n-6* PUFA | $169_{(\pm 171.6)} + 82x_{(\pm 46.04)}$ | **(0.23)** | | | $5.96_{(\pm 5.11)} + 2.73x_{(\pm 1.46)}$ | **(0.24)** | $0.86_{(\pm 1.32)} + 0.74x_{(\pm 0.36)}$ | **(0.53)** |
| Total *trans* MUFA | $338_{(\pm 89.9)} - 31.6\,x_{(\pm 9.43)}$ | **(0.38)** | $11.3_{(\pm 3.79)} - 0.85x_{(\pm 0.36)}$ | **(0.22)** | $12.6_{(\pm 3.25)} - 0.98x_{(\pm 0.36)}$ | **(0.18)** | $2.51_{(\pm 0.74)} - 0.26x_{(\pm 0.07)}$ | **(0.44)** |

Table A.4. Equations linearly relating relative changes in milk fatty acid proportions (g/100 g milk fat) to relative changes in methane emissions (g/d, g/kg DMI, g/kg milk, g/kg $BW^{0.75}$/d). Regression coefficients ± standard error. $R^2$ presented in brackets and in bold at the end of each equation. Intercept is not presented as none of them was different from zero

| Parameter | g/d | g/kg DMI | g/kg milk | g/kg $BW^{0.75}$/d |
|---|---|---|---|---|
| C6:0 | $0.90x_{(\pm0.21)}$ **(0.57)** | $0.50x_{(\pm0.215)}$ **(0.47)** | $0.58x_{(\pm0.19)}$ **(0.19)** | $0.98x_{(\pm0.23)}$ **(0.34)** |
| C8:0 | $0.84x_{(\pm0.23)}$ **(0.52)** | $0.56x_{(\pm0.219)}$ **(0.46)** | $0.62x_{(\pm0.19)}$ **(0.19)** | $0.88x_{(\pm0.26)}$ **(0.30)** |
| C10:0 | $0.60x_{(\pm0.26)}$ **(0.46)** | | $0.39x_{(\pm0.2)}$ **(0.07)** | $0.66x_{(\pm0.28)}$ **(0.24)** |
| C13:0 | $0.72x_{(\pm0.33)}$ **(0.59)** | | | |
| *Iso* C14:0 | $1.10x_{(\pm0.34)}$ **(0.49)** | | | $1.21x_{(\pm0.38)}$ **(0.38)** |
| *Iso* C15:0 | $2.63x_{(\pm0.47)}$ **(0.50)** | | | $2.90x^{(\pm0.52)}$ **(0.31)** |
| C15:0 | $1.52x_{(\pm0.41)}$ **(0.51)** | | $1.01x_{(\pm0.42)}$ **(0.30)** | $2.09x^{(\pm0.4)}$ **(0.45)** |
| *Trans*-9 C16:1 | $-0.36x_{(\pm0.14)}$ **(0.65)** | $-0.48x_{(\pm0.203)}$ **(0.66)** | $-0.68x_{(\pm0.12)}$ **(0.60)** | $-0.49^{(\pm0.07)}$ **(0.80)** |
| *cis*-9 C17:1 | | $-0.98x_{(\pm0.361)}$ **(0.78)** | $-0.93x_{(\pm0.42)}$ **(0.56)** | |
| C17:0 | $0.73x_{(\pm0.39)}$ **(0.34)** | | $0.64x_{(\pm0.32)}$ **(0.12)** | |
| *Cis*-11 C18:1 | $-0.80x_{(\pm0.25)}$ **(0.36)** | $-0.61x_{(\pm0.138)}$ **(0.14)** | $-0.62x_{(\pm0.29)}$ **(0.16)** | $-0.89x_{(\pm0.28)}$ **(0.17)** |
| *Cis*-13 C18:1 | $-0.30x_{(\pm0.06)}$ **(0.53)** | $-0.16x_{(\pm0.078)}$ **(0.35)** | $-0.19x_{(\pm0.09)}$ **(0.27)** | $-0.33x_{(\pm0.07)}$ **(0.34)** |
| *Trans*-6 + 7 + 8 C18:1 | $-0.30x_{(\pm0.09)}$ **(0.45)** | | | $-0.34x_{(\pm0.1)}$ **(0.27)** |
| *Trans*-10 C18:1 | $-0.15x_{(\pm0.05)}$ **(0.43)** | | | $-0.16x_{(\pm0.06)}$ **(0.23)** |
| *Trans*-11 C18:1 | $-0.11x_{(\pm0.01)}$ **(0.21)** | $-0.07x_{(\pm0.006)}$ **(0.19)** | $-0.09x_{(\pm0.01)}$ **(0.06)** | $-0.12x_{(\pm0.01)}$ **(0.12)** |
| *Trans*-12 C18:1 | $-0.35x_{(\pm0.09)}$ **(0.48)** | $-0.23x_{(\pm0.099)}$ **(0.22)** | $-0.31x_{(\pm0.12)}$ **(0.23)** | $-0.39x_{(\pm0.11)}$ **(0.27)** |
| *Trans*-13 + 14 C18:1 | $-0.67x_{(\pm0.21)}$ **(0.48)** | $-0.47x_{(\pm0.201)}$ **(0.35)** | $-0.61x_{(\pm0.257)}$ **(0.40)** | $-0.74x_{(\pm0.23)}$ **(0.38)** |
| C20:0 | $0.58x_{(\pm0.26)}$ **(0.55)** | | | $0.65x_{(\pm0.29)}$ **(0.37)** |
| *n-6* C20:2 | $3.08x_{(\pm1.33)}$ **(0.80)** | $5.96x_{(\pm0.809)}$ **(0.79)** | $3.41x_{(\pm1.38)}$ **(0.31)** | $3.64x_{(\pm1.42)}$ **(0.91)** |
| *n-6* C20:3 | $0.89x_{(\pm0.28)}$ **(0.33)** | $0.75x_{(\pm0.234)}$ **(0.36)** | | $0.99x_{(\pm0.31)}$ **(0.29)** |
| *n-6* C20:4 | $0.34x_{(\pm0.11)}$ **(0.27)** | $0.29x_{(\pm0.09)}$ **(0.29)** | | $0.38x_{(\pm0.12)}$ **(0.27)** |
| *n-3* C20:5 (EPA) | $0.66x_{(\pm0.2)}$ **(0.35)** | $0.58x_{(\pm0.168)}$ **(0.37)** | | $0.73x_{(\pm0.22)}$ **(0.28)** |
| *n-3* C22:5 | $0.19x_{(\pm0.08)}$ **(0.33)** | $0.19x_{(\pm0.058)}$ **(0.37)** | | $0.21\ x_{(\pm0.09)}$ **(0.34)** |
| Total FA < C16 | $0.55x_{(\pm0.19)}$ **(0.23)** | | | $0.80\ x_{(\pm0.22)}$ **(0.18)** |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| Total FA > C16 | | | | | | | – 1.57$x$ (±0.71) | **(0.10)** |
| Total MUFA | –2.28$x$ (±0.62) | **(0.45)** | –1.48$x$ (±0.629) | **(0.39)** | – 1.42$x$ (±0.68) | **(0.14)** | – 2.55$x$ (±0.68) | **(0.29)** |
| Total SFA | 1.89$x$ (±0.46) | **(0.46)** | 1.01$x$ (±0.503) | **(0.40)** | 0.99$x$ (±0.49) | **(0.05)** | 2.10$x$ (±0.53) | **(0.27)** |
| Total OBCFA | 1.92$x$ (±0.65) | **(0.62)** | | | 1.21$x$ (±0.57) | **(0.28)** | 2.12$x$ (±0.71) | **(0.46)** |
| Total *trans* MUFA | –0.30$x$ (±0.11) | **(0.45)** | | | | | – 0.33$x$ (±0.12) | **(0.24)** |
| Total *trans* C18:1 | | | | | | | – 0.54$x$ (±0.18) | **(0.02)** |
| Total *n-6* PUFA | 0.70$x$ (±0.23) | **(0.20)** | 0.58$x$ (±0.207) | **(0.38)** | | | 0.77$x$ (±0.27) | **(0.19)** |

Table A.5. Summary statistics of data used for modelling

| Variable | Mean | SD | Minimum | Maximum |
|---|---|---|---|---|
| $CH_4$ (g/d) | 360 | 70.7 | 198 | 547 |
| $CH_4$ (g/kg DMI) | 18.5 | 3.28 | 12.3 | 28.0 |
| $CH_4$ (g/kg milk) | 16.7 | 5.32 | 9.02 | 46.2 |
| $CH_4$ (g/kg $BW^{0.75}$/d) | 2.86 | 0.532 | 1.60 | 4.17 |
| *Milk Fatty Acids (g/100 g Fat)* | | | | |
| C4:0 | 3.30 | 0.267 | 2.55 | 4.13 |
| C5:0 | 0.03 | 0.010 | 0.01 | 0.06 |
| C6:0 | 2.16 | 0.242 | 1.25 | 2.54 |
| C7:0 | 0.02 | 0.009 | 0.00 | 0.09 |
| C8:0 | 1.26 | 0.221 | 0.61 | 1.61 |
| C9:0 | 0.03 | 0.010 | 0.00 | 0.06 |
| C10:0 | 2.80 | 0.634 | 1.11 | 4.04 |
| C10:1 | 0.30 | 0.073 | 0.11 | 0.45 |
| C11:0 | 0.04 | 0.018 | 0.01 | 0.11 |
| C12:0 | 3.27 | 0.772 | 1.22 | 4.90 |
| C12:1 | 0.09 | 0.028 | 0.02 | 0.15 |
| *Iso* C14:0 | 0.09 | 0.053 | 0.01 | 0.47 |
| C14:0 | 11.3 | 1.715 | 6.24 | 13.9 |
| *Iso* C15:0 | 0.22 | 0.038 | 0.12 | 0.29 |
| *Anteiso* C15:0 | 0.39 | 0.058 | 0.24 | 0.51 |
| C14:1 | 1.08 | 0.263 | 0.43 | 1.78 |
| C15:0 | 0.96 | 0.136 | 0.57 | 1.27 |
| *Iso* C16:0 | 0.21 | 0.038 | 0.09 | 0.29 |
| C16:0 | 35.8 | 5.749 | 20.7 | 54.0 |
| *Trans*-9 C16:1 | 0.06 | 0.018 | 0.02 | 0.13 |
| *Iso* C17:0 | 0.28 | 0.082 | 0.14 | 0.53 |
| *Cis*-7 C16:1 | 0.21 | 0.088 | 0.03 | 0.40 |
| *Cis*-9 C16:1 | 1.83 | 0.417 | 1.08 | 3.38 |
| *Anteiso* C17:0 | 0.40 | 0.058 | 0.22 | 0.58 |
| *Cis*-11 C16:1 | 0.04 | 0.014 | 0.01 | 0.13 |
| *Trans*-14 C16:1 | 0.03 | 0.021 | 0.01 | 0.11 |
| *Cis*-13 C16:1 | 0.19 | 0.091 | 0.01 | 0.52 |
| C17:0 | 0.55 | 0.120 | 0.25 | 0.83 |
| *Iso* C18:0 | 0.05 | 0.032 | 0.01 | 0.30 |
| *Cis*-9 C17:1 | 0.19 | 0.072 | 0.07 | 0.54 |
| C18:0 | 8.61 | 1.528 | 4.76 | 14.0 |
| *Trans*-6 + 7 + 8 C18:1 | 0.24 | 0.114 | 0.12 | 0.91 |
| *Trans*-9 C18:1 | 0.19 | 0.080 | 0.11 | 0.80 |
| *Trans*-10 C18:1 | 0.34 | 0.251 | 0.13 | 1.90 |
| *Trans*-11 C18:1 | 0.96 | 0.592 | 0.28 | 5.26 |
| *Trans*-12 C18:1 | 0.28 | 0.126 | 0.06 | 0.48 |
| *Trans*-13 C18:1 | 0.51 | 0.221 | 0.10 | 1.02 |
| *Cis*-9 C18:1 | 16.4 | 2.936 | 7.41 | 31.5 |
| *Trans*-15 C18:1 | 0.27 | 0.137 | 0.05 | 1.20 |
| *Cis*-11 C18:1 | 0.40 | 0.217 | 0.12 | 1.65 |
| *Cis*-12 C18:1 | 0.26 | 0.136 | 0.13 | 1.18 |
| *Cis*-13 C18:1 | 0.07 | 0.033 | 0.02 | 0.24 |

| | | | | |
|---|---|---|---|---|
| *Cis*-14 + *trans*-16 C18:1 | 0.41 | 0.233 | 0.16 | 1.92 |
| *Cis*-15 C18:1 | 0.23 | 0.469 | 0.05 | 4.19 |
| *Trans*-11, *cis*-15 C18:2 | 0.13 | 0.246 | 0.03 | 1.97 |
| *n-6* C18:2 | 1.32 | 0.206 | 0.97 | 1.86 |
| *n-6* C18:3 + C20:0 | 0.12 | 0.039 | 0.01 | 0.24 |
| *n-3* C18:3 | 0.39 | 0.071 | 0.28 | 0.64 |
| *Cis*-9, *trans*-11 CLA | 0.46 | 0.263 | 0.16 | 2.21 |
| *n-6* C20:3 | 0.07 | 0.034 | 0.04 | 0.41 |
| *n-3* C20:3 | 0.15 | 0.260 | 0.01 | 1.30 |
| *n-6* C20:4 | 0.26 | 0.293 | 0.01 | 1.12 |
| *n-3* C20:5 | 0.05 | 0.022 | 0.01 | 0.14 |
| *n-6* C22:5 | 0.04 | 0.031 | 0.01 | 0.21 |
| *n-3* C22:5 | 0.08 | 0.058 | 0.01 | 0.31 |
| *n-3* C22:6 | 0.04 | 0.025 | 0.01 | 0.15 |

Table A.6. Pearson correlation between MFA and methane

| | g $CH_4$/d | | g $CH_4$/kg DMI | | g $CH_4$/kg milk | | g $CH_4$/$BW^{0.75}$/d | |
|---|---|---|---|---|---|---|---|---|
| MFA | Corr | P value | Corr | P value | Corr | P value | Corr | P value |
| C4:0 | -0.02 | 0.80 | -0.09 | 0.28 | -0.36 | 0.00 | 0.01 | 0.90 |
| C5:0 | 0.20 | 0.02 | -0.09 | 0.31 | -0.18 | 0.03 | 0.22 | 0.01 |
| C6:0 | 0.06 | 0.46 | -0.51 | 0.00 | -0.44 | 0.00 | -0.07 | 0.39 |
| C7:0 | 0.11 | 0.21 | -0.17 | 0.04 | -0.18 | 0.03 | 0.10 | 0.23 |
| C8:0 | 0.11 | 0.19 | -0.48 | 0.00 | -0.32 | 0.00 | -0.04 | 0.66 |
| C9:0 | 0.28 | 0.00 | -0.25 | 0.00 | -0.21 | 0.01 | 0.21 | 0.01 |
| C10:0 | 0.13 | 0.11 | -0.45 | 0.00 | -0.25 | 0.00 | 0.00 | 0.96 |
| C10:1 | 0.15 | 0.07 | -0.36 | 0.00 | -0.25 | 0.00 | -0.02 | 0.77 |
| C11:0 | 0.24 | 0.00 | -0.22 | 0.01 | -0.09 | 0.26 | 0.19 | 0.02 |
| C12:0 | 0.18 | 0.03 | -0.41 | 0.00 | -0.20 | 0.02 | 0.04 | 0.64 |
| C12:1 | 0.32 | 0.00 | -0.16 | 0.05 | -0.06 | 0.49 | 0.17 | 0.04 |
| *Iso* 14:0 | 0.13 | 0.13 | 0.25 | 0.00 | 0.15 | 0.08 | 0.18 | 0.03 |
| C14:0 | -0.02 | 0.78 | -0.61 | 0.00 | -0.39 | 0.00 | -0.19 | 0.02 |
| *Iso* C15:0 | 0.14 | 0.09 | -0.42 | 0.00 | -0.26 | 0.00 | 0.01 | 0.89 |
| *Anteiso* C15:0 | 0.02 | 0.84 | -0.35 | 0.00 | -0.22 | 0.01 | -0.07 | 0.38 |
| *Cis*-9 C14:1 | 0.06 | 0.48 | -0.26 | 0.00 | -0.07 | 0.41 | -0.12 | 0.16 |
| C15:0 | 0.14 | 0.10 | -0.38 | 0.00 | -0.20 | 0.02 | 0.05 | 0.52 |
| *Iso* C16:0 | -0.02 | 0.82 | -0.30 | 0.00 | -0.15 | 0.07 | -0.15 | 0.08 |
| C16:0 | 0.20 | 0.02 | 0.34 | 0.00 | 0.16 | 0.05 | 0.26 | 0.00 |
| *Trans*-9 C16:1 | -0.27 | 0.00 | -0.05 | 0.52 | -0.12 | 0.15 | -0.26 | 0.00 |
| *Iso* C17:0 | -0.34 | 0.00 | -0.30 | 0.00 | 0.05 | 0.51 | -0.34 | 0.00 |
| *Cis*-7 C16:1 | 0.09 | 0.26 | 0.24 | 0.00 | -0.02 | 0.79 | 0.09 | 0.28 |
| *Cis*-9 C16:1 | 0.25 | 0.00 | 0.36 | 0.00 | 0.29 | 0.00 | 0.23 | 0.00 |
| *Anteiso* C17:0 | -0.13 | 0.13 | -0.47 | 0.00 | -0.32 | 0.00 | -0.23 | 0.00 |
| *Cis*-11 C16:1 | -0.27 | 0.00 | -0.19 | 0.02 | -0.12 | 0.14 | -0.29 | 0.00 |
| *Trans*-14 C16:1 | -0.02 | 0.78 | 0.51 | 0.00 | 0.45 | 0.00 | 0.13 | 0.13 |
| *Cis*-13 C16:1 | -0.32 | 0.00 | -0.27 | 0.00 | -0.25 | 0.00 | -0.38 | 0.00 |
| C17:0 | -0.16 | 0.05 | -0.67 | 0.00 | -0.33 | 0.00 | -0.24 | 0.00 |
| *Iso* C18:0 | -0.44 | 0.00 | -0.25 | 0.00 | -0.19 | 0.02 | -0.39 | 0.00 |
| *Cis*-9 C17:1 | -0.60 | 0.00 | -0.37 | 0.00 | -0.21 | 0.01 | -0.58 | 0.00 |
| C18:0 | -0.30 | 0.00 | -0.22 | 0.01 | -0.10 | 0.24 | -0.27 | 0.00 |
| *Trans*-6+7+8 C18:1 | 0.06 | 0.49 | 0.30 | 0.00 | 0.27 | 0.00 | 0.14 | 0.09 |
| *Trans*-9 C18:1 | 0.05 | 0.58 | 0.32 | 0.00 | 0.26 | 0.00 | 0.14 | 0.09 |
| *Trans*-10 C18:1 | 0.10 | 0.25 | 0.36 | 0.00 | 0.33 | 0.00 | 0.19 | 0.02 |
| *Trans*-11 C18:1 | -0.04 | 0.65 | 0.21 | 0.01 | 0.10 | 0.22 | 0.06 | 0.45 |
| *Trans*-12 C18:1 | -0.52 | 0.00 | -0.44 | 0.00 | -0.21 | 0.01 | -0.54 | 0.00 |
| *Trans*-13 C18:1 | -0.50 | 0.00 | -0.34 | 0.00 | -0.09 | 0.29 | -0.48 | 0.00 |
| *Cis*-9 C18:1 | -0.23 | 0.00 | 0.04 | 0.62 | 0.07 | 0.43 | -0.20 | 0.01 |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| *Trans*-15 C18:1 | 0.36 | 0.00 | 0.30 | 0.00 | 0.31 | 0.00 | 0.34 | 0.00 |
| *Cis*-11 C18:1 | -0.35 | 0.00 | -0.07 | 0.40 | -0.04 | 0.63 | -0.26 | 0.00 |
| *Cis*-12 C18:1 | -0.02 | 0.79 | 0.27 | 0.00 | 0.22 | 0.01 | 0.07 | 0.42 |
| *Cis*-13 C18:1 | -0.26 | 0.00 | -0.04 | 0.65 | -0.08 | 0.33 | -0.22 | 0.01 |
| *Cis*-14 + *trans*-16 C18:1 | 0.01 | 0.90 | 0.29 | 0.00 | 0.25 | 0.00 | 0.10 | 0.22 |
| *Cis*-15 C18:1 | 0.15 | 0.07 | 0.46 | 0.00 | 0.30 | 0.00 | 0.27 | 0.00 |
| *Trans*-11, *cis*-15 C18:2 | 0.05 | 0.52 | 0.36 | 0.00 | 0.29 | 0.00 | 0.18 | 0.03 |
| *n-6* C18:2 | -0.18 | 0.03 | -0.20 | 0.01 | -0.16 | 0.06 | -0.18 | 0.03 |
| *n-6* C18:3 + C20:0 | -0.24 | 0.00 | -0.60 | 0.00 | -0.42 | 0.00 | -0.37 | 0.00 |
| *n-3* C18:3 | -0.27 | 0.00 | -0.18 | 0.03 | -0.09 | 0.30 | -0.18 | 0.03 |
| *Cis*-9, *trans*-11 CLA | -0.04 | 0.60 | 0.27 | 0.00 | 0.15 | 0.06 | 0.05 | 0.54 |
| *n-6* C20:3 | -0.23 | 0.01 | -0.15 | 0.07 | -0.10 | 0.23 | -0.21 | 0.01 |
| *n-3* C20:3 | -0.19 | 0.02 | -0.29 | 0.00 | -0.03 | 0.71 | -0.17 | 0.04 |
| *n-6* C20:4 | -0.22 | 0.01 | -0.34 | 0.00 | -0.10 | 0.23 | -0.26 | 0.00 |
| *n-3* C20:5 | -0.09 | 0.30 | 0.24 | 0.00 | -0.06 | 0.50 | -0.04 | 0.64 |
| *n-6* C22:5 | -0.07 | 0.41 | -0.25 | 0.00 | 0.02 | 0.82 | -0.05 | 0.53 |
| *n-3* C22:5 | 0.30 | 0.00 | 0.00 | 0.99 | -0.24 | 0.00 | 0.22 | 0.01 |
| *n-3* C22:6 | 0.12 | 0.15 | 0.05 | 0.53 | 0.21 | 0.01 | 0.18 | 0.03 |

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Joaquín is author of a number of publications, and has taken part in several national and international congresses and symposia.

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