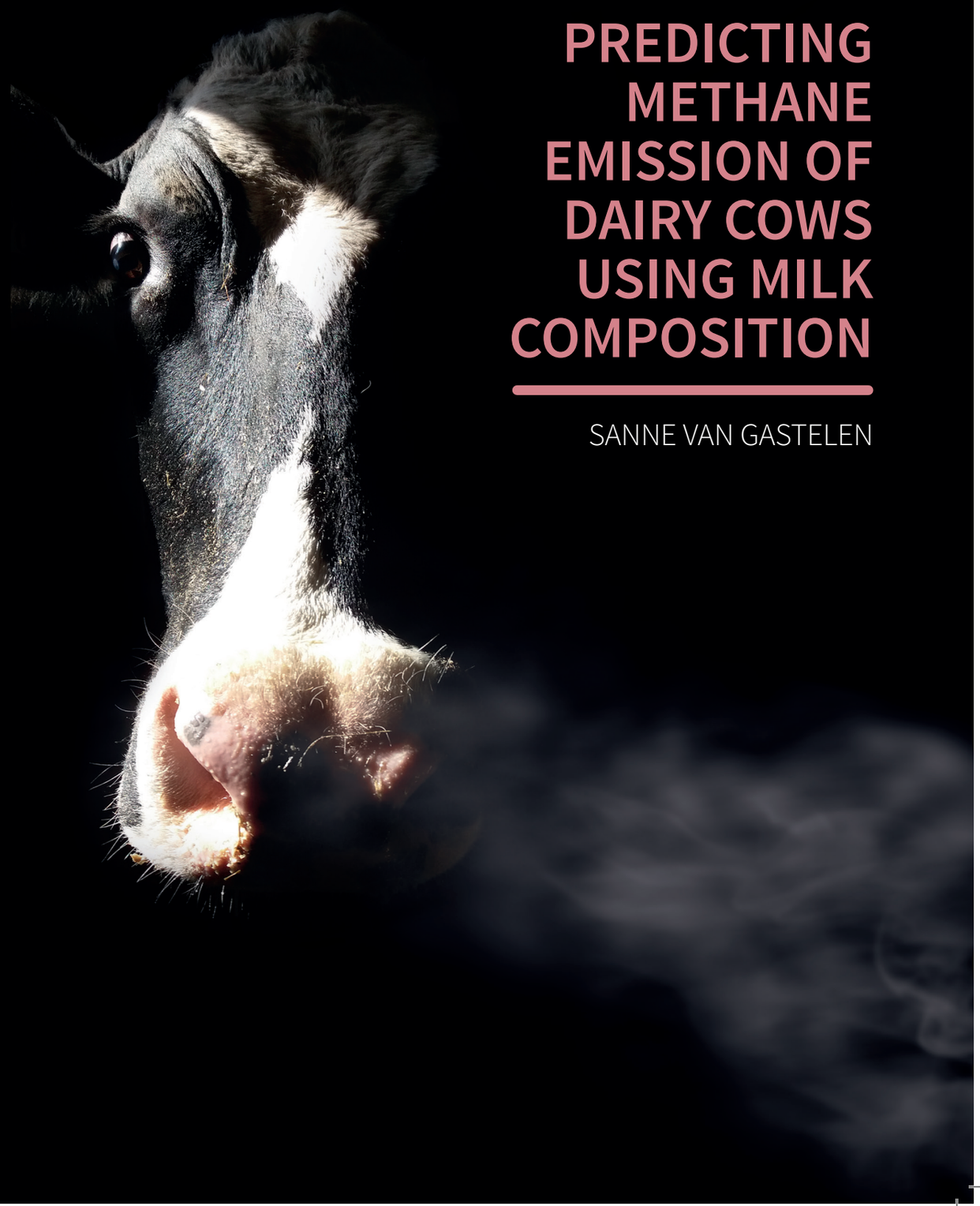
PREDICTING METHANE EMISSION OF DAIRY COWS USING MILK COMPOSITION

SANNE VAN GASTELEN 2017



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PROPOSITIONS

1. A 'one-size fits all' indicator for methane emission that can be measured in bovine milk is not achievable.
   (this thesis)

2. Counterintuitively, combining indicators for methane emission that can be measured in bovine milk does not result in a better estimation of methane emission of dairy cows fed a wide range of roughage-based diets.
   (this thesis)

3. The second challenge of interdisciplinary research is understanding each other's jargon, concepts, and reasoning.

4. Reviewers of scientific articles are sometimes like children – you should not want to win every battle.

5. Introducing yourself as a biologist comes with unrealistic expectations.

6. To derive more robust conclusions from animal research, stimulating their natural behavior is needed, which counteracts with the reduction in the number of animals used.

7. Typical driving behavior is contrary to evolutionary beneficial behavior.

Propositions belonging to the thesis, entitled
Predicting methane emission of dairy cows using milk composition

Sanne van Gastelen
Wageningen, 22 December 2017

Predicting methane emission of dairy cows using milk composition

Sanne van Gastelen

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Predicting methane emission of dairy cows using milk composition

Sanne van Gastelen

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Chapter 1

General introduction

ENTERIC FERMENTATION

The rumen harbors a diverse microbial population comprising mainly bacteria, protozoa, fungi, and archaea. These microbes reside in the rumen symbiotically with the host and grow through the process of microbial fermentation of feed ingested by the host, also called enteric fermentation. With this process, ruminants are able to effectively turn human inedible biomass, such as coarse plant material, into high quality protein in the form of milk and meat for human consumption (Gerber et al., 2015).

Enteric fermentation occurs in the gastrointestinal tract of ruminants, predominantly within the rumen (~87%) and to a small extent in the large intestines (~13%) (Murray et al., 1976). Microorganisms in the rumen hydrolyze protein and carbohydrates into amino acids and sugars, which in turn are fermented into amongst others volatile fatty acids (**VFA**), hydrogen (**H_2**), and carbon dioxide (**CO_2**). The VFA are partly absorbed through the rumen wall and are the main energy supply required for maintenance and productive functions of the ruminant (Boadi et al., 2004). As a final step, methanogenic archaea generate metabolic energy in the form of ATP for their maintenance and growth, by forming methane (**CH_4**) using mainly CO_2 and H_2 (Ellis et al., 2008; McAllister and Newbold, 2008). This process of methanogenesis is essential for a good performance of the rumen because it assures a low concentration of H_2 in the rumen, allowing the ruminal microbial population to function under optimal conditions to support the continuation of substrate fermentation (McAllister and Newbold, 2008). Van Lingen et al. (2016) however demonstrated that not all main fermentation processes, viz. glucose fermentation pathways, are controlled by the ruminal concentrations of H_2. The CH_4 produced by the ruminal methanogens is predominately released into the environment through eructation and breath, and as a greenhouse gas (**GHG**) significantly contributes to global warming.

THE ENVIRONMENTAL IMPACT OF ENTERIC FERMENTATION

Methane is, together with CO_2 and nitrous oxide, one of the three main GHG and has a global warming potential of 28 CO_2 equivalents (Myhre et al., 2013). Methane originates from natural sources, such as wetlands, and from anthropogenic sources, such as natural gas production, landfills, and agriculture (Lassey, 2008). The livestock sector was estimated to be responsible for approximately 14.5% of total global anthropogenic GHG emissions (Gerber et al., 2013). Enteric fermentation is the main source of GHG emissions from dairy cattle, representing 46% of the total emissions in the dairy supply chain (Gerber et al., 2013). Furthermore, based on the expected farming and consumer lifestyle practices, global CH_4 emissions from enteric fermentation is expected to increase by 70% in 2055, compared with 1995 (Popp et al., 2010). This makes enteric CH_4 emission one of the main targets of the GHG mitigation objectives of the dairy cattle sector (Hristov et al., 2013a). At present, there are several strategies to mitigate CH_4 emissions. For example, increased animal productivity, which can be achieved through improvements in animal genetics, feeding, reproduction, health, and overall management, may allow a reduction of the number of animals needed to maintain constant output with a reduced CH_4 emission (Hristov et al., 2013a). Additionally, several altered feeding strategies as well as other farm management practices are available to mitigate CH_4 emissions, which have been extensively reviewed by, for example, Hristov et al. (2013a,b), Montes et al.

(2013), and Knapp et al. (2014). The effect of a mitigation strategy may vary depending on the unit in which enteric CH_4 production can be expressed. See Textbox 1 for a description of the different units to express enteric CH_4 production.

TECHNIQUES TO QUANTIFY AND MEASURE ENTERIC METHANE PRODUCTION

Accurate and repeatable measurements of CH_4 emission from individual dairy cows are required to assess the efficacy of possible mitigation strategies, to decrease uncertainties associated with national GHG inventories, and to develop protocols for genetic selection for cows with reduced CH_4 emission (Hammond et al., 2016). There are several techniques to estimate or measure enteric CH_4 production of dairy cows, including mathematical models, the *in vitro* gas production technique, and several *in vivo* measurement techniques.

A wide range of mathematical models have been developed to estimate CH_4 emission from ruminants using nutrient intake data as input. These include dynamic mechanistic models, which estimate CH_4 emission based on a representation of microbial fermentation processes that occur in the rumen and hindgut, and empirical (or statistical) models, which relate nutrient intake to CH_4 emission directly (Bannink et al., 2011). Dynamic mechanistic models may be more successful in predicting observed variation in CH_4 emission than empirical models, but they require detailed dietary inputs which may not be commonly available at the national level or at the individual farm level (Alemu et al., 2011). Because mechanistic models have, in comparison with empirical models, a more detailed representation of the underlying mechanisms of microbial activity and methanogenesis, they have an advantage in terms of evaluating the effectiveness of CH_4 mitigation options that may be implemented on farm. Empirical models are however very useful because of their simplicity and ease of use. The accuracy of empirical models to evaluate specific dietary mitigation measures is generally lower than that of mechanistic models because no diet specific information is included. Subsequently, use of empirical models may introduce errors into the accounting of mitigation measures in inventories of GHG emissions and lead to incorrect mitigation recommendations (Ellis et al., 2010).

Various techniques are available to measure CH_4 emission. The *in vitro* gas production technique has been widely used to evaluate the nutritive value of feeds for ruminants, and in the last decade to assess the CH_4 production potential of different feeds as well (Yáñez-Ruiz et al., 2016). As recently reviewed by Yáñez-Ruiz et al. (2016), *in vitro* and *in vivo* results, however, are poorly related. Therefore, *in vitro* CH_4 production results with well-buffered and standardized fermentation conditions should be interpreted with care and may not reflect the *in vivo* CH_4 production. According to Yáñez-Ruiz et al. (2016), for the *in vitro* gas production technique, one should only use rumen fluid from donor animals that were fed the same diet as incubated or should be of similar nutrient composition, because using rumen fluid from adapted *versus* non-adapted animals significantly affects *in vitro* CH_4 production. The importance of using rumen fluid of adapted animals has also been demonstrated by Klop et al. (2017). However, as demonstrated by Hatew et al. (2015), even inoculum obtained from specifically adapted animals may still lead to a large difference between CH_4 production observed *in vitro* and *in vivo*.

Text box 1. Different units to express enteric methane production

There is currently limited consensus on which unit of CH_4 emission to use for evaluating the CH_4 mitigation potential of altered feeding strategies or for lowering the carbon footprint of milk production through genetic selection (Negussie et al., 2017). It could be either of the three units CH_4 production, CH_4 yield, or CH_4 intensity. When referring to CH_4 production, the typical unit is mass (g) or volume (L) per unit of time (e.g., day), per animal. The obvious problem with this unit of CH_4 emission is that it is highly correlated with the dry matter intake (**DMI**) of the animal (De Haas et al., 2017). Most of the CH_4 production originates from enteric fermentation, hence more fermentation due to a higher DMI will increase the total CH_4 production per day. Additionally, CH_4 production is also highly correlated with the production trait of interest: in sheep or beef, meet production; in dairy, milk production (De Haas et al., 2017). A higher milk yield is often associated with a higher DMI (Garnsworthy et al., 2012), and, as already explained above, a higher DMI is often associated with a higher daily CH_4 production (e.g., Hristov et al., 2013). Hence, a positive association between milk yield and CH_4 production exists in dairy cows.

For breeding purposes, CH_4 production might be the best phenotype of CH_4 emission to breed for (Lassen and Løvendahl, 2016; De Haas et al., 2017; Negussie et al., 2017). Not only does it represent the direct goal, namely the trait of interest which needs to be improved (Herd et al., 2013), but also the most correct way to breed for reduced CH_4 emission, because the relationship with feed intake or milk production could be accounted for by including these in the final selection index or scheme (De Haas et al., 2017; Negussie et al., 2017). It is however questionable whether it might be more effective or accurate to directly use feed intake-corrected CH_4 emission or milk production-corrected CH_4 emission (e.g., CH_4 yield or CH_4 intensity) as the breeding goal.

When referring to CH_4 yield, the typical unit is g or L of CH_4 per kg DMI. To exclude the effect of feed intake in the expression of CH_4 emission, Dijkstra et al. (2011) proposed that the evaluation of nutritional mitigation strategies should be based on CH_4 production relative to feed intake as this avoids the confounding effect of DMI. There are, however, uncertainties in measuring DMI at the farm level, making an accurate relation of CH_4 to DMI difficult in practice (Bannink et al., 2011). In addition, the nutritional value of feed can affect animal productivity despite a similar DMI. Therefore, another unit of expression refers to CH_4 intensity (g or L of CH_4 per unit of product yield). The CH_4 intensity for dairy cattle is usually expressed as CH_4 production per unit of fat- and protein-corrected milk. As clearly demonstrated by Warner et al. (2015), CH_4 intensity takes the value and characteristics (i.e., digestibility) of gross energy intake by dairy cows into account, illustrating that this unit of CH_4 emission has great value. In the context of global food supply and efficient use of resources it is important to consider the latter two units, CH_4 yield and CH_4 intensity, in particular.

The need for high throughput measurements of enteric CH_4 emission has led to the development of a variety of approaches for measuring this emission *in vivo*, many of which have been reviewed by Hammond et al. (2016). Enclosure techniques, tracer gas, and short-term measurements are among those techniques and are briefly described below. The open-circuit respiration chambers are a 'gold standard' in terms of accuracy and precision under the condition that they are routinely calibrated and gas recovery approximates 100% (Hammond et al., 2016). However, CH_4 measurements are conducted under highly controlled conditions which do not exist under practical farming. Additionally, the costs of construction and operation are high and the throughput capacity of the system is limited, making this technique unsuitable for large scale measurements. Therefore, alternative high throughput measurement techniques have been developed.

A commonly used CH_4 measurement technique is the sulfur hexafluoride (**SF_6**) tracer technique; a technique suitable for penned as well as free ranging and grazing animals (Hammond et al., 2016). Although the SF_6 tracer technique allows for measurement of CH_4 emission from many individual animals whilst in their natural environment, the SF_6 tracer technique provides a mean CH_4 emission that can differ from that obtained for the same animals in respiration chambers (Hammond et al., 2016). Also, within- and between-animal variation is larger when using the SF_6 tracer technique for dairy cattle compared to the respiration chamber technique (Grainger et al., 2007). More recently, however, Deighton et al. (2014) demonstrated that a modified SF_6 tracer technique (e.g., a constant sample collection rate) reduced errors associated with SF_6 release, sample collection, and analysis. Therefore, these authors concluded that their modified SF_6 tracer technique can be an accurate and versatile research tool for measuring CH_4 emission of ruminants. Relative to the climate respiration chambers, the SF_6 tracer technique has a higher throughput in terms of animal measurements obtained relative to time and cost, but this technique is labor intensive and dependent on implementation and technical skill to minimize experimental error (Hammond et al., 2016).

Other techniques that have been developed involve the short-term measurement of CH_4 emission with spot measurements of exhaled CH_4 at certain time points (e.g., at milking or during feeding). These techniques are usually automated, non-invasive, and non-intrusive, allowing a high throughput of animals, such as the GreenFeed system, so-called 'sniffer' techniques, CH_4:CO_2 ratio techniques, and the handheld laser CH_4 detector (Hammond et al., 2016). Methane emission from an animal is, however, not constant throughout the day, with diurnal patterns affected by the diet, feed allowance and feeding pattern. The timing and duration of sampling of the short-term measurement techniques is therefore critical for accuracy as well as precision, and there is in principle a high potential of biased measurement (or estimates derived from those measurements) of CH_4 emission. Hence, serious concerns regarding the accuracy, repeatability, and precision of the data obtained with such short-term measurement techniques exist (Hammond et al., 2016).

PROXIES

As described above, in the last few years efforts have been made to develop direct, reliable, and low-cost measurement techniques for CH_4 emissions of individual animals. However, progress has not been as fast as desired, mainly because direct measurement of CH_4 on an individual-animal basis is still difficult and expensive (Pickering et al., 2015). This has stimulated researchers to look for proxies for CH_4 emission of dairy cattle as alternatives for direct CH_4 measurement techniques.

Proxies for CH_4 emission of dairy cows are indicators or indirect traits that are correlated with enteric CH_4 production. There are several criteria that a proxy needs to adhere to, in order to actually be valuable. From a technical point of view, it is important that a proxy is both accurate and precise when estimating CH_4 emission. Accuracy refers to how closely the model-predicted value(s) is (are) to the true observed value(s). If a proxy is not accurate, it could result in a biased prediction (over- or underprediction) of CH_4 emission and thus systematically deviates from the reality (Tedeschi, 2006). Precision refers to the magnitude of the scatter around the average mean. If a proxy is not precise, the proxy is most likely not able to detect differences among model predictions (Tedeschi, 2006). Ideally, in terms of accuracy and precision, the proxy should be able to estimate CH_4 emission of both individual cows and of dairy herds, with a certain level of robustnesst (also accurate and precise CH_4 estimates under different dietary regimes, environmental condition, farming systems, and so on), to support farmers in their management to reduce CH_4 emission. If the precision and accuracy of such proxies is satisfactory, they might serve as the much-needed alternative to expensive direct CH_4 measurements. To achieve this, a proxy should also be valuable from a practical points of view, such as easy to measure at relatively low costs on a large scale. These practical issues can be assigned to the attributes simplicity, costs, invasiveness, and throughput (Negussie et al., 2017). Simplicity refers to the ease with which proxies can be measured. Costs refer to all costs associated with the measurement of the proxy, including the costs of construction, operation, and analysis. Invasiveness is the intensity of animal handling that is required to measure the proxy, and throughput is the number of observations within in a given period per animal.

Negussie et al. (2017) assessed existing potential proxies for CH_4 emissions of dairy cows both in terms of statistical and practical aspect, including proxies related to (1) feed intake and feeding behavior, (2) rumen function, metabolites, and microbiome, (3) milk production and composition, (4) hindgut and feces, and (5) measurements at the level of the whole animal (e.g., body condition score, body weight, and lactation stage). To illustrate, results of Negussie et al. (2017) indicate that proxies based on samples from the rumen or related to rumen sources are poorly to moderately related to enteric CH_4 production (i.e., statistical aspect). Moreover, these proxies were considered too costly and difficult for routine on-farm implementation (i.e., practical aspect). Proxies related to body weight, milk yield, and milk composition (e.g., milk fatty acids) appeared to be moderately to highly accurate predictors of enteric CH_4 production (i.e., statistical aspect) and are relatively simple, inexpensive, and easy to implement in practice (i.e., practical aspect). Hence, one can imagine that latter type of proxies are more suitable proxies for CH_4 emission than the rumen related proxies. In particular, milk mid-infrared spectroscopy is a promising proxy; accurate, cheap, and easily implemented in routine milk analysis at no extra

cost (Negussie et al., 2017). The latter authors emphasized that no single proxy may accurately predict enteric CH_4 production, and that combining proxies may be the best way forward. Combining proxies for CH_4 emission will allow improved description of independent sources of variations in CH_4 emissions and result in the most accurate prediction of CH_4 emissions in dairy cows (Negussie et al., 2017). Examples of combinations of proxies include prediction of CH_4 emission based on diet-specific milk fatty acid composition or milk mid-infrared spectroscopy combined with lactation stage.

It is important to note though that enteric CH_4 production is influenced by many factors, including dietary factors (such as the type and the amount of feed), animal factors (such as milk yield, body weight, activity, lactation stage, and genetic traits), management factors (such as feeding frequency), and environmental factors (such as seasons and temperature) (e.g., Hristov et al., 2013a,b). These factors together result in large variation in CH_4 emission of dairy cattle, making it a challenge to develop a universal CH_4 proxy.

RESEARCH OBJECTIVES

As outlined above, proxies might serve as a good alternative to quantify CH_4 emission of dairy cattle. Therefore, the general objective of the PhD study described in this thesis was to develop a proxy for CH_4 emission that can be measured in milk of dairy cows. To this end, a large range of chemical analyses was performed on milk samples obtained from cows fed a wide range of roughage-based diets while housed in climate respiration chambers. These data on milk composition were subsequently used to examine relationships between the chemical composition of milk and the CH_4 emitted by the cows. This PhD study builds further on a CH_4 prediction model recently proposed by Dijkstra et al. (2011) which is exclusively based on the fatty acid composition of milk. It is hypothesized that the addition of other metabolites in this prediction model will enhance its predictive power and thus will lead to a better indicator in milk for enteric CH_4 production of dairy cows. For the identification of these components (i.e., fatty acids, volatile metabolites, and non-volatiles metabolites) in milk, gas chromatography, gas chromatography-mass spectroscopy, and nuclear magnetic resonance equipment, respectively, were required. These techniques are, however, not suitable for large-scale measurements. Therefore, to apply the indicator in practice, a method based on Fourier-transform infrared spectroscopy has been used in this PhD study as well. Overall, the specific objectives of this PhD study were:

1. to quantify the relation between enteric CH_4 production and individual milk fatty acids, volatile metabolites, and non-volatile metabolites based on data of dairy cows fed diets with increasing amounts of corn silage at the expense of grass silage;
2. to determine the CH_4 prediction potential of milk fatty acids alone, volatile metabolites alone, non-volatile metabolites alone, and the combination of these three component groups;
3. to determine the CH_4 prediction potential of milk Fourier-transform infrared spectroscopy;

4. to evaluate the robustness of the established relationships between enteric CH_4 production and milk fatty acids, volatile metabolites, and non-volatile metabolites upon linseed oil supplementation in the diet of dairy cows with a different *DGAT1* K232A polymorphism.

The research presented in this thesis was part of the TI Food and Nutrition project entitled 'Reduced methane emissions of dairy cows' (see Textbox 2 for a brief project description).

Textbox 2. TI Food and Nutrition program *Reduced methane emissions from dairy cows: towards sustainable dairy cattle production by increased understanding of genetic variation and rumen functioning*

This multi-disciplinary project aimed to increase our knowledge regarding CH_4 emission by dairy cows in order to decrease the ecological footprint of dairy production and to contribute to the goal of a 30% decrease in greenhouse gas emission from the Dutch dairy sector by 2020. For this purpose, a proxy for CH_4 emission from individual cows based on milk metabolite composition was developed, with the use of data from climate respiration chamber experiments originating from the Dutch 'Low Emission Animal Feed' research program. Another proxy was developed based on gases expelled by cows, and was used to explore the genetic variation in CH_4 emission between cows. These two proxies can be used as simple and inexpensive quantification tools for estimating enteric CH_4 emissions from dairy cattle under field conditions. The understanding of processes related to CH_4 production has increased by characterization of the composition and functioning of micro-organisms and the metabolites produced in the rumen of the cow in response to feed composition and diurnal patterns of feed intake. The interaction between diet, microbiome composition, and genotype of animals has been explored to obtain a more holistic understanding of factors affecting ruminal CH_4 production. Various modeling approaches have been applied to improve the systematic understanding of rumen fermentation. These approaches and their results provided a profound basis for relating CH_4 production to feeding regime and feed composition.

The multidisciplinary project team was comprised of experts in Animal Breeding and Genetics, Animal Nutrition, Dairy Science and Technology, and Microbiology. The team was based at Wageningen University and collaborated with researchers from the industrial parties CRV, Lely Industries, and Qlip. Financial support was obtained from the Centraal Bureau Levensmiddelenhandel (CBL), Cooperative cattle improvement organization CRV, Federatie Nederlandse Levensmiddelen Industrie (FNLI), Lely Industries NV, Dutch Ministry of Economic Affairs, Qlip BV, Wageningen University & Research, and ZuivelNL.

OUTLINE OF THE THESIS

The research in this thesis focuses on the development of a proxy for CH_4 emission that can be measured in milk of dairy cows. **Chapter 2** provides an overview of recent research that relates milk fatty acids with CH_4 emission, and discusses the opportunities and limitations of using milk mid-infrared spectroscopy to estimate CH_4 emissions of dairy cattle. **Chapter 3** describes the effects of replacing grass silage with corn silage on enteric CH_4 production, rumen VFA concentrations, milk production, and milk composition including the fatty acid profile. Based on the data from the experiment described in Chapter 3, the relation between enteric CH_4 production and individual volatile metabolites and non-volatile metabolites is quantified and described in **Chapter 4**. **Chapter 5** describes the CH_4 prediction potential of milk fatty acids alone, volatile metabolites alone, non-volatile metabolites alone, and the combination of these three (also using data from the experiment described in Chapter 3). In **Chapter 6**, the relation between enteric CH_4 production and the individual milk metabolites as well as the CH_4 prediction potential of the milk metabolites is described, using a larger dataset comprising 6 experiments and a wide range of roughage-based diets. **Chapter 7** describes the effect of dietary linseed oil, the *DGAT1* K232A polymorphism, and their interaction, on enteric CH_4 production, rumen VFA concentrations, milk production, and milk composition including fatty acid profile. In **Chapter 8** the robustness is evaluated of the relationship between enteric CH_4 production and the fatty acids, volatile metabolites, and non-volatile metabolites in milk, upon linseed oil supplementation in the diet of dairy cows with a different *DGAT1* K232A polymorphism. In **Chapter 9**, the CH_4 prediction potential of milk Fourier-transform infrared spectroscopy is determined and compared with the prediction potential milk fatty acids. Finally, **Chapter 10** comprises a general discussion of the results in this thesis, including suggestions for future research, and providing general conclusions on the applicability and development of milk proxies for enteric CH_4 emission.

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Chapter 2

Prediction of methane emission from lactating dairy cows using milk fatty acids and mid-infrared spectroscopy

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ABSTRACT

Enteric methane (**CH_4**) production is among the main targets of greenhouse gas mitigation practices for the dairy industry. A simple, robust and inexpensive measurement technique applicable on large scale to estimate CH_4 emission from dairy cattle would therefore be valuable. Milk fatty acids (**MFA**) are related to CH_4 production because of the common biochemical pathway between CH_4 and fatty acids in the rumen. A summary of studies that investigated the predictive power of MFA composition for CH_4 emission indicated good potential, with predictive power ranging between 47 and 95%. Until recently, gas chromatography (**GC**) was the principal method used to determine the MFA profile, but GC is unsuitable for routine analysis. This has led to the application of mid-infrared (**MIR**) spectroscopy. The major advantages of using MIR spectroscopy to predict CH_4 emission include its simplicity and potential practical application at large scale. Disadvantages include the inability to predict important MFA for CH_4 prediction, and the moderate predictive power for CH_4 emission. It may not be sufficient to predict CH_4 emission based on MIR alone. Integration with other factors, like feed intake, nutrient composition of the feed, parity, and lactation stage may improve the prediction of CH_4 emission using MIR spectra.

Keywords: mid-infrared spectroscopy, milk fatty acids, methane emission, dairy cows

INTRODUCTION

Enteric methane (**CH_4**) is produced in the gastrointestinal tract of ruminants, mainly the rumen, by methanogenic *archaea*. Enteric CH_4 comprises 17% of global CH_4, and is therefore the single largest source of anthropogenic CH_4 (Knapp et al., 2014). In addition to its relevance of environmental impact, CH_4 represents an energy loss, making CH_4 emission one of the main targets of greenhouse gas (**GHG**) mitigation practices for the dairy industry (Hristov et al., 2013). The quantification of CH_4 emission is important to understand factors that contribute to the variation and to identify effective CH_4 mitigation strategies. Several techniques, such as the climate respiration chambers, the sulfur hexafluoride tracer (**SF_6**) technique, and mathematical models, have been developed to estimate CH_4 emission, many of which have been reviewed by Kebreab et al. (2006) and Storm et al. (2012). However, a simple, robust and inexpensive measurement technique applicable on large scale to estimate CH_4 emission from dairy cattle in commercial practice is still missing and would be valuable for the dairy industry (Van Lingen et al., 2014). Therefore, the potential of various metabolites in milk as biomarkers of CH_4 emission gained interest, including milk fatty acids (**MFA**; Fievez et al., 2012). The aim of this review is to provide an overview of the recent research that relates MFA with CH_4 emission, and to discuss the opportunities and limitations of using mid-infrared (**MIR**) spectroscopy to estimate, direct and indirect, CH_4 emission of dairy cattle.

MILK FATTY ACIDS AND METHANE EMISSION

Several studies have related individual MFA (g/100 g fatty acids; **FA**) to CH_4 emission in dairy cows (Chilliard et al., 2009; Castro-Montoya et al., 2011; Dijkstra et al., 2011; Mohammed et al., 2011; Van Lingen et al., 2014; Williams et al., 2014; Dijkstra et al., 2016; Rico et al., 2016). Straight short- and medium-chain fatty acids (**SMCFA**) in milk arise almost exclusively from *de*

novo synthesis in the mammary gland from acetate and β-hydroxybutyrate produced in the rumen (Bernard et al., 2008). Ruminal acetate and butyrate are positively associated with enteric CH_4 emission (Boadi et al., 2004; Ellis et al., 2008). Hence, a positive relationship between CH_4 emission and SMCFA can be assumed due to the common biochemical pathway (Ellis et al., 2008; Chilliard et al., 2009). Odd- and branched-chain fatty acids (**OBCFA**) in milk can also be used to predict CH_4 emission (Fievez et al., 2012). Propionate is a substrate for *de novo* synthesis of C15:0 and C17:0 in the mammary gland, and given the negative relation between propionate and CH_4 emission (Boadi et al., 2004; Ellis et al., 2008), a negative relation between CH_4 emission and these linear odd-chain FA in the milk can be assumed. In addition, milk OBCFA are of microbial origin in the rumen, which in turn relate directly to CH_4 emission. Fibrolytic bacteria are generally enriched in *iso* FA, whereas amylolytic bacteria contain high amounts of linear odd-chain FA and *anteiso* FA (Vlaeminck et al., 2006). Hence, a positive relation between CH_4 emission and *iso* FA can be assumed, as well as a negative relation between CH_4 emission and linear odd-chain FA and *anteiso* FA (Fievez et al., 2012).

A higher content of dietary unsaturated fatty acids (**UFA**) is negatively associated with CH_4 emission (Patra et al., 2013; Van Lingen et al., 2014). This CH_4 suppressing effect may be related to the intermediary metabolic products resulting from biohydrogenation (**BHG**) of UFA in the rumen, such as C18:1 and C18:2 isomers (Mohammed et al., 2011). Because several long-chain UFA in milk originate from dietary UFA and their BHG products formed in the rumen, a negative relation can be assumed between long-chain UFA in milk and CH_4 emission (Van Lingen et al., 2014).

A negative relation can also be expected between CH_4 emission and BHG intermediates in milk, because certain dietary strategies, including low-fiber diets and high-concentrate diets, alter the rumen environment and lower ruminal pH (Boadi et al., 2004). This often results in microbial population shifts, which have been associated with modifications in the BHG pathways. With a lower ruminal pH, BHG becomes more incomplete (i.e., concentrations of BHG intermediates increase) and C18:1 *trans*-10 replaces C18:1 *trans*-11 as the predominant *trans* C18:1 isomer of milk fat (Bauman and Griinari, 2003). Furthermore, a lower ruminal pH reduces the activity of rumen methanogens, and inhibits fiber fermentation, whereas starch fermentation is not reduced. Hence, propionate production is favored, thereby reducing H_2 availability for the production of CH_4 (Bannink et al., 2008).

Table 2.1. The use of milk fatty acids to predict CH_4 emission of dairy cows

Study	Number of studies, observations, MFA[A] identified	Method to determine CH_4 emission	Best prediction equation with only MFA	Relation individual MFA and CH_4 emission[B]	
				Negative relation	Positive relation
Chilliard et al. (2009)[C]	N[D] = 1 O[E] = 32 MFA[F] = 64	SF_6-tracer technique	CH_4 (g/d) = -21.2 + 9.46 × C16:0 - 97.6 × C18:1 *cis*-14+*trans*-16 + 13.3 × forage DMI[G] - 78.3 × C14:1 *cis*-9 + 77.4 × C18:2n-6 (R^2 = 0.95)	C16:1 *trans*-11 C18:1 *cis*-9 C18:1 *cis*-10 C18:1 *cis*-13 C18:1 *cis*-14 + *trans*-16 C18:1 *cis*-15 + *trans*-17 C18:1 *trans*-6+7+8 C18:1 *trans*-10 C18:1 *trans*-12 C18:1 *trans*-13+14 C18:2 *cis*-9, *trans*-13 C18:2 *trans*-11, *cis*-15	C4:0 C6:0 C8:0 C9:0 C10:0 C10:1 C11:0 C12:0 C12:1 C14:0 C15:0 C16:0 C17:0 C20:4n-6
Dijkstra et al. (2011)[H]	N = 3 O = 50 MFA = 40	Climate respiration chamber	CH_4 (g/kg DMI) = 24.6 + 8.74 × *anteiso* C17:0 - 1.97 × C18:1 *trans*-10+11 - 9.09 × C18:1 *cis*-11 + 5.07 × C18:1 *cis*-13 (R^2 = 0.73)	*iso* C17:0 C17:1 *cis*-9 C18:1 *cis*-9 C18:1 *cis*-11 C18:1 *cis*-12 C18:1 *cis*-14 + *trans*-16 C18:1 *trans*-10+11 C18:2 *cis*-9, *trans*-11	C8:0 C10:0 C11:0 *iso* C14:0 *iso* C15:0 C16:0 *anteiso* C17:0
Mohammed et al. (2011)	N = 1 O = 32 MFA = 61	Climate respiration chamber	CH_4 (g/d) = 272.4 - 486.2 × C17:1 *cis*-9 - 122.7 × C18:1 *cis*-11 + 2,220 × CLA *trans, trans* - 11.76 × ∑ *trans* C18:1 + 260.1 × *anteiso* C15:0 (R^2 = 0.74)	C16:1 *trans*-6+7+8 + *iso* C17:0 C17:1 *cis*-9 C18:1 *cis*-11 C18:1 *cis*-13 C18:1 *cis*-16	C8:0

Table 2.1. Continued

Study	Number of studies, observations, MFA identified	Method to determine CH_4 emission	Best prediction equation with only milk fatty acids	Negative relation	Positive relation
				C18:1 *trans*-6+7+8 C18:1 *trans*-10 C18:2 *cis*-9,*trans*-13+ *trans*-8,*cis*-12 C18:2n-6 C18:3n-3	
Van Lingen et al. (2014)[H]	N = 8 O = 146 MFA = 29	Climate respiration chamber	CH_4 (g/kg DMI) = 23.39 + 9.74 × *iso* C16:0 - 1.06 × C18:1 *trans*-10+11 -1.75 × C18:2n-6 (R^2 = 0.54)	C18:1 *cis*-11 C18:1 *cis*-12 C18:1 *trans*-6+7+8+9 C18:1 *trans*-10+11	C16:0
Van Lingen et al. (2014)[H]	N = 8 O = 146 MFA = 29	Climate respiration chamber	CH_4 (g/kg FPCM[I]) = 21.13 - 1.38 × C4:0 + 8.53 × *iso* C16:0 - 0.22 × C18:1 *cis*-9 - 0.59 × C18:1 *trans*-10+11 (R^2 = 0.47)	C4:0 C18:0 C18:1 *cis*-9 C18:1 *cis*-11 C18:2n-6	C10:0 C12:0 C14:0 C14:1 *cis*-9 C15:0 C16:0
Rico et al. (2016)[J]	N = 3 O = 81 MFA = 88	Climate respiration chamber	CH_4 (g/d) = 669.1 + 838.7 × C14:1 *cis*-11 - 493.2 × C17:1 *cis*-9 - 44.2 × C18:1 *cis*-11 - 963.7 × C18:2 *trans*-8, *cis*-13 (R^2 = 0.80)	*anteiso* C13:0 C14:1 *cis*-9 C15:0 C16:1 *cis*-9 C16:1 *cis*-11 C17:0 *iso* C17:0 C17:1 *cis*-9 C18:1 *trans*-4 C18:1 *trans*-5	C10:0 C12:0 C14:0 C14:1 *cis*-11 *iso* C16:0

Table 2.1. Continued

Study	Number of studies, observations, MFA identified	Method to determine CH_4 emission	Best prediction equation with only milk fatty acids	Negative relation	Positive relation
				C18:1 *trans*-6	
				C18:1 *trans*-10	
				C18:1 *trans*-12	
				C18:1 *trans*-13+14	
				C18:1 *cis*-11	
				C18:1 *cis*-13	
				C18:2 *trans*-8, *cis*-12	
				C18:2 *trans*-8, *cis*-13	
				C18:2 *cis*-9, *trans*-12	
				C20:1 *cis*-9	
				C18:3n-3	
				C18:2 *trans*-10, *cis*-12	
				C18:4n-3	
				C20:3n-3	
				C22:3 *cis*-13,16,19	
				C22:5n-6	
				C22:6n-3	

[A] Milk fatty acids in g/100 g fatty acids.

[B] The unit of CH_4 emission is similar to the CH_4 emission unit of the column with prediction equations in the corresponding row.

[C] Only correlations >0.7 or <−0.7 ($P < 0.001$) between individual MFA and CH_4, and no prediction equation with only MFA are reported in this study.

[D] Number of studies (N).

[E] Number of observations (O).

[F] Number of milk fatty acids identified (MFA).

[G] Dry matter intake (kg/d).

[H] The reported R^2 is adjusted for study effect.

[I] Fat- and Protein-Corrected Milk (FPCM)= (0.337 + 0.116 × fat % + 0.06 × protein %) × milk yield (kg/d).

[J] The reported R^2 is adjusted for cow, period, and study effect.

The relation between MFA and CH_4 emission has resulted in the suggestion that MFA composition can be used to predict CH_4 emission in lactating dairy cows. Table 2.1 summarizes the studies that have investigated the predictive power of MFA composition for CH_4 emission and derived multivariate models to predict CH_4 emission (Chilliard et al., 2009; Dijkstra et al., 2011; Mohammed et al., 2011; Van Lingen et al., 2014; Rico et al., 2016). In all studies, MFA profile was elucidated using gas chromatography (**GC**), and detailed information regarding the GC method used to determine the MFA profile is provided in the respective studies. In general, the significant correlations found between individual MFA and CH_4 emission are moderate (correlation coefficient ranging between 0.3 and 0.7), with the exception of the ones reported by Chilliard et al. (2009). Four studies (Chilliard et al., 2009; Dijkstra et al., 2011; Van Lingen et al., 2014; Rico et al., 2016) associated OBCFA with CH_4 emission, with varying results; C15:0 was negatively related with CH_4 production (g/d) in one study (Rico et al., 2016), positively related with CH_4 production (g/d) and intensity (g/kg fat- and protein-corrected milk; **FPCM**) in two studies (Chilliard et al., 2009; Van Lingen et al., 2014), and not related with CH_4 yield (g/kg dry matter intake; **DMI**) in another study (Dijkstra et al., 2011). All studies have relative similar results for the C18:1 and C18:2 isomers, which were generally found to be negatively related to CH_4 emission, and all studies have relative similar results for the SMCFA, which were generally found to be positively related to CH_4 emission. However, the specific SMCFA positively associated with CH_4 emission differ between studies, with C10:0 and C16:0 having a positive association with CH_4 emission in four studies each, but C4:0 having a positive relation with CH_4 production (g/d; Chilliard et al., 2009) or a negative relation with CH_4 intensity (g/kg FPCM; Van Lingen et al., 2014). Williams et al. (2014) (not included in the table) studied the relation between CH_4 production (g/d) and both C8:0 and total C18 FA in milk and concluded that the concentrations of C8:0 and total C18 FA in milk do not enable accurate prediction of CH_4 production (g/d). The variation between the studies regarding the SMCFA and individual C18 FA (Table 2.1), may explain why Williams et al. (2014) did not find a significant association between both C8:0 and total C18 FA in milk and CH_4 production (g/d). It should be noted here that the studies used different units to express CH_4 emission (CH_4 production in g/d, CH_4 yield in g/kg DMI, and CH_4 intensity in g/kg FPCM; Table 2.1), which may affect the relationships as well. For example, Van Lingen et al. (2014) and Dijkstra et al. (2016) found strong negative relations between CH_4 yield (g/kg DMI) and certain *trans* C18:1 FA (e.g., C18:1 *trans*-10 or C18:1 *trans*-10+11), but these were not observed for CH_4 intensity (g/kg FPCM). This can be explained by the various BHG intermediates in milk being associated with a reduction of CH_4 yield (g/kg DMI), as well as with milk fat depression. This negatively affects the amount of FPCM, resulting in the absence of a significant relationship between these various MFA and CH_4 intensity (g/kg FPCM) despite a strong negative relation with CH_4 yield (g/kg DMI).

Although these studies, with exception of Williams et al. (2014), show that MFA hold potential to reflect changes in rumen fermentation, due to discrepancies between studies, it remains unclear which MFA have the greatest potential as biomarker for CH_4 emission. Similar reservations hold for the CH_4 prediction equations given in several studies (Chilliard et al., 2009; Dijkstra et al., 2011; Mohammed et al., 2011; Van Lingen et al., 2014; Rico et al., 2016). The predictive power of the prediction equations range between 47% and 95% (Table 2.1), but the MFA included in these equations often differ between studies, with only C17:1 *cis*-9 (Mohammed

et al., 2011; Rico et al., 2016) and C18:1 *cis*-11 (Dijkstra et al., 2011; Mohammed et al., 2011; Rico et al., 2016) appearing in two or more equations. The discrepancies between the studies might be the result of the different CH_4 measurement techniques and analytical methods used to determine the MFA profile, the unit in which CH_4 is expressed, and the number of experiments used to determine the relation between MFA and CH_4. Overall, the predictive power seems higher when CH_4 is expressed as yield (g/kg DMI) or as production (g/d) with feed intake included as explanatory variable. This is because feed intake is a principal predictor of CH_4 production (g/d) (Moraes et al., 2014). However, in practice feed intake is usually unknown and therefore CH_4 intensity (g/kg FPCM) is of interest.

The most extensive dataset (i.e., number of studies and observations) was used by Van Lingen et al. (2014) with a wide variety of diets in order to assess the potential of MFA as an indicator for CH_4 emission (Table 2.1). Despite using a similar CH_4 measurement technique and a large number of experiments, Van Lingen et al. (2014) concluded that MFA have moderate potential to predict CH_4 emission, because the predictive power of the best CH_4 prediction equation was 0.47 for CH_4 intensity (g/kg FPCM) and 0.54 for CH_4 yield (g/kg DMI). Because these prediction equations were developed on a wide range of dietary treatments, the results of Van Lingen et al. (2014) suggest that one prediction equation for CH_4 emission may not be realistic. This is in agreement with independent evaluations (Mohammed et al., 2011; Dijkstra et al., 2016). Mohammed et al. (2011) compared observed CH_4 emission with CH_4 emission predicted by the equations of Chilliard et al. (2009) and Dijkstra et al. (2011). Estimating CH_4 emission using the other equations resulted in overprediction of CH_4 emission. Dijkstra et al. (2016) compared observed CH_4 emission of dairy cattle fed grass- and grass silage-based diets with CH_4 emission predicted by the equations of Van Lingen et al. (2014). It was concluded that these prediction equations could not accurately predict CH_4 yield (g/kg DMI) and intensity (g/kg FPCM), indicating that the relation between MFA profile and CH_4 emission in dairy cows fed grass- and grass silage-based diets differ from those determined for other types of diets. This suggests that diet specific prediction equations may have to be developed.

Although the relation between MFA and CH_4 emission seems moderate and diet specific, it might provide a simple method to predict CH_4 emission from dairy cattle on large scale. Because enteric CH_4 emission is among the main targets of GHG mitigation practices for the dairy industry (Hristov et al., 2013), it is worthwhile to further explore the application of this biomarker technique.

MID-INFRARED TO MEASURE MILK FATTY ACIDS

Until recently, GC was the principal method for MFA analysis as GC measures a large number of MFA precise and accurately, even those present at low concentrations in milk fat. However, the GC method is unsuitable for routine milk recording (Soyeurt et al., 2011). Infrared spectroscopy techniques are inexpensive, non-destructive, rapid, and multi-parametric (Coppa et al., 2014). Both near-infrared spectroscopy and MIR show good prediction performance for MFA concentrations (either in g/100 g FA or g/kg milk) allowing their use for routine MFA composition recording (Coppa et al., 2014). At present, MIR spectroscopy is routinely used in milk recording systems worldwide to predict fat, protein, lactose, and urea contents in dairy milk

(Coates, 2000) to assist in farm management decisions and for breeding purposes. Because MIR is already a major tool in dairy science and therefore easily implementable for estimating CH_4 emission, this review focuses only on MIR.

Several studies have investigated the potential use of MIR spectroscopy to predict MFA composition in dairy cattle (Soyeurt et al., 2006, 2011; Rutten et al., 2009; De Marchi et al., 2011; Ferrand et al., 2011; Maurice-Van Eijndhoven et al., 2012), which have been extensively reviewed by De Marchi et al. (2014). In general, these studies find a clear relationship between MFA concentration (g/100 g FA) and the accuracy of the MIR spectroscopy prediction models; the accuracy of the MIR spectroscopy prediction models for major MFA is higher compared with minor MFA. The accuracy of MIR spectroscopy prediction models is also higher for individual saturated fatty acids (**SFA**) than individual UFA. When dividing UFA in two groups, namely mono unsaturated fatty acids (**MUFA**) and poly unsaturated fatty acids (**PUFA**), good accuracy is achieved for MIR spectroscopy prediction models for MUFA, whereas it is not for PUFA (De Marchi et al., 2014).

The results from these studies (i.e., Soyeurt et al., 2006, 2011; Rutten et al., 2009; De Marchi et al., 2011; Ferrand et al., 2011; Maurice-Van Eijndhoven et al., 2012) confirm the potential of MIR spectroscopy for accurate prediction of several individual, in particular major, MFA and groups of MFA, but a considerable number of lower abundant MFA cannot be predicted by MIR. In addition, Eskildsen et al. (2014) investigated whether the predictions of individual MFA using MIR spectroscopy rely on direct association or indirect correlations, which are confined to covariance structures in the dataset. It was concluded that the prediction of MFA with MIR spectroscopy is indirect and based primarily on covariation between individual MFA and total fat content of the milk. This indicates that the implementation of MIR spectroscopy MFA predictions in milk recording systems must account for the universal validity of these indirect correlations, because the ratio between individual MFA and total fat content found in calibration milk samples may not be conserved in future milk samples resulting in incorrect and biased predictions for future milk samples (Eskilden et al., 2014). Therefore, MIR spectroscopy predicted MFA in calibration milk samples always need to be cross validated with the use of an external and independent dataset. Overall, MIR spectroscopy is an interesting alternative in the dairy sector for providing indications of the MFA profile of dairy cows (Soyeurt et al., 2006).

MID-INFRARED TO ESTIMATE METHANE EMISSION

In general, CH_4 emission is linked to MFA profile. As MIR spectroscopy reflects the MFA profile, it is logical to assume that MIR spectroscopy could estimate CH_4 emission from dairy cows. Van Lingen et al. (2014) evaluated, indirectly via MFA composition, the use of MIR spectroscopy to estimate CH_4 emission of dairy cows and developed prediction models with restricted selection of MFA based on the MIR results of Soyeurt et al. (2011) and of Rutten et al. (2009). The prediction equations for CH_4 yield (g/kg DMI) decreased in predictive power from $R^2 = 0.54$ when using all MFA to $R^2 = 0.43$ when using the accurately MIR determined MFA reported by Soyeurt et al. (2011) and to $R^2 = 0.29$ when using the accurately MIR determined MFA reported by Rutten et al. (2009). Similarly, the predictive power for CH_4 emission intensity (g/kg FPCM) decreased from $R^2 = 0.47$ when using all MFA to $R^2 = 0.36$

when using the accurately MIR determined MFA reported by Soyeurt et al. (2011) and to R^2 = 0.28 when using the accurately MIR determined MFA reported by Rutten et al. (2009). These results indicate that the performance of MIR spectroscopy limits the potential for estimating CH_4 emission based on MFA, compared with GC, because several lower abundant MFA that appear in various CH_4 prediction equations published (Chilliard et al., 2009; Dijkstra et al., 2011; Mohammed et al., 2011; Van Lingen et al., 2014; Rico et al., 2016). are not available when MFA is determined using MIR spectroscopy (Van Lingen et al., 2014).

Kandel et al. (2015) assessed indirectly whether MIR spectrometry can predict CH_4 production (g/d) from dairy cows by the use of four CH_4 prediction equations, each developed by Chilliard et al. (2009). The predicted CH_4 production (g/d) was within the expected range from 350 ± 40 to 449 ± 65 g CH_4/d, and Kandel et al. (2015) concluded that it is feasible to use MIR spectroscopy to predict CH_4 production (g/d). However, only the CH_4 prediction equations developed by Chilliard et al. (2009) were considered, because these were developed from abundant MFA which have a high MIR prediction accuracy (Soyeurt et al., 2011). This highlights, similar to Van Lingen et al. (2014) that the performance of MIR spectroscopy is limited compared with GC, because lower abundant MFA important for the prediction of CH_4 emission are not available when MFA is determined using MIR spectroscopy.

At present, there are two studies that investigated directly with no intermediate steps (i.e., MFA profile) if MIR spectroscopy can predict CH_4 emission from individual cows. Dehareng et al. (2012) used two experiments involving 11 lactating Holstein cows and three dietary treatments, in which CH_4 emission was measured using the SF_6-tracer technique, and MIR spectroscopy prediction models were developed using average milk spectra from morning and evening milk samples. The accuracy of the different developed MIR spectroscopy prediction models for CH_4 production (g/d) and CH_4 intensity (g/kg milk) on this small dataset is rather high; the cross-validation coefficient of determination ranges from 0.68 to 0.79. However, according to Vanlierde et al. (2015), the predicted CH_4 emission using the MIR spectroscopy prediction models from Dehareng et al. (2012) was lowest in early lactation to increase thereafter, which is biologically not meaningful (Vanlierde et al., 2015). Therefore, Vanlierde et al. (2015) developed lactation stage dependent predictions of CH_4 emission from MIR spectra, using a total of 446 CH_4 measurements of 142 Holstein, Jersey and Holstein-Jersey cows, measured with the SF_6 tracer technique. Methane predictions using MIR spectra only were compared with CH_4 predictions using MIR spectra and days in milk (**DIM**). The average CH_4 production (g/d) predicted by both models hardly differed (both models, standard error of calibration of 63 g CH_4/d; observed mean of 416 g CH_4/d). However, in contrast to the predictions based on MIR spectra only, the predictions that included DIM showed biologically meaningful behavior throughout lactation (an increase in CH_4 production (g/d) after calving up to some 100 DIM, followed by gradual decline to end of lactation). Both studies (Dehareng et al., 2012; Vanlierde et al., 2015) show the potential to estimate CH_4 emission directly using MIR spectroscopy, in particular in combination with other characteristics such as DIM.

The inclusion of other milk constituents may also result in better CH_4 emission prediction. Moraes et al. (2014) identified milk fat proportion as key explanatory variable for CH_4 emission. This component can be swiftly and easily determined. In addition, there are new

developments to include other milk constituents. Van Gastelen et al. (2015) show the potential to use volatile and non-volatile metabolites in milk to quantify CH_4 emission. However, the techniques for identifying volatile (i.e., gas chromatography-mass spectrometry) and non-volatile metabolites (i.e., nuclear magnetic resonance) are not suitable for large-scale measurements.

IMPLICATIONS AND CONCLUSIONS

The predicted power of MFA-based equations indicates good potential for CH_4 emission prediction, but the GC method used to determine the MFA profile is unsuitable for routine analysis. The use of MIR spectroscopy appears to be a promising approach to predict CH_4 emission routinely at large scale. MIR spectroscopy is able to predict CH_4 emission directly or indirectly by prediction of a number of MFA, which in turn can be used to estimate CH_4 emission. The major advantages of using MIR spectroscopy to predict CH_4 emission include its simplicity and potential practical application at large scale. Disadvantages include the inability to predict important MFA for CH_4 prediction, and the moderate predictive power for CH_4 emission, both direct and indirect. It may not be sufficient to predict CH_4 emission based on MIR alone. Integration with other factors, like feed intake, nutrient composition of the feed, parity, and lactation stage may improve the prediction of CH_4 emission using MIR spectra.

More research is needed, including cross-validation with external and independent data to account for the universal validity of indirect correlations, more observations and a larger variation in dietary treatments, to establish the robustness, accuracy and repeatability of MIR spectroscopy to predict CH_4 emission of dairy cows directly and indirectly, and to make MIR spectroscopy more reliable and potentially implementable.

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Chapter 3

Enteric methane production, rumen volatile fatty acid concentrations, and milk fatty acid composition in lactating Holstein-Friesian cows fed grass silage- or corn silage-based diets

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ABSTRACT

The objective of this study was to determine the effects of replacing grass silage (**GS**) with corn silage (**CS**) in dairy cow diets on enteric methane (CH_4) production, rumen volatile fatty acid concentrations, and milk fatty acid (**FA**) composition. A completely randomized block design experiment was conducted with 32 multiparous lactating Holstein-Friesian cows. Four dietary treatments were used, all having a roughage-to-concentrate ratio of 80:20 based on dry matter (**DM**). The roughage consisted of either 100% GS, 67% GS and 33% CS, 33% GS and 67% CS, or 100% CS (all DM basis). Feed intake was restricted (95% of *ad libitum* DM intake) to avoid confounding effects of DM intake on CH4 production. Nutrient intake, apparent digestibility, milk production and composition, nitrogen (**N**) and energy balance, and CH4 production were measured during a 5-d period in climate respiration chambers after adaptation to the diet for 12 d. Increasing CS proportion linearly decreased neutral detergent fiber and crude protein intake and linearly increased starch intake. Milk production and milk fat content (on average 23.4 kg/d and 4.68%, respectively) were not affected by increasing CS inclusion, whereas milk protein content increased quadratically. Rumen variables were unaffected by increasing CS inclusion, except the molar proportion of butyrate, which increased linearly. Methane production (g/d), yield (% gross energy intake), and intensity (g/kg fat- and protein-corrected milk; FPCM) decreased quadratically with increasing CS inclusion, and decreased linearly when expressed as yield (g CH_4 / kg DM intake; **DMI**). In comparison with 100% GS, CH_4 yield (g/kg DM intake) and CH_4 intensity (g/kg **FPCM**) were 11 and 8% reduced for the 100% CS diet, respectively. Nitrogen efficiency increased linearly with increased inclusion of CS. The concentration of *trans* C18:1 FA, C18:1 *cis*-12, and total CLA increased quadratically, and *iso* C16:0, C18:1 *cis*-13, and C18:2n-6 increased linearly, whereas the concentration of C15:0, *iso* C15:0, C17:0, and C18:3n-3 decreased linearly with increasing inclusion of CS. No differences were found in short- and medium-straight, even-chain FA concentrations, with the exception of C4:0 which increased linearly with increased inclusion of CS. Replacing GS with CS in a common forage-based diet for dairy cattle offers an effective strategy to decrease enteric CH_4 production without negatively affecting dairy cow performance, although a critical level of starch in the diet seems to be needed.

Keywords: dairy cow, enteric methane production, grass silage, corn silage

INTRODUCTION

Developing strategies to reduce enteric methane (CH_4) emissions from ruminants has received increasing interest recently, as it reduces the ecological footprint of milk production and potentially improves feed efficiency. Dietary manipulation seems to be the most direct and effective approach for reducing CH_4 production from ruminants (Beauchemin et al., 2009) because CH_4 production depends greatly on the level of feed intake and dietary composition, in particular the type of carbohydrates (Beauchemin et al., 2008; Ellis et al., 2008). Including various inhibitors or electron receptors in ruminant diets can reduce CH4 production up to 50%, but in view of effectiveness and safety issues (e.g., issues with nitrates include potential

toxicity from intermediate products), reductions of 10 to 30% are more likely in commercial practice (Hristov et al., 2013). Roughage represents the major component in dairy cow diets and, therefore, it is interesting to investigate the reduction of CH_4 production using roughage-based diets.

Replacing fiber-rich roughage with starch-rich roughage has potential to reduce CH_4 emissions (Brask et al., 2013; Hassanat et al., 2013). Fermentation of starch favors the ruminal production of propionate at the expense of acetate and decreases rumen pH, which reduces hydrogen availability and activity of rumen methanogens (Van Kessel and Russell, 1996; Hook et al., 2011). The scientific evidence for this particular dietary replacement strategy is limited and does not always reflect diets used in practice. Staerfl et al. (2012) investigated this strategy, but the corn silage (**CS**) used had a net energy content some 10% lower than that of the grass silage (**GS**), which is uncommon in many countries. Brask et al. (2013) also investigated the effect of this dietary strategy, but the CS used had a starch content of only 150 g/kg of DM, which is low and uncommon compared with the reported starch content of CS at comparable DM contents (Sutton et al., 2000; Mc Geough et al., 2010a); therefore, the difference in starch content between the CS- (141 g/kg of DM) and the GS-diet (43 g/kg of DM) was not large.

When manipulating dairy cow diet for CH_4 reduction, one should be aware that the composition of milk can also change. Several studies observed that changes in dietary proportion of GS and CS can alter milk FA composition (Ferlay et al., 2006; Nielsen et al., 2006; Kliem et al., 2008). These studies were mainly interested in altering milk FA from a human health perspective, because milk and dairy products are an important source of fat and specific FA in the human diet (Van Valenberg et al., 2013). In terms of dietary CH_4 mitigation strategies, differences in milk FA are interesting because they reflect the variations in the amount and composition of carbohydrate between GS and CS (Nielsen et al., 2006), which influences both rumen environment and biohydrogenation of unsaturated FA (Kliem et al., 2008). Consequently, milk FA composition has been suggested as a method to predict enteric CH_4 output in lactating dairy cattle (Dijkstra et al., 2011).

Roughages are nutritionally and economically important (Hassanat et al., 2013). Therefore, it is imperative to investigate dietary strategies using roughage-based diets to mitigate CH_4 production and to determine its effect on milk FA composition. Although GS and CS represent the major conserved roughages and are commonly used in dairy production (Wilkinson et al., 1996), to the best of our knowledge no study has investigated the effect of replacing GS with CS on enteric CH_4 production, rumen VFA concentrations, and milk production and composition, including milk FA composition together. Thus, the objectives of our study were (1) to gain more scientific evidence for the CH_4 mitigation strategy of replacing fiber-rich GS with starch-rich CS, (2) to examine the changes in ruminal VFA concentration and pH when replacing GS with CS, and (3) to determine the effects of replacing GS with CS on milk production and milk FA composition.

MATERIALS AND METHODS

Experimental design

The experiment was conducted from October to December 2012 in accordance with Dutch law and approved by the Animal Care and Use Committee of Wageningen University & Research. The experiment followed a completely randomized block design with 4 dietary treatments and 32 multiparous lactating Holstein-Friesian cows with an average milk production of 34.0 ± 5.71 kg/d and 192 ± 87 DIM at the start of the experiment. Cows were blocked in groups of 4 according to lactation stage, parity, milk production, and presence of a rumen cannula (12 cows), and within each block cows were randomly assigned to 1 of 4 dietary treatments; treatment periods, 8 in total, lasted 17 d.

Table 3.1. Ingredient and chemical composition of experimental diets

	Treatment[1]			
Item	GS100	GS67	GS33	GS0
Ingredient (g/kg DM)				
Grass silage[2]	800	533	267	-
Corn silage[3]	-	267	533	800
Concentrate[4]	200	200	200	200
Chemical composition (g/kg DM)				
Organic matter	924	931	938	945
Crude protein	192	182	172	163
Crude fat	22	22	21	21
Gross energy (MJ/kg DM)	18.8	18.7	18.6	18.5
Neutral detergent fiber	431	396	360	325
Acid detergent fiber	233	219	204	190
Acid detergent lignin	14	14	15	15
Starch	5	91	177	262
Reducing sugars	130	98	66	34

[1] Treatments had a roughage:concentrate ratio of 80:20 (DM basis). Concentrate was similar for all treatments.

[2] Dry matter = 471 g/kg, chemical composition (g/kg DM): CP = 112, fat = 22, ash = 68, gross energy = 18.8 MJ/kg DM, net energy for lactation = 6.5 MJ/kg DM, NDF = 510, sugar = 133, ensiling characteristics (g/kg DM): acetic acid = 22, lactic acid = 47, ammonia = 2, and pH = 4.8.

[3] Dry matter = 320 g/kg, chemical composition (g/kg DM): CP = 76, fat = 20, ash = 42, gross energy = 18.5 MJ/kg DM, net energy for lactation = 6.7MJ/kg DM, NDF = 377, starch = 322, sugar = 14, ensiling characteristics (g/kg DM): ammonia = 1, and pH = 3.8.

[4] Contained (g/kg DM): solvent extracted soybean meal 502, formaldehyde treated soybean meal 300, citrus pulp 80, molasses 50, urea 30, $CaCO_3$ 15, NaCl 8, trace mineral and vitamin premix 8, and MgO 7; dry matter = 882 g/kg; chemical composition (g/kg DM): CP = 510, fat = 23, ash = 109, gross energy = 18.6 MJ/kg DM, net energy for lactation = 7.4 MJ/kg DM, NDF = 116, starch = 24, sugar = 116.

Diets and feeding

All dietary treatments had a roughage-to-concentrate ratio of 80:20 based on DM content. The composition of the compound feed was the same for all 4 treatments, whereas the roughage was GS, CS, or a mixture of both. The ingredient and chemical composition of the 4 diets are presented in Table 3.1. Dietary treatments were (ingredient as percentage of the total amount of roughage in the diet; DM basis): (1) 100% GS (**GS100**); (2) 67% GS and

33% CS (**GS67**); (3) 33% GS and 67% CS (**GS33**); and (4) 100% CS (**GS0**).

Cows were fed individually and feed refusals collected to determine DMI throughout the experiment. The cows received their feed twice daily in equal portions before milking, with compound feed supplied on top of the roughage. The cows were fed ad libitum during the first 7 d of the adaptation period in the tiestalls. From d 8 to 17 [i.e., last 5 d of the adaptation period and the 5-d period in the climate respiration chambers (**CRC**)], feed intake was restricted to 95% of the ad libitum DMI of the cow within a block consuming the lowest amount of feed during d 5 to 8, as described previously by Van Zijderveld et al. (2011a).

Samples of GS, CS, and compound feed were obtained when fresh feed was prepared (i.e., twice a week). These samples were subsequently pooled per period and subsampled for analyses. Orts, when present during the 5-d period in the CRC, were collected and pooled per cow and a representative sample was collected. The samples of GS, CS, compound feed, and orts were stored at −20°C until further analyses.

Housing and climate respiration chambers

In each treatment period, 4 cows of 1 block were individually housed in tiestalls for a 12-d period to become accustomed to the diet and restriction in movement. After the adaptation period, the cows were housed in identical CRC for a 5-d period to determine gaseous exchange, energy and nitrogen (**N**) balance, and apparent digestibility. Clean drinking water was ad libitum provided and cows were milked and fed twice daily at 0600 and 1600 h during the entire experiment. Cows were exposed to 16 h of light per day (from 0530 to 2130 h).

Two large CRC were used, each containing 2 individual airtight compartments. The CRC were equipped with thin walls with windows, to ensure cows could see and hear each other to minimize the effect of social isolation on cow behavior and performance. The principles of the CRC are described in detail by Verstegen et al. (1987). The ventilation rate within the CRC was 42 m^3/h per compartment. Each compartment had an area of 11.8 m^2 and a volume of 34.5 m^3, and the relative humidity was maintained at 70% and temperature at 16°C by 2 computer-controlled air conditioning units. The relative humidity was monitored by 1 relative humidity sensor (Novasina Hygrodat100, Novasina AG, Lachen, Switzerland), and the temperature was monitored by 5 PT100 temperature sensors (Sensor Data BV, Rijswijk, the Netherlands) evenly distributed over the chamber at animal height.

Air from outside was pumped into each compartment via a gas volume meter (Itron Delta 2080 G100, Itron GmbH, Karlsruhe, Germany). Exhaust air exited through a duct with an iris valve controlling the pressure inside the compartment. Within each compartment, a positive pressure of 120 Pa was maintained. The inlet and exhaust air of each compartment was sampled for gas analysis (CH_4, O_2, and CO_2). Gas analyzers (ABB Advance Optima AO2000 systems, ABB, Berlin, Germany) were setup in series with analysis of CO_2 and CH_4 concentration using a nondispersive infrared method, and O_2 concentration using a paramagnetic method.

The volumes of inlet and exhaust air of the CRC were corrected for pressure, temperature, and humidity to arrive at standard temperature pressure dewpoint volumes. Inlet

and exhaust volumes of CH_4, O_2, and CO_2 were calculated by multiplying the respective gasconcentrations with the standard temperature pressure dewpoint volumes of inlet and exhaust air. Production of CO_2 and CH_4 and consumption of O_2 was calculated from the difference between inlet and exhaust gas volumes. This was measured with 10-min intervals because the 4 compartments share 1 gas analysis system. Computer-controlled valves direct the air sample from the 4 compartments in sequence (i.e., inlet air, exhaust air compartment A, B, C, and D) to the gas analysis system. Sampled air was flushed through the gas analysis system for 120 s and the average gas concentration of the last 30 s was stored in a computer database. After 120 s the air valves switched to the next compartment.

Once a day, calibration gasses were sampled for gas analysis instead of the inlet air. The analyzed and actual values of these calibration gasses were used to correct the measured gas concentrations from the inlet air and exhaust air of the 4 compartments. In addition, before the experiment started, compartments were checked by releasing known amounts of CO_2 in each compartment and comparing these values with the data from the gas analysis system to calculate the recovery. The recovered amounts of CO_2 were between 98 and 100%.

Staff entered each CRC compartment twice daily at 0600 and 1600 h for approximately 30 min for milking and feeding. The gas measurements during the opening of the CRC were not used for data analysis; CH_4 and CO_2 production and O_2 consumption during these periods was assumed to be linear between the last data point before opening and the first data point after closing the CRC.

For the CH_4 and CO_2 production and O_2 consumption, 3 full 24-h periods were used (i.e., starting at 0800 h of d 14 until 0800 h of d 17). For N and energy balance, manure of each cow of the complete measuring period in the CRC (i.e., starting at 1500 h on d 13 until 0900 h on d 17) was quantitatively collected in the CRC, weighed, mixed, sampled for analyses, and stored at −20°C until further analyses. Cows were weighed immediately after entering and before leaving the CRC. All data presented in the current paper refer to the period the cows were in the CRC. In contrast to other experiments previously performed in the CRC of Wageningen University & Research where 2 cows were housed in 1 large CRC (e.g., Van Zijderveld et al., 2011b), in the present experiment the experimental unit is the individual cow because cows were individually housed in the compartments of the CRC.

Milk yield and composition

Milk yield was recorded during each milking. In the CRC, a milk sample (10 mL) of each milking was collected in a tube containing sodium azide (5 μL) for preservation. These samples, 8 in total per cow, were analyzed for fat, protein, and lactose content by mid-infrared spectroscopy, and for MUN using the pH difference technique (ISO 14637; ISO, 2004) at Qlip (Zutphen, the Netherlands). Milk composition was corrected for differences in milk yield between individual milkings and the average was used for data analysis.

A representative sample (5 g/kg of milk production) was obtained at each milking from each cow, pooled per cow for the entire period in the CRC, and stored at −20°C pending analyses for gross energy (**GE**) and N. For milk FA composition, another representative sample was obtained (5 g/kg of milk production at each milking from each cow).

Sodium azide (0.05% wt/wt) was added afterwards to the pooled sample of the first 4 milkings, followed by the same procedure for milking 5 to 8 in a separate bottle. Both bottles were stored at 5°C. After the last milking, these 2 subsamples (milkings 1–4 and 5–8) were pooled and stored at −40°C until FA composition analysis.

Rumen fermentation parameters and VFA

Samples of rumen fluid were taken from the rumen cannulated cows on d 10 and 11 to determine VFA concentration and pH levels. Rumen fluid samples (approximately 200 mL) were collected 1 h before, and 1, 2, 4, 6, and 8 h after morning feeding on both days. Rumen fluid samples were obtained as described by Van Zijderveld et al. (2011b), and collected in 3 equal amounts from the front and middle of the ventral sac and from the cranial sac of the rumen. After collection, the collected rumen fluid was thoroughly mixed, pH was measured using an electronic pH meter (HI9024C, Hanna Instruments, IJsselstein, the Netherlands), and 2 rumen fluid samples (600 μL each) were taken and acidified with an equal volume of 0.85% *M* ortho-phosphoric acid containing 19.68 m*M* isocaproic acid as internal standard. These 2 rumen fluid samples were directly frozen (−20°C) to stop microbial fermentation and stored at −20°C until VFA analysis.

Analytical procedures

Prior to analyses, GS, CS, and compound feed samples were thawed at room temperature, air-dried at 60°C, ground to pass a 1-mm screen using a Wiley mill (Peppink 100AN, Olst, the Netherlands), and analyzed for DM, ash, crude fat, starch (except for GS samples), reducing sugars (all carbohydrates with reducing properties and soluble in 40% ethanol), NDF, ADF, ADL, GE, and N. Orts were analyzed for DM, ash, GE, and N.

The manure samples (i.e., feces plus urine combined) were analyzed for DM, ash, N, NDF, GE, crude fat, and starch. Prior to analyses, these samples were thawed at room temperature, air-dried at 60°C, and ground to pass a 1-mm screen. In addition, to determine the N balance, the total amount of condensed water (i.e., collected from the heat exchanger) produced, and the increase in 25% sulfuric acid solution (wt/wt; i.e., through which the outflowing air was led to trap aerial ammonia) of each CRC compartment was measured (both in grams). Samples of both condensed water and 25% sulfuric acid solution were analyzed for N.

Ash, DM, N, crude fat, starch, reducing sugars (all carbohydrates with reducing properties and soluble in 40% ethanol), NDF, ADF, and ADL of feed and manure samples were analyzed as described by Abrahamse et al. (2008a). Bomb calorimetry (ISO 9831; ISO, 1998) was used to determine GE. Crude protein was calculated as N × 6.25. Starch, NDF, and crude fat were assumed to be absent in urine, allowing for calculation of apparent digestibility of these components from analysis of starch, NDF, and crude fat in the combined mixture of feces and urine and in feed.

Milk FA composition was analyzed through gas chromatograph analysis by Qlip. Milk fat was extracted from the milk samples and FAME were prepared from fat fractions (ISO 15884; ISO, 2002a). Methyl esters were analyzed (ISO 15885; ISO, 2002b) on a TRACE

Ultra gas chromatograph (Thermo Electron Corporation, Waltham, MA) with a split/splitless injector operated in split mode (split ratio 1:100), at a temperature of 270°C, using a Varian WCOT fused silica column with CP-select CB for FAME as stationary phase (100 m × 0.25 mm i.d.; Varian Inc., Palo Alto, CA) and hydrogen as carrier gas, and fitted to a flame ionization detector (**FID**; 250°C). The initial temperature was held at 65°C for 1 min, increased to 225°C at 3°C/min, and held at 225°C for 5 min. A volume of 1 μL was injected. Peaks were identified and quantified using pure methyl esters (Sigma-Aldrich, Zwijndrecht, the Netherlands; Larodan, Malmö, Sweden; Lipidox). Results of FA were corrected for FID response and expressed as grams per 100 g of total FA.

For determination of VFA, the rumen fluid samples were thawed and centrifuged for 5 min at 14,000 × *g* at room temperature. The clear supernatant (1 μL) was injected onto a gas chromatograph (Fisons HRGC Mega 2, CE Instruments, Milan, Italy) with a split/splitless injector operated in split mode (split ratio 1:10), at a temperature of 225°C, using a capillary column (EC-1000, Alltech, Deerfield, IL; 30 m, i.d. = 0.53mm, film thickness = 1 um) and helium as carrier gas, and fitted to an FID. The initial temperature of the column was held at 110°C for 2 min, increased to 200°C at 18°C/min, and held at 200°C for 2 min. Identification and quantification was conducted with a chemical standard solution (0.85% *M* ortho-phosphoric acid), including an internal standard (19.681 m*M* isocaproic acid) for correction.

Statistical analysis

All parameters related to feed, milk production, and milk composition while cows were housed in the CRC were averaged over a 4-d period. The parameters related to energy and N balance were expressed per kilogram of metabolic body weight (**$BW^{0.75}$**) per day. One cow (receiving diet GS0) was excluded from the experiment because large feed residuals while housed in the CRC resulted in much lower DMI compared with the last 4 d in the tiestall and the other cows in that block. Cow was considered the experimental unit for all parameters. Data were analyzed using the MIXED procedure in SAS (version 9.2, SAS Institute Inc., Cary, NC).

The model included dietary treatment as a fixed effect and period (which is equal to block) as a random effect. For all analyses, the fixed effect of CRC was initially included in the model, but was removed because it was found not significant. Autoregressive 1, variance component, compound symmetry, and unstructured covariance structures were tested for each analysis, and the covariance structure with the lowest overall Akaike's information criterion values (i.e., variance component) was selected. The rumen variables were averaged per time point per cow and subjected to repeated-measures ANOVA to take repeated samples within the same animal into account. This model included cow and period as random effects and diet, time of sampling, and the interaction of diet and time of sampling as fixed effects. To take the unbalanced sampling time intervals into account, the spatial covariance structure was selected.

Both models had unequal variances, therefore, the Kenward-Roger option was used to estimate the denominator degrees of freedom. Orthogonal polynomial contrasts (linear and

quadratic) were used to examine treatment effect on response variables. A significant effect of treatment on least squares means was declared when $P \leq 0.05$.

RESULTS

Feed intake and digestibility

Increasing the proportion of CS in the diet resulted in a linear increase of DMI (Table 3.2). No difference was found when comparing the DMI of the cows housed in the CRC with the DMI of the cows housed in the tiestall during the 4 d before entering the CRC ($P = 0.153$, data not shown). Intakes of CP, NDF, ADF, and reducing sugars declined linearly ($P < 0.002$), whereas intake of starch increased linearly ($P < 0.001$) as the dietary proportion of CS increased. Apparent total-tract digestibility of NDF decreased quadratically ($P < 0.001$), whereas apparent total-tract digestibility of fat increased quadratically ($P < 0.001$) and of starch linearly ($P < 0.001$) as the CS proportion in the diet increased.

Milk production and composition

Milk production, fat- and protein-corrected milk production (**FPCM**), milk fat content, and fat yield were unaffected by increasing proportion of CS in the diet (Table 3.3). A quadratic increase was observed for milk protein and milk lactose content ($P < 0.001$) when CS proportion of diet increased. Protein yield increased linearly ($P < 0.019$), whereas MUN decreased linearly ($P < 0.001$) with an increasing proportion of CS in the diet.

Table 3.2. Intake and apparent total tract digestibility of nutrients in lactating dairy cows fed different proportions of grass silage in the diet

	Treatment[1]					*P*-value	
Item	GS100	GS67	GS33	GS0	SEM	Linear	Quadratic
Intake (kg/d)							
Dry matter	16.2	16.7	16.6	17.5	0.41	0.001	0.858
Organic	14.8	15.4	15.4	16.4	0.47	0.020	0.776
Crude protein	3.11	3.05	2.90	2.86	0.101	0.002	0.790
Crude fat	0.36	0.36	0.35	0.36	0.013	0.324	0.915
Gross energy	303.7	311.2	308.7	322.5	9.34	0.222	0.863
NDF	6.97	6.58	5.97	5.68	0.202	< 0.001	0.926
ADF	3.78	3.64	3.39	3.32	0.114	< 0.001	0.873
ADL	0.23	0.24	0.24	0.26	0.013	0.159	0.831
Starch	0.08	1.51	2.92	4.59	0.070	< 0.001	0.105
Reducing	2.11	1.65	1.10	0.61	0.130	< 0.001	0.776
Apparent digestibility (% of intake)							
Fat	48.9	56.4	61.8	68.4	1.43	< 0.001	< 0.001
NDF	73.1	71.4	62.6	48.4	1.42	< 0.001	< 0.001
Starch	13.8	94.4	97.5	98.6	1.52	< 0.001	0.716

[1] Treatments had a roughage:concentrate ratio of 80:20 (DM basis). Concentrate was similar for all treatments. Roughage consisted of (all DM basis) 100% grass silage for GS100; 67% grass silage and 33% corn silage for GS67; 33% grass silage and 67% corn silage for GS33; 100% corn silage for GS0 (n = 8 for GS100, GS67, and GS33; n = 7 for GS0).

Table 3.3. Milk production and milk composition of lactating dairy cows fed different proportions of grass silage in the diet

	Treatment[1]					*P*-value	
Item	GS100	GS67	GS33	GS0	SEM	Linear	Quadratic
Milk production (kg/d)	22.6	23.2	24.2	23.6	1.19	0.457	0.185
FPCM[2] (kg/d)	24.0	24.9	25.7	25.6	0.93	0.125	0.297
Milk fat content (%)	4.61	4.77	4.72	4.62	0.148	0.885	0.317
Milk protein content (%)	3.44	3.49	3.34	3.67	0.104	0.003	< 0.001
Milk lactose content (%)	4.39	4.55	4.60	4.61	0.041	< 0.001	< 0.001
Fat yield (g/d)	1,019	1,069	1,106	1,080	40.0	0.165	0.190
Protein yield (g/d)	771	782	781	833	19.0	0.019	0.245
MUN (mg/dL)	14.6	11.9	11.5	10.3	0.80	< 0.001	0.288

[1] Treatments had a roughage:concentrate ratio of 80:20 (DM basis). Concentrate was similar for all treatments. Roughage consisted of (all DM basis) 100% grass silage for GS100; 67% grass silage and 33% corn silage for GS67; 33% grass silage and 67% corn silage for GS33; 100% corn silage for GS0 (n = 8 for GS100, GS67, and GS33; n = 7 for GS0).

[2] Fat- and protein-corrected milk = (0.337 + 0.116 × fat% + 0.06 × protein%) × milk yield (kg/d).

Methane emission

Methane yield (g/kg of DMI) decreased linearly ($P = 0.010$) with increasing dietary CS proportion (Table 3.4). A quadratic decrease ($P < 0.001$) was observed for CH_4 production (g/d), intensity (g/kg FPCM), and yield as a percent of GE intake (**GEI**). The total decrease in CH_4 emission observed when the dietary GS proportion was replaced with CS was 11, 8, and 7% for CH_4 yield (g/kg DMI), intensity (g/kg FPCM), and yield as a percent of GEI, respectively.

Rumen VFA concentrations and pH

All rumen variables were affected by time of rumen sampling ($P \leq 0.002$) and the interaction between diet and time of rumen sampling ($P < 0.001$; data not shown). In general, rumen pH initially decreased after morning feeding and increased again several hours later, whereas VFA concentration showed the opposite pattern. Butyrate molar proportions increased linearly ($P = 0.006$) when the dietary proportion of CS increased (Table 3.5). No other rumen variables responded linearly or quadratically upon increasing the dietary CS proportion.

Energy and nitrogen balance

All parameters related to the energy balance and expressed per kilograms of $BW^{0.75}$ per day [i.e., GEI, CH_4 production, metabolizable energy intake (**MEI**), heat production, energy retention (**ER**) total, ER protein, ER fat, and energy output in milk] were unaffected by increasing the proportion of CS in the diet (Table 3.6). Nitrogen intake and N output in manure decreased linearly ($P < 0.001$) with increasing dietary CS proportion (Table 3.6), whereas N output in milk and the N balance were unaffected by dietary CS proportion. A linear increase was observed for N efficiency ($P < 0.001$).

Table 3.4. Methane production of lactating dairy cows fed different proportions of grass silage in the diet

	Treatment[1]					*P*-value	
Item	GS100	GS67	GS33	GS0	SEM	Linear	Quadratic
CH_4 (g/d)	399	414	411	387	12.8	0.028	< 0.001
CH_4 (g/kg DMI)	24.6	25.0	24.5	22.0	0.38	0.010	0.107
CH_4 (g/kg	16.6	17.0	16.2	15.3	0.50	< 0.001	< 0.001
CH_4 (% of GEI[3])	6.96	7.17	7.11	6.45	0.107	< 0.001	< 0.001

[1] Treatments had a roughage:concentrate ratio of 80:20 (DM basis). Concentrate was similar for all diets. Roughage consisted of (all DM basis) 100% grass silage for GS100; 67% grass silage and 33% corn silage for GS67; 33% grass silage and 67% corn silage for GS33; 100% corn silage for GS0 (n = 8 for GS100, GS67, and GS33; n = 7 for GS0).

[2] Fat- and protein-corrected milk.

[3] Gross energy intake.

Table 3.5. Rumen pH, total VFA concentration, and VFA molar proportions of fistulated lactating dairy cows fed different proportions of grass silage in the diet[1]

	Treatment[2]					*P*-value	
Item	GS100	GS67	GS33	GS0	SEM	Linear	Quadratic
pH	6.77	6.74	6.73	6.72	0.100	0.671	0.917
Total VFA (mM)	103	100	98	98	6.6	0.580	0.804
VFA (% of total VFA)							
Acetate	65.6	66.0	65.8	63.6	1.23	0.126	0.127
Propionate	18.9	17.8	17.7	17.1	1.10	0.141	0.590
Butyrate	11.7	12.5	13.0	15.2	0.41	0.006	0.577
Isobutyrate	1.06	1.05	0.96	1.05	0.033	0.756	0.138
Valerate	1.54	1.33	1.28	1.37	0.138	0.338	0.227
Isovalerate	1.20	1.32	1.30	1.73	0.069	0.061	0.313
Acetate :	3.55	3.83	3.97	3.78	0.318	0.426	0.241

[1] Data shown are the mean of values on d 10 and d 11.

[2] Treatments had a roughage:concentrate ratio of 80:20 (DM basis). Concentrate was similar for all diets. Roughage consisted of (all DM basis) 100% grass silage for GS100; 67% grass silage and 33% corn silage for GS67; 33% grass silage and 67% corn silage for GS33; 100% corn silage for GS0 (n = 8 for GS100, GS67, and GS33; n = 7 for GS0).

Milk fatty acid composition

The total SFA, total MUFA, and total PUFA concentrations in milk fat were unaffected by dietary CS proportion (Table 3.7). Concentrations of C18:3n-3 decreased linearly ($P < 0.001$), whereas C18:2n-6 concentration increased linearly ($P = 0.003$) and the n-6-to-n-3 ratio increased quadratically ($P = 0.005$) with an increasing proportion of CS in the diet. Concentration of C18:1 *cis*-13 increased linearly ($P = 0.01$), and the concentrations of C18:1 *cis*-12, C18:1 *trans*-9, C18:1 *trans*-10, C18:1 *trans*-11, and total CLA increased quadratically ($P < 0.026$) with increasing proportion of dietary CS. A quadratic response was observed for C18:0 ($P = 0.022$). Most of the short- and medium-chain milk FA were unaffected, whereas some odd- and branched-chain FA were affected by dietary treatment (Table 3.7). Concentration of *iso* C15:0 decreased linearly ($P < 0.001$), whereas *iso* C16:0 concentrations increased linearly ($P < 0.001$) with increasing dietary CS proportion. A quadratic response was observed for *iso* C14:0 concentrations ($P = 0.011$). Concentrations of C15:0 and C17:0 decreased linearly ($P < 0.001$) and *anteiso* C15:0 concentration decreased quadratically ($P = 0.027$) with increasing dietary CS proportion.

Table 3.6. Energy balance and nitrogen balance of lactating dairy cows fed different proportions of grass silage in the diet

	Treatment[1]					*P*-value	
Item	GS100	GS67	GS33	GS0	SEM	Linear	Quadratic
Metabolic BW[2] ($kg^{0.75}$)	123	128	126	126	1.1	0.124	0.035
Energy balance (kJ/kg of $BW^{0.75}$/d)							
GEI[3]	2,587	2,515	2,551	2,644	79.2	0.671	0.297
CH_4 production	180	179	181	171	6.5	0.110	0.172
MEI[4]	1,647	1,638	1,634	1,651	45.8	0.923	0.834
MEI:GEI ratio	63.8	65.1	64.1	62.5	0.74	0.154	0.057
Heat production	896	876	893	922	21.0	0.265	0.113
Energy in milk	590	622	629	620	30.8	0.507	0.435
ER total[5]	161	138	113	110	39.7	0.261	0.738
ER protein[6]	52	60	67	51	10.5	0.989	0.175
ER fat[7]	109	78	45	59	39.0	0.217	0.415
Nitrogen balance (mg/kg of $BW^{0.75}$/d)							
N intake[8]	4,155	3,942	3,827	3,694	141.0	< 0.001	0.971
N manure	2,748	2,487	2,330	2,251	108.0	< 0.001	0.410
N milk	993	1,000	987	1,059	44.6	0.370	0.479
N condense + acid	59	45	53	38	8.2	0.044	0.857
N balance	355	409	457	347	71.1	0.989	0.175
N efficiency[9]	0.24	0.25	0.26	0.29	0.008	< 0.001	0.380

[1] Treatments had a roughage:concentrate ratio of 80:20 (DM basis). Concentrate was similar for all diets. Roughage consisted of (all DM basis) 100% grass silage for GS100; 67% grass silage and 33% corn silage for GS67; 33% grass silage and 67% corn silage for GS33; 100% corn silage for GS0 (n = 8 for GS100, GS67, and GS33; n = 7 for GS0).

[2] The mean BW per cow per balance period was used to calculate metabolic BW.

[3] GEI = Gross energy intake.

[4] MEI (Metabolizable energy intake) = GEI – methane production – energy in feces + urine.

[5] Energy retention total = MEI – heat production – energy in milk.

[6] Energy retention protein = protein gain (N x 6.25) x 23.6 kJ/g (energetic value of body protein).

[7] Energy retention fat = energy retention total – energy retention protein.

[8] N = nitrogen.

[9] N efficiency = N milk/N feed.

Table 3.7. Milk fatty acid composition of lactating dairy cows fed different proportions of grass silage in the diet

	Treatment[1]					*P*-value	
Fatty acid, g/100 g FA	GS100	GS67	GS33	GS0	SEM	Linear	Quadratic
C4:0	3.22	3.30	3.44	3.53	0.108	0.033	0.967
C6:0	2.18	2.19	2.20	2.27	0.050	0.247	0.619
C8:0	1.21	1.18	1.18	1.22	0.040	0.967	0.408
C10:0	2.80	2.68	2.62	2.69	0.136	0.498	0.478
C12:0	3.37	3.17	3.07	3.19	0.193	0.404	0.426
C14:0	11.57	10.89	11.13	11.22	0.335	0.594	0.256
iso C14:0	0.09	0.08	0.08	0.09	0.005	0.260	0.011
C14:1 *cis*-9	1.23	1.18	1.11	1.27	0.107	0.965	0.297
C15:0	1.23	1.05	0.93	0.91	0.061	< 0.001	0.221
iso C15:0	0.30	0.24	0.24	0.21	0.009	< 0.001	0.183
anteiso C15:0	0.45	0.39	0.39	0.41	0.017	0.255	0.027
C16:0	35.73	35.50	34.40	34.86	1.107	0.398	0.755
iso C16:0	0.16	0.15	0.18	0.19	0.006	< 0.001	0.057
C16:1 *cis*-9	2.11	1.98	1.87	1.97	0.112	0.246	0.205
C16:1 *trans*-9	0.19	0.20	0.22	0.23	0.012	0.012	0.825
C17:0	0.70	0.66	0.59	0.52	0.021	< 0.001	0.396
iso C17:0	0.38	0.37	0.37	0.37	0.012	0.499	0.530
anteiso C17:0	0.41	0.40	0.40	0.42	0.018	0.776	0.292
C17:1 *cis*-9	0.29	0.30	0.28	0.26	0.014	0.036	0.378
C18:0	7.10	8.04	8.16	7.16	0.438	0.781	0.022
C18:1 *cis*-9[2]	17.38	18.49	19.06	17.66	1.178	0.740	0.272
C18:1 *cis*-12	0.12	0.13	0.20	0.34	0.050	< 0.001	< 0.001
C18:1 *cis*-13	0.10	0.11	0.11	0.13	0.007	0.010	0.155
C18:1 *trans*-9	0.11	0.12	0.14	0.19	0.009	< 0.001	0.026
C18:1 *trans*-10	0.12	0.13	0.20	0.45	0.021	< 0.001	< 0.001
C18:1 *trans*-11	0.72	0.74	0.77	1.18	0.098	0.001	0.025
C18:1 *trans*-15 + C18:1 *cis*-11	0.52	0.56	0.64	0.69	0.040	0.001	0.870
Total CLA[3]	0.38	0.35	0.37	0.64	0.059	0.004	0.012
C18:2n-6	1.35	1.37	1.55	1.65	0.085	0.003	0.553
C18:3n-3	0.54	0.44	0.38	0.23	0.073	< 0.001	0.210
C18:3n-6	0.07	0.07	0.08	0.09	0.003	0.001	0.115
C20:0	0.13	0.13	0.13	0.10	0.004	< 0.001	0.006
C20:1 *cis*-11	0.04	0.05	0.05	0.04	0.003	0.242	0.008
C20:2n-6	0.05	0.05	0.05	0.04	0.002	0.140	0.462
C20:3n-6	0.09	0.08	0.09	0.10	0.006	0.269	0.028
C20:4n-3	0.08	0.06	0.07	0.07	0.014	0.484	0.529
C20:4n-6	0.13	0.12	0.13	0.15	0.009	0.063	0.069
C20:5n-3	0.08	0.06	0.06	0.05	0.004	< 0.001	0.533
C22:0	0.09	0.07	0.06	0.04	0.004	< 0.001	0.104
C22:5n-3	0.09	0.09	0.09	0.09	0.006	0.685	0.536
C24:0	0.06	0.05	0.05	0.03	0.004	< 0.001	0.117
SFA[4]	71.20	70.59	69.62	69.43	1.232	0.238	0.876
MUFA[5]	22.70	23.65	24.22	23.94	1.096	0.370	0.580
PUFA[6]	2.86	2.68	2.86	3.10	0.139	0.126	0.107
n-6 to n-3 ratio[7]	2.16	2.58	3.22	4.83	0.210	< 0.001	0.005

Table 3.7. Continued

[1] Treatment had a roughage:concentrate ratio of 80:20 (DM basis). Concentrate was similar for all diets. Roughage consisted of (all DM basis) 100% grass silage for GS100; 67% grass silage and 33% corn silage for GS67; 33% grass silage and 67% corn silage for GS33; 100% corn silage for GS0 (n = 8 for GS100, GS67, and GS33; n = 7 for GS0).
[2] C18:1 *cis*-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12, since these two FA could not be separated in the analysis. The portion of C18:1 *trans*-12 is considered to be negligible, since this FA is always present in small contents.
[3] Total CLA consists mainly of C18:2 *cis*-9, *trans*-11.
[4] SFA = saturated fatty acids, sum of all SFA reported in this table.
[5] MUFA = mono unsaturated fatty acids, sum of all MUFA reported in this table.
[6] PUFA = poly unsaturated fatty acids, sum of all PUFA reported in this table.
[7] Ratio between the sum of C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, and C20:4n-6 and the sum of C18:3n-3, C20:4n-3, C20:5n-3, and C22:5n-3.

DISCUSSION

Milk production and composition

Replacing GS with CS resulted in a lower CP content in the diets and a lower CP intake, but did not affect milk production. Law et al. (2009) observed that increasing dietary protein content from 14.4 to 17.3% has beneficial effects on milk production for cows in early lactation, but not for cows in late lactation. The cows in the current experiment were in mid lactation (average 192 DIM); the effect of replacing GS with CS on milk production in the present study (CP content of 19.2 and 16.3%, respectively) may have been different (i.e., lower milk production) for cows in early lactation. For the current experiment, protein intake was sufficient because MUN decreased linearly with increasing CS inclusion but remained within the range of MUN values commonly observed in practice (5.0–15.0 mg/dL) and above a minimum value (10.0 mg/dL) considered to indicate possible shortage of protein (Spek et al., 2013). Milk fat content and milk fat yield did not change when GS was replaced by CS, which is in agreement with Brask et al. (2013). A decrease in milk fat content was expected, because an increase in starch intake coupled with a decrease in NDF intake is, in general, associated with a decrease in milk fat content (Nielsen et al., 2006; Abrahamse et al., 2008b). However, feeding CS- compared with GS-based diets is not always associated with a significant reduction of milk fat content (e.g., Fitzgerald and Murphy, 1999; Kliem et al., 2008). The differences in results can probably be ascribed to variations in chemical composition of the CS, especially NDF and starch content; physical characteristics of the silages (i.e., particle size) may also be important (Griinari and Bauman, 2006).

Both milk protein content and milk protein yield increased with increasing CS proportion at the expense of GS. Other studies have also reported increases in milk protein content when GS was replaced by CS (Abrahamse et al., 2008b; Kliem et al., 2008). An increase in milk protein concentration may be attributed to microbial protein synthesis being energetically more efficient on CS- rather than GS-based diets (Givens and Rulquin, 2004). In addition, in contrast to GS, CS supplies rumen-resistant starch that is digested postruminally; this results in glucose absorption, which is associated with an increase in milk protein concentration likely mediated through changes in arterial insulin concentrations (Rius et al., 2010).

Energy and nitrogen balance

Replacing GS with CS did not affect any of the energy balance parameters. The ratio of MEI to GEI varied between 62.5 and 65.1%, which reflects the high quality of the silages used. Replacing GS with CS resulted in a higher DMI, whereas GEI and MEI were unaffected. This explains why milk production did not increase with the increasing DMI. Total ER was positive, which was expected given that cows were, on average, in midlactation. The mean N balance was 49 g/d and unaffected by dietary treatment. This positive N balance is in line with the average N balance (39 g of N/d) reported by Spanghero and Kowalski (1997) in a review on dairy cattle N balance trials. Replacing GS with CS decreased N intake; this, in combination with an unaffected milk N secretion, resulted in a greater efficiency of dietary N utilization for milk N production. This increased N efficiency with increased starch content and decreased protein content in the diet is in agreement with Hassanat et al. (2013) and Benchaar et al. (2014).

Milk fatty acid composition

In the present study, replacing GS with CS did not affect total concentration of SFA, MUFA, and PUFA contents in milk fat. This is in agreement with Chilliard et al. (2001), who indicated that PUFA content in milk of cows fed either GS or MS does not differ to a large extent; this is also in agreement with Kliem et al. (2008), who did not find an a difference in SFA content in the milk when replacing GS with CS.

Replacing GS with CS in the present study resulted in an increase of C18:1 *trans*-9, C18:1 *trans*-10, C18:1 *trans*-11, and total CLA, suggesting that rumen biohydrogenation was less complete. During biohydrogenation in the rumen, unsaturated FA are converted to C18:0, with an array of *trans* C18:1 isomers and CLA as major intermediates. The isomer profile formed during biohydrogenation can influence milk FA profile. Previous studies (Ferlay et al., 2006; Nielsen et al., 2006; Chilliard et al., 2007) have reported that CS-based diets increase milk fat CLA content compared with GS-based diets. Nielsen et al. (2006) also found increased C18:1 *trans*-10 and C18:1 *trans*-11 and Kliem et al. (2008) reported an increase in C18:1 *trans*-10 and total *trans* C18:1 FA when replacing GS with CS.

Replacing GS with CS did not affect short- and medium-chain FA contents (defined here as straight, even-chain FA up to 16 carbon chain length) in milk fat, suggesting that de novo synthesis of milk FA in the mammary gland was unaffected, which is in line with the results found for acetate. Acetate is the major carbon source for de novo synthesized FA (Bauman and Griinari, 2003) and was unaffected in the present study by replacing GS with CS. Only C4:0 in milk fat increased linearly when CS dietary proportions increased. The presence of odd- and branched-chain FA in milk can be used to identify shifts in the rumen microbial population, as most of them are of bacterial origin (Kliem et al., 2008). In the present study, *iso* C15:0 concentration decreased linearly, whereas *iso* C14:0 and *iso* C16:0 concentrations increased (quadratically and linearly, respectively) when GS was replaced by CS. The results for *iso* C14:0 and *iso* C16:0 were unexpected, because Vlaeminck et al. (2006) reported that diets rich in starch decrease *iso* C14:0, *iso* C15:0, and *iso* C16:0 in milk fat. Shingfield et al. (2005) observed similar shifts as Vlaeminck et al. (2006) when replacing GS with CS, except for *iso*

C16:0, suggesting *iso* C14:0 and *iso* C15:0 originate from rumen fibrolytic bacteria. However, Kliem et al. (2008) reported that replacing GS with CS did not affect the proportion of *iso* C14:0 and *iso* C16:0 and showed a quadratic relationship for *iso* C15:0, with the lowest *iso* C15:0 content in the diet with GS only.

In the present study, C15:0, *anteiso* C15:0, and C17:0 decreased when GS was replaced by CS. These results are in contrast with Vlaeminck et al. (2006), who suggested that amylolytic bacteria contain high amounts of linear odd-chain FA and *anteiso* FA. Higher starch content in the diet enhances the growth of amylolytic bacteria, potentially leading to an increase in linear odd-chain FA and *anteiso* FA leaving the rumen. Thus, one might expect that with increasing CS inclusion, milk C15:0, *anteiso* C15:0, C17:0, and *anteiso* C17:0 would increase. The results of C15:0 and C17:0 in the present study are, however, in agreement with Kliem et al. (2008), who found a linear decrease in milk C15:0 and C17:0 content when replacing GS with CS, and with Dijkstra et al. (2011), who found an decrease in these linear odd-chain FA when CH_4 decreased.

Methane production and ruminal VFA concentrations

Dry matter intake is a major determinant for CH_4 production (Ellis et al., 2008). Despite restricted feeding with the aim of similar DMI for all treatments, DMI increased linearly when GS was replaced by CS, which was caused by 2 reasons. First, there was a difference in the DM content of silages measured in a sample of both silages before the start of the experiment (used to calculate the amounts of silage to be mixed in the total diet fed to the cows) and the DM content of silages actually fed during the experiment (determined after the trial was finished). Second, there was a difference in the amount of feed refusals between the treatments (data not shown). Feed refusals were not expected, as the cows were fed a restricted diet. Despite the linear increase in DMI in the present study, CH_4 production (g/d) decreased quadratically when GS was replaced by CS. This decline in CH_4 production might be partially explained by passage rate of digesta in the gastrointestinal tract. The rumen residence time decreases with increased feed intake, thereby reducing the extent of the rumen fermentation and shifting digestion from the rumen to the small intestine (Aluwong et al., 2011). If the cows in the current study were fed ad libitum, results may have differed. According to Abrahamse et al. (2008b), DMI is higher for CS- compared with GS- based diets, and higher DMI has been associated with a higher absolute CH_4 production (Ellis et al., 2008). When fed ad libitum, increasing the inclusion of CS in the diet would result in a higher DMI and, therefore, may not have reduced CH_4 production (g/d). However, CH_4 yield (g/kg DMI or as a percent of GEI) would again be lower for the CS- compared with the GS-based diet.

Replacing GS with CS resulted in decreased CH_4 emission (in g/d, g/kg of DMI, g/kg of FPCM, and % of GEI), which is in agreement with Staerfl et al. (2012) and Brask et al. (2013). In general, decreased CH_4 emission is associated with decreased rumen acetate proportion and increased propionate proportion (Johnson and Johnson, 1995). In the present study, however, acetate and propionate proportions in the rumen were unaffected when replacing GS by CS. A decrease in acetate proportion when replacing GS with CS was

expected because NDF intake and apparent total-tract digestibility of NDF decreased and because acetate is a major end product of NDF fermentation (Bannink et al., 2008). In addition, an increase in propionate proportion when replacing GS with CS was expected because starch intake and apparent total-tract digestibility of starch increased and diets with high starch content are often associated with increased propionate in the rumen (Ellis et al., 2008). Overall, total VFA concentration was unaffected when replacing GS with CS, which is consistent with the absence of difference in rumen pH. However, ruminal pH measurements in the current study seemed high. According to Dijkstra et al. (2012), pH is expected be around 6.2 with a VFA concentration of 100 m*M*. In the present study the pH ranged between 6.72 and 6.77 with a VFA concentration of approximately 100 m*M*. Although pH seems high, in combination with the roughage-based diets used, it helps explain the absence of an effect when replacing GS with CS on ruminal propionate proportion. According to Bannink et al. (2008), with roughage-based diets and high rumen pH, the proportion of starch fermented to propionic acid per unit glucose fermented is only marginally higher than the proportion of fiber fermented to propionic acid per unit glucose fermented. Hence, with the present high-roughage diets, no or small differences in molar proportion of propionic acid may be expected. The increased butyrate proportion when replacing GS with CS was unexpected. In general, fermentation of fiber favors the production of acetate and butyrate (Johnson et al., 1996). However, Benchaar et al. (2014) also found an increase in butyrate proportions and a decline in CH_4 production when barley silage was replaced with CS. This increase in butyrate proportion was accompanied by a linear increase in protozoa numbers in the rumen, which is consistent with protozoa being associated with butyrate production (Morgavi et al., 2012).

The quadratic decrease in CH_4 emission observed suggests that a critical dietary concentration of starch is required to decrease CH_4 emission. A similar response in daily CH_4 output (g/d) was observed by Mc Geough et al. (2010b) in beef cattle fed whole-crop wheat silages with increasing grain content ($P = 0.004$, quadratic response), by Hassanat et al. (2013) in dairy cattle fed diets in which alfalfa silage was replaced with CS ($P < 0.01$, quadratic response), and by Benchaar et al. (2014) in dairy cattle fed diets where barley silage was replaced with CS ($P = 0.07$, quadratic response).

It appears that CH_4 mitigation with roughage-based diets is more difficult than grain-based diets. Mc Geough et al. (2010a) showed that increasing the starch content of roughage can decrease CH_4 production, but CH_4 production of roughage-fed cattle is still considerably higher than for concentrate-fed cattle. Opportunities to use compound feed to lower CH_4 emission from the dairy sector is limited, as milk quality is negatively affected once compound feed exceeds approximately 50% of the diet (Beauchemin et al., 2008), and it ignores the importance of ruminants in converting fibrous feeds, unsuitable for direct human consumption, to the high-quality protein source milk (Gill et al., 2010). Roughages represent a major source of ingredients in dairy cow diets and are nutritionally and economically important (Hassanat et al., 2013). Therefore, it is important to investigate dietary strategies to mitigate CH_4 emission using roughage-based diets.

CONCLUSIONS

Our results showed that replacing GS with CS in a common roughage-based diet for dairy cattle can be an effective strategy to decrease enteric CH_4 emission, without negative consequences for milk production and milk composition, and improve N efficiency. A critical dietary concentration of starch seems to be required to decrease CH_4 emission.

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Chapter 4

Milk metabolome relates enteric methane emission to milk synthesis and energy metabolism pathways

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ABSTRACT

Methane (**CH_4**) emission of dairy cows contributes significantly to the carbon footprint of the dairy chain; therefore, a better understanding of CH_4 formation is urgently needed. The present study explored the milk metabolome by gas chromatography-mass spectrometry (milk volatile metabolites) and nuclear magnetic resonance (milk non-volatile metabolites) to better understand the biological pathways involved in CH_4 emission in dairy cattle. Data were used from a randomized block design experiment with 32 multiparous Holstein-Friesian cows and 4 diets. All diets had a roughage:concentrate ratio of 80:20 (DM basis) and the roughage was grass silage (**GS**), corn silage (**CS**), or a mixture of both (67% GS, 33% CS; 33% GS, 67% CS). Methane emission was measured in climate respiration chambers and expressed as CH_4 yield (per unit of dry matter intake) and CH_4 intensity (per unit of fat- and protein-corrected milk; **FPCM**). No volatile or non-volatile metabolite was positively related to CH_4 yield and acetone (measured as a volatile and as a non-volatile metabolite) was negatively related to CH_4 yield. The volatile metabolites 1-heptanol-decanol, 3-nonanone, ethanol, and tetrahydrofuran were positively related to CH_4 intensity. None of the volatile metabolites was negatively related to CH_4 intensity. The non-volatile metabolites acetoacetate, creatinine, ethanol, formate, methylmalonate, and *N*-acetylsugar A were positively related to CH_4 intensity, and uridine diphosphate (**UDP**)-hexose B and citrate were negatively related to CH_4 intensity. Several volatile and non-volatile metabolites that were correlated with CH_4 intensity also were correlated with FPCM and not significantly related to CH_4 intensity anymore when FPCM was included as covariate. This suggests that changes in these milk metabolites may be related to changes in milk yield or metabolic processes involved in milk synthesis. The UDP-hexose B was correlated with FPCM, whereas citrate was not. Both metabolites were still related to CH_4 intensity when FPCM was included as covariate. The UDP-hexose B is an intermediate of lactose metabolism, and citrate is an important intermediate of Krebs cycle–related energy processes. Therefore, the negative correlation of UDP-hexose B and citrate with CH_4 intensity may reflect a decrease in metabolic activity in the mammary gland. Our results suggest that an integrative approach including milk yield and composition, and dietary and animal traits will help to explain the biological metabolism of dairy cows in relation to CH_4 emission.

Keywords: dairy cow, milk metabolome, enteric methane emission, energy metabolism

INTRODUCTION

Enteric methane (**CH_4**) production in ruminants mainly occurs in the rumen and is a natural byproduct of microbial feed fermentation and degradation, an essential process to provide nutrients to the animal. An increase of DMI results in a higher CH_4 production because more substrate is available for rumen microbiota to degrade, but diet characteristics, including the type of carbohydrates and fat content, can also have a large effect on CH_4 production (Kirchgebner et al., 1995). Due to the large contribution (approximately 52%) of CH_4 emission to the total greenhouse gas (**GHG**) emissions of the dairy sector (Gerber et al., 2013), mitigation strategies have been widely investigated (Hristov et al., 2013). Dietary changes to influence CH_4 emission are among the most direct CH_4 mitigation strategies (Knapp

et al., 2014). Their importance increases because they are also candidates for implementation at dairy farms. According to Dijkstra et al. (2011), evaluating dietary mitigation strategies should be based on CH_4 production relative to feed intake because it avoids confounding effects of DMI on total CH_4 production (CH_4 produced per animal). However, uncertainties in measuring DMI at farm level makes an accurate relation of CH_4 to DMI difficult in practice (Bannink et al., 2011). Others have related CH_4 mitigation strategies to their effect on the product (milk) of a dairy farm (Knapp et al., 2014).

To assess GHG emissions by the dairy chain, it is also possible to relate CH_4 production per unit of milk [usually expressed per unit of ECM or per unit of fat- and protein-corrected milk (**FPCM**)]. Higher production levels related to nutritional and nonnutritional management strategies may reduce CH_4 emissions per unit of milk (FAO, 2010). Emissions per unit of animal product reflect the accuracy of management practices on the composite of feed intake, GHG emission, and animal productivity (FAO, 2010). Therefore, evaluating CH_4 production in relation to feed intake and in relation to milk production are complementary.

Many studies have focused on the effect of CH_4 mitigation strategies on milk composition, but mainly on the macro constituents level (Mohammed et al., 2011; Hart et al., 2015). Less attention has been paid to individual metabolites of milk, with the exception of milk fatty acids (**MFA**; Odongo et al., 2007; Chilliard et al., 2009). This focus on MFA is because of the relation between MFA and ruminal activity with respect to microbial metabolism and type of VFA formed (Vlaeminck and Fievez, 2005). Changes in feeding can result in clear changes in MFA, which are partly related to how feed is degraded in the rumen (Halmemies-Beauchet-Filleau et al., 2014). Although MFA may predict CH_4 emission accurately within a limited range of dietary variation (e.g., variation in lipid source only; Chilliard et al., 2009), MFA cannot accurately predict the differences in CH_4 emission on a wider range of diets (Van Lingen et al., 2014; Williams et al., 2014).

Milk volatile metabolite and non-volatile metabolite profiles can be used to monitor animal health, feeding regimens, and metabolism in dairy cows. Based on different feeding regimens, indole and skatole present in the volatile fraction of milk were pointed out as indicative of the feeding regimen of dairy cows (Toso et al., 2002; Croissant et al., 2007). Further, Hettinga et al. (2008) used the milk volatile metabolite profile to detect and differentiate mastitis caused by different pathogens. Also, Klein et al. (2012) indicated the ratio of the non-volatiles glycerophosphocholine and choline as possible predictor for developing ketosis in dairy cows and Lu et al. (2013), showed that phosphate sugars can be related to energy balance of the cow, due to a different organization of the epithelial membrane in relation to energy balance. These authors also showed that determining milk components using different techniques simultaneously can be useful for a more integrated understanding of the metabolism of cows (Klein et al., 2010; Lu et al., 2013).

Many fields of research analyze the same bio-matrix with different methods and integrate the resulting information to better monitor, predict, and interpret biological processes. Although milk volatile metabolite and non-volatile metabolite profiles have been used to monitor digestion and metabolism in dairy cows, to the best of our knowledge these

profiles have not been related to CH_4 emission. The present study explores the milk metabolome by gas chromatography – mass spectroscopy (**GC-MS** metabolomics; milk volatile metabolites) and proton nuclear magnetic resonance (**^{1}H-NMR** metabolomics; milk non-volatile metabolites) to better understand the biological pathways involved in CH_4 emission.

MATERIALS AND METHODS

Experimental design

Data from a completely randomized block design experiment were used with a total of 32 multiparous lactating Holstein-Friesian cows fed 4 diets that differed in grass silage (**GS**) and corn silage (**CS**) content. The experiment was fully described by Van Gastelen et al. (2015). The experiment was conducted in 2012 in accordance with Dutch law and approved by the Animal Care and Use Committee of Wageningen University & Research (Wageningen, the Netherlands).

The 4 diets had a roughage:concentrate ratio of 80:20 based on DM content. The composition of the concentrate was similar for all diets, whereas the roughage consisted of 100% GS, 67% GS and 33% CS, 33% GS and 67% CS, and 100% CS (ingredient as percentage of the total amount of roughage in the diet, all DM basis). Feed intake was restricted (95% of ad libitum DMI) to avoid confounding effects of DMI on CH_4 production. After an adaptation period of 12 d, on d 13, cows were housed in climate respiration chambers (**CRC**) for a 5-d period. Cows were milked and fed twice daily. Production of CH_4 was determined in 10 min intervals during 3 full 24-h periods in the CRC. The details of the CRC used in this experiment are extensively described by Van Gastelen et al. (2015).

Milk yield and composition

Milk yield was recorded during each milking, and a milk sample (10 mL) was collected for analyses of fat, protein, and lactose content by mid-infrared spectroscopy by Qlip (Zutphen, the Netherlands). In addition, a representative milk sample (5 g/kg of milk production) was obtained at each milking from each cow. The first milk sample was collected on d 13 in the afternoon and the last milk sample was collected on d 17 in the morning, when cows were housed in the CRC. Sodium azide (0.05% wt/wt) was added to the pooled samples of the first 4 milkings, followed by the same procedure for milking 5 to 8 in a separate bottle. Both bottles were stored at 5°C. After the last milking, these 2 sub-samples (milkings 1 to 4 and 5 to 8) were pooled, and stored in 10-mL aliquots at −40°C for milk composition analyses.

Analytical procedures

Volatile metabolites. To determine the volatile metabolite profile, GC-MS metabolomics was performed based on the method described by Hettinga et al. (2008) and Settachaimongkon et al. (2014). Milk samples were thawed overnight in a refrigerator (7°C). A 5-mL milk sample was preheated in 10-mL vials sealed with silicon/Teflon septa and magnetic caps for 1 min at 60°C. Volatile metabolites were extracted from the headspace for

5 min with a 75-μm PDMS-carboxen SPME fiber (Supelco, Bellefonte, PA) using the Triplus autosampler (CTC Analytics Ag, Zwingen, Switzerland). The volatile metabolites were thermally desorbed from the fiber by heating it in a Best PTV injector (Thermo-Finnigan, San Jose, CA) with an empty liner for 5 min at 250°C. The fiber was subsequently cleaned for 10 min at 290°C. Vials without milk (only air) were used as blank samples.

Gas chromatography separation of volatile metabolites was performed on a Trace GC/MS (Thermo-Fisher Scientific, Waltham, MA). Volatiles were separated on a polar Stabilwax column of 30 m length, 0.32 mm, and 1-μm film thickness (Restek, Breda, the Netherlands). Oven temperature was kept at 40°C for 3 min, after which it was increased to 220°C at 15°C/min, with 1 min holding at 220°C. Helium at a flow rate of 1.5 mL/min was used as a carrier gas. Mass spectrometry analysis was performed in electron impact mode (70 eV) in the range of 33 to 250 m/z, with 2 scans/s; the mass range of m/z 33 to 250 was used. The ion source was kept at 225°C.

The resulting chromatograms were analyzed using the AMDIS software (NIST, Gaithersburg, MD); data were deconvoluted to obtain pure mass spectra for improved peak identification. Identification of volatile metabolites was based on AMDIS software referred to NIST/EPA/NIH database (http://www.nist.gov/srd/nist1a.cfm) and matching mass spectra and retention time with an in-house library based on previous milk analyses (Hettinga et al., 2009). Fragment patterns were not specific enough to identify the chain length of 3 alkanes and these were labeled as alkane A, B, and C. Peak integration was subsequently performed using the XCalibur software package (Thermo-Scientific, Austin, TX). Peak area of the milk samples was corrected for the peak area of the blank samples, resulting in a peak area in arbitrary units that was used for statistical analyses.

Non-volatile metabolites. To determine the non-volatile metabolite profile of the milk samples, ^{1}H-NMR metabolomics was performed. The procedure is described in detail by Lu et al. (2013). In short, milk samples were ultra-centrifuged to isolate milk serum. One-dimensional nuclear Overhauser enhancement spectroscopy (1-D-NOESY) spectra were obtained for all milk serum samples, using a nuclear magnetic resonance Bruker spectrometer Avance III with a 600 MHz/54 mm UltraShielded Plus magnet equipped with a CryoPlatform cryogenic cooling system, a BCU-05 cooling unit, an ATM automatic tuning and matching unit (Bruker, Rheinstetten, Germany). To assign milk serum nonoverlapping metabolite resonances, comparisons were made with published literature (Klein et al., 2010, 2012), the Human Metabolome Database version 2.0 online library (http://www.hmdb.ca), as well as internal standards. The peak area of each assignment is relative to the calibration standard 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate, resulting in a relative peak area in arbitrary units that was used for statistical analyses.

Statistical analysis

One cow was excluded from the experiment, because of large feed residuals while housed in the CRC, which resulted in much lower DMI compared with the last 4 d in the tie-stall and the other cows in that block (Van Gastelen et al., 2015). In addition, the results of GC-MS metabolomics of another cow could not be used because these results were

considered outliers (i.e., 9 from 25 volatile metabolites were not found in the milk of this cow, and acetone was 10 times more present in the milk of this cow compared with the milk of other cows). Last, the results of ^{1}H-NMR metabolomics of a third cow could not be used because the sample showed repeatedly a high background interference, which impaired peak integration. Therefore, relations between metabolites measured with both techniques were based on 29 samples, whereas all remaining data analyses of volatile and non-volatile metabolites, CH_4 emission, and production traits were based on 30 samples.

Data on DMI, milk production, milk composition, and MFA are described by Van Gastelen et al. (2015). The summary statistics of the volatile metabolites and non-volatile metabolites are presented in Supplemental Tables S4.1 and S4.2, respectively. All statistical analyses were done using SPSS version 21 (SPSS Inc., Chicago, IL). The relations between individual volatile metabolites or non-volatile metabolites, and CH_4 intensity (g/kg of FPCM) or CH_4 yield (g/kg of DMI) were established by linear regression with CH_4 intensity and CH_4 yield as dependent variables and milk volatile or non-volatile metabolites as independent variables. All coefficients were calculated over all diets. To evaluate the influence of FPCM on the established relations between individual volatile metabolites or non-volatile metabolites and CH_4 intensity, FPCM was included as a covariate in the linear regressions. To evaluate the influence of diet on the established relations between individual volatile metabolites or non-volatile metabolites, and CH_4 yield and intensity, dietary treatment was included as a covariate in the linear regressions. Pearson correlation coefficients between milk (non)volatile metabolites and milk and animal traits were determined, with a 2-tailed test for significance ($P < 0.05$). Multivariate analysis of the data was done by principal component analysis in R version 3.2.3. (R Core Team, 2013).

RESULTS AND DISCUSSION

Relation between individual metabolites and methane intensity or yield

Volatile metabolites. In the present study, a total of 25 volatile metabolites were identified. These included ketones, aldehydes, alcohols, hydrocarbons, sulfur compounds, esters, and terpenes. The relations between each volatile metabolite and CH_4 intensity (g/kg of FPCM) and CH_4 yield (g/kg of DMI) are shown in Tables 4.1 and 4.2, respectively. The volatile metabolites 1-heptanol-decanol, 3-nonanone, ethanol, and tetrahydrofuran were positively related ($P < 0.044$) to CH_4 intensity, and none of the volatile metabolites were negatively related to CH_4 intensity. None of the volatile metabolites were positively related to CH_4 yield, whereas acetone was negatively related ($P = 0.043$) to CH_4 yield.

The relations between each volatile metabolite and CH_4 intensity including FPCM as a covariate are shown in Table 4.1. Including FPCM as a covariate in the regression model resulted in no relationship between the volatile metabolites and CH_4 intensity (Table 4.1). This suggests that the previous relations were due to a relation between the volatile metabolites and FPCM.

Non-volatile metabolites. In the present study, 30 resonances could be assigned either to a compound or to a member of a class of compounds (Supplemental Table S4.3). The relations between each non-volatile metabolite and CH_4 intensity or CH_4 yield are shown

in Tables 4.3 and 4.4, respectively. The non-volatile metabolites, acetoacetate, creatinine, ethanol, formate, methylmalonate, and *N*-acetylsugar A were positively related ($P < 0.030$) to CH_4 intensity. Citrate and uridine diphosphate (**UDP**)-hexose B were negatively related ($P < 0.026$) to CH_4 intensity. None of the non-volatile metabolites were positively related to CH_4 yield, whereas acetone was the only non-volatile metabolite negatively related ($P = 0.046$) to CH_4 yield.

The relations between each non-volatile metabolite and CH_4 intensity, including FPCM as a covariate, are shown in Table 4.3. When including FPCM as a covariate in the regression model, none of the non-volatile metabolites were positively related to CH_4 intensity (Table 4.3). This suggests that the relation between CH_4 intensity and acetoacetate, creatinine, ethanol, formate, methyl-malonate, and *N*-acetylsugar A were due to the relation between these non-volatiles metabolites and FPCM. Citrate and UDP-hexose B remained negatively related ($P < 0.026$) to CH_4 intensity upon including FPCM as a covariate in the regression model. The significant relationship with CH_4 intensity in the presence of FPCM as a covariate suggests that the metabolic pathways in which these metabolites are involved relate to CH_4 production in dairy cows, independent of FPCM yield. Our results suggest that relating milk metabolites to CH_4 intensity without including FPCM as a covariate may identify milk metabolites that are also or exclusively related to FPCM rather than CH_4 emission. This dependency seems to be true for both volatile and non-volatile metabolites, and indicates that an increase in milk yield may reduce the concentration of some milk constituents. Bovenhuis et al. (2015) reported similar findings showing the genetic polymorphism in the *DGAT1* gene related to a higher milk yield and reduced milk protein content. However, changes in milk yield do not account for all the variation in milk composition. Hristov et al. (2015) reported that without changes in milk yield, milk of cows with a lower CH_4 production (g/d) due to feeding of 3-nitrooxypropanol had a higher content of de novo synthesized fatty acids. Due to the relation between rumen VFA and de novo synthesized fatty acids, the results of Hristov et al. (2015) suggest that milk composition also depends on blood-derived compounds and mammary gland metabolic activity (Bauman and Griinari, 2003; Jenkins and McGuire, 2006). Taken together, these 2 processes contribute to changes in milk composition, and in our data set it was difficult to distinguish between a dilution effect due to higher milk yield or a higher metabolic activity related to higher milk yield. Therefore, throughout the discussion we will consider them both.

Comparison of both techniques. Milk metabolites can be present in solution and be measured by ^{1}H-NMR. Upon heating, some milk metabolites can be volatized and measured by GC-MS. Ethanol and acetone were the only compounds detected both by ^{1}H-NMR and GC-MS. A positive correlation was found between the (relative) areas of ethanol ($P = 0.031$, $R^2 = 0.401$; $n = 29$) and acetone ($P < 0.001$, $R^2 = 0.684$; $n = 29$) measured by both methods. This is an important prerequisite for the combined analysis of milk metabolome. In our data set, both volatile and non-volatile metabolites are generally better correlated with CH_4 intensity than with CH_4 yield. Further, non-volatile metabolites are also better related to CH_4 intensity than volatile metabolites, and the relationship of 2 non-volatile metabolites (UDP-hexose B and citrate) remained significant after including FPCM as a

covariate in the regression model. The weaker relation between volatile metabolites and CH_4 intensity might be due to the direct transfer of volatile compounds from diet to milk or the interaction between volatile metabolites and rumen metabolism (Urbach, 1990; Désage et al., 1996; Toso et al., 2002) that are not related to the ruminal pathways leading to CH_4 production.

Multivariate analysis. Principal component analysis was conducted to identify general differences in volatile and non-volatile metabolite profiles. The extraction of 2 components explained only 46.2% of the variation of volatile metabolites and 45.0% of the variation of non-volatile metabolites (Supplementary Figures S4.1A and S4.1B, respectively). Further, no clear correlation was found between the groups of variables and the factors.

Changes in methane intensity may be related to the 1-carbon metabolism and energy metabolism pathways

Formate is positively related to CH_4 intensity; however, when FPCM is included in the regression model the relation is no longer significant. This may be explained by the negative correlation between formate and FPCM ($P = 0.024$, data not shown). The relation between formate and CH_4 intensity may therefore be explained by the milk synthesis processes, more specifically the 1-C metabolism in postabsorption pathways. One-carbon donors, including formate, are important in eukaryotic 1-C metabolism as they connect parallel mitochondrial and cytosolic pathways. Activated 1-C compounds, such as formate, are produced by the mitochondria, after which an enzymatic cascade in the cytoplasm will allow formate to be further incorporated in 1-C metabolism (Appling, 1991; Christensen and MacKenzie, 2006). The 1-C metabolism is a housekeeping process involving diverse mechanisms such as biosynthesis of lipids and proteins as well as methylation reactions (Christensen and MacKenzie, 2006). These processes are entwined with milk synthesis (Bian et al., 2015) as in healthy cows, milk metabolites may be secreted into milk via transcellular routes (McManaman and Neville, 2003). Therefore, a negative relation between formate and FPCM might reflect a change in postabsorption 1-C metabolism, possibly in the mammary gland.

Table 4.1. Linear regression between methane intensity (g/kg of fat- and protein-corrected milk; FPCM[1]) and milk volatile metabolites (peak area[2]) and between methane intensity (g/kg of FPCM) and milk volatile metabolites, including FPCM as a covariate[3]

Volatile metabolite (peak area)	Linear regression between methane intensity and milk volatile metabolites[4]				Linear regression between methane intensity and milk volatile metabolites, including FPCM as a covariate[5]						
	Slope	SE	Slope *P*	R^2	Slope	SE	Slope *P*	Slope	SE	Slope *P*	R^2
1-heptanol	-2.47×10^{-7}	1.000×10^{-6}	0.855	0.001	1.79×10^{-7}	1.07×10^{-6}	0.868	-0.366	0.089	<0.001	0.388
1-heptanol-decanol	3.35×10^{-6}	2.000×10^{-6}	0.044	0.137	1.22×10^{-6}	1.48×10^{-6}	0.417	-0.334	0.096	-0.002	0.403
1-pentanol	4.42×10^{-7}	3.755×10^{-7}	0.249	0.047	2.16×10^{-7}	3.09×10^{-7}	0.490	-0.355	0.089	<0.001	0.398
2-butanone	4.49×10^{-8}	5.527×10^{-8}	0.424	0.023	3.02×10^{-8}	4.43×10^{-8}	0.501	-0.362	0.088	<0.001	0.398
2-heptanone	1.60×10^{-7}	1.271×10^{-7}	0.218	0.054	2.51×10^{-8}	1.04×10^{-7}	0.384	-0.354	0.089	<0.001	0.405
3-nonanone	2.47×10^{-6}	6.561×10^{-7}	0.001	0.336	1.28×10^{-6}	8.16×10^{-7}	0.129	-0.250	0.113	0.035	0.439
Acetone	4.55×10^{-10}	3.928×10^{-9}	0.909	0.001	2.03×10^{-9}	3.16×10^{-6}	0.527	-0.377	0.090	<0.001	0.397
Alkane A	9.76×10^{-7}	1.000×10^{-6}	0.493	0.017	1.12×10^{-6}	1.10×10^{-6}	0.321	-0.369	0.087	<0.001	0.410
Alkane B	2.11×10^{-6}	2.000×10^{-6}	0.309	0.037	1.25×10^{-7}	1.73×10^{-6}	0.943	-0.365	0.093	0.001	0.388
Alkane C	-1.95×10^{-7}	3.831×10^{-7}	0.614	0.009	5.38×10^{-8}	3.10×10^{-7}	0.986	-0.366	0.090	<0.001	0.388
Benzene alkane	2.47×10^{-6}	2.000×10^{-6}	0.184	0.062	$-5.40\times10^{-}$	1.68×10^{-6}	0.751	-0.382	0.100	0.001	0.390
Benzene compound	6.16×10^{-7}	6.454×10^{-7}	0.348	0.031	7.34×10^{-7}	5.04×10^{-7}	0.157	-0.373	0.086	<0.001	0.432
Butanoic acid	6.28×10^{-9}	8.263×10^{-9}	0.454	0.020	4.94×10^{-9}	6.59×10^{-9}	0.460	-0.363	0.088	<0.001	0.400
Cyclohexane	1.10×10^{-5}	7.000×10^{-6}	0.139	0.076	$-7.53\times10^{-}$	6.77×10^{-6}	0.912	-0.372	0.100	0.001	0.388
Dimethyl sulfone	-3.26×10^{-7}	3.190×10^{-7}	0.316	0.036	$-1.69\times10^{-}$	2.60×10^{-7}	0.520	-0.358	0.089	<0.001	0.397
Ethanol	5.71×10^{-7}	2.166×10^{-7}	0.013	0.199	3.20×10^{-7}	1.98×10^{-7}	0.118	-0.312	0.091	0.002	0.442
Ethyl acetate	1.95×10^{-7}	2.269×10^{-7}	0.398	0.026	6.75×10^{-8}	1.86×10^{-6}	0.719	-0.361	0.090	<0.001	0.391
Hexanal	-2.13×10^{-9}	2.763×10^{-8}	0.939	<0.001	1.21×10^{-8}	2.22×10^{-8}	0.589	-0.374	0.089	<0.001	0.394
Hexanoic acid	6.96×10^{-9}	5.109×10^{-9}	0.184	0.062	5.34×10^{-9}	4.10×10^{-9}	0.204	-0.356	0.086	<0.001	0.424
Hydrogen azide	5.22×10^{-8}	2.180×10^{-8}	0.170	0.170	2.51×10^{-8}	2.01×10^{-8}	0.222	-0.321	0.094	0.002	0.421
Ketone A	1.36×10^{-6}	8.571×10^{-7}	0.124	0.082	9.42×10^{-7}	6.98×10^{-7}	0.187	-0.349	0.087	<0.001	0.427
Limonene	-6.09×10^{-7}	1.400×10^{-5}	0.966	<0.001	8.60×10^{-6}	1.13×10^{-5}	0.454	-0.380	0.089	<0.001	0.400
Octanoic acid	1.09×10^{-8}	6.681×10^{-9}	0.113	0.087	7.84×10^{-9}	5.42×10^{-9}	0.160	-0.349	0.086	<0.001	0.431
Tetrahydrofuran	3.22×10^{-8}	1.283×10^{-8}	0.018	0.184	9.14×10^{-9}	1.35×10^{-8}	0.503	-0.327	0.106	0.005	0.398

[1] Fat- and protein-corrected milk (FPCM; kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d).

[2] Numbers are area values (arbitrary units).

Table 4.1. Continued

[3] Milk volatiles are ordered alphabetically, n = 30.

[4] Parameters in these table were extracted from the equation: CH_4 intensity = a + b × volatile metabolite + e, where a is the intercept of the regression line, b is the slope of the regression line associated with the volatile metabolite and e is the error.

[5] Parameters in these table were extracted from the equation: CH_4 intensity = a + b × volatile metabolite + c × FPCM + e, where a is the intercept of the regression line, b is the slope of the regression line associated with the volatile metabolite, c is the slope of the regression line associated with FPCM and e is the error.

Acetoacetate, together with volatile 3-nonane, are ketone bodies that are positively correlated with CH_4 intensity (Tables 4.3 and 4.1, respectively). The presence of ketones bodies in milk is often used to monitor changes in energy metabolism of dairy cows (Enjalbert et al., 2001). A higher amount of ketone bodies in blood plasma and subsequently in milk occurs when there is a surplus of acetyl-CoA for the tricarboxylic acid (**TCA**) cycle (Enjalbert et al., 2001; Wellen and Thompson, 2012). In general, diets relatively rich in fiber that promote production of the ketogenic VFA, acetic acid, and butyric acid in the rumen may give rise to higher levels of ketone bodies in blood than glucogenic diets (diets relatively rich in rumen bypass starch delivering glucose in the small intestine or rich in rapidly fermentable carbohydrates that promote production of propionic acid in the rumen; Van Knegsel et al., 2007). Diet composition is known to be related to CH_4 production in the rumen, with fiber generally resulting in higher CH_4 production per unit substrate degraded in comparison with starch (Ellis et al., 2008; Hristov et al., 2013). Hence, a positive relationship between ketone bodies in milk and CH_4 intensity might be explained from the ketogenic or glucogenic nutrient supply to the rumen and its effect on rumen fermentation. However, higher concentrations of these ketone bodies in milk were not significantly related to CH_4 yield, indicating that such differences in ketogenic or lipogenic supply to the rumen and effects on rumen fermentation do not have a role in the observed relationships with CH_4 intensity. The correlation between acetoacetate, 3-nonanone, and CH_4 intensity disappears when FPCM is included as a covariate in the regression model. In fact, acetoacetate and 3-nonanone are negatively related ($P = 0.022$, $P < 0.001$, respectively, data not shown) to FPCM. This may indicate a dilution effect of certain milk metabolites due to a higher milk yield.

The TCA cycle in the mitochondria is of paramount significance to the metabolic efficiency of the cell and therefore the metabolism of the cow. In the mitochondria, ATP is produced from acetyl-CoA originating from glucose, fatty acids, lactate, pyruvate, and AA (Hardie and Carling, 1997). The production and utilization of ATP is therefore a flexible situation in which molecules are interconverted depending on the needs of the cell. In this situation, molecules with a high-energy phosphate, such as phosphocreatine, have been widely studied. Together with its precursor creatine, they form an important pool of energy in the cell, which can be used by the mitochondria. A consequence of this metabolism is the formation of creatinine, which is often monitored for energy status of tissues (Wyss and Kaddurah-Daouk, 2000). In our data set, a positive correlation is present between creatinine and CH_4 intensity ($P = 0.007$, Table 4.4) and a negative correlation between creatinine and milk yield expressed as FPCM ($P = 0.026$, data not shown). Further, the positive correlation between creatinine and CH_4 intensity disappears when FPCM is included as a covariate in the regression model. Therefore, in our data set, changes in creatinine concentration in milk seem related to changes in milk yield and, therefore, milk synthesis. This may indicate a change in the metabolic activity fueled by the mitochondria. As discussed in this section, a negative relation was found between milk formate, acetoacetate, 3-nonanone, creatinine, and milk yield, expressed as FPCM. This supports the idea of a possible dilution effect on milk metabolites. Further, when FPCM is included as a covariate in the regression models, the above mentioned metabolites are no longer related to CH_4 intensity. An increase in CH_4 intensity

resulting from a lower milk yield may therefore be associated with a lower metabolic rate, explaining the changes in milk metabolites related to milk synthesis and energy metabolism.

Table 4.2. Linear regression between methane yield (g/kg DMI) and milk volatile metabolites (peak area[1])[2]

Volatile metabolite[3] (peak area)	Intercept	SE	Slope	SE	Slope P	R^2
1-heptanol	24.8	0.59	-1.72×10^{-6}	1.000×10^{-6}	0.184	0.062
1-heptanol-decanol	23.4	1.33	9.52×10^{-7}	2.000×10^{-6}	0.569	0.012
1-pentanol	23.8	0.98	1.18×10^{-7}	3.734×10^{-7}	0.755	0.004
2-butanone	23.9	0.57	2.33×10^{-8}	5.418×10^{-8}	0.671	0.007
2-heptanone	24.1	0.49	5.11×10^{-9}	1.270×10^{-7}	0.968	<0.001
3-nonanone	24.3	1.43	-8.54×10^{-8}	7.828×10^{-7}	0.914	<0.001
Acetone	25.1	0.54	-7.53×10^{-9}	3.545×10^{-9}	0.043	0.139
Alkane A	23.9	0.93	3.54×10^{-7}	1.000×10^{-6}	0.799	0.002
Alkane B	24.4	1.32	-4.64×10^{-7}	2.000×10^{-6}	0.819	0.002
Alkane C	24.6	0.61	-3.53×10^{-7}	3.682×10^{-7}	0.346	0.032
Benzene alkane	24.4	1.31	-4.02×10^{-7}	2.000×10^{-6}	0.827	0.002
Benzene compound	23.9	0.43	5.53×10^{-7}	6.290×10^{-7}	0.387	0.027
Butanoic acid	24.0	0.47	3.61×10^{-9}	8.088×10^{-9}	0.659	0.007
Cyclohexane	24.7	1.16	-4.00×10^{-6}	7.000×10^{-6}	0.587	0.011
Dimethyl sulfone	23.3	0.71	3.98×10^{-7}	3.067×10^{-7}	0.205	0.057
Ethanol	23.2	1.04	2.13×10^{-7}	2.318×10^{-7}	0.366	0.029
Ethyl acetate	25.3	1.12	-2.44×10^{-7}	2.187×10^{-7}	0.273	0.043
Hexanal	24.6	0.50	-3.53×10^{-8}	2.602×10^{-8}	0.186	0.062
Hexanoic acid	23.9	0.42	4.08×10^{-9}	5.071×10^{-9}	0.428	0.023
Hydrogen azide	23.4	1.18	1.41×10^{-8}	2.310×10^{-8}	0.548	0.013
Ketone A	23.9	0.48	6.62×10^{-7}	8.608×10^{-7}	0.449	0.021
Limonene	23.5	1.23	6.85×10^{-6}	1.400×10^{-5}	0.620	0.009
Octanoic acid	23.9	0.43	5.25×10^{-9}	6.727×10^{-9}	0.442	0.021
Tetrahydrofuran	23.8	0.70	6.98×10^{-9}	1.374×10^{-8}	0.615	0.009

[1] Numbers are peak area values (arbitrary units).

[2] Milk volatile metabolites are ordered alphabetically, n = 30.

[3] Parameters were extracted from the equation: CH_4 yield = a + b × volatile metabolite + e, where a is the intercept of the regression line, b is the slope of the regression line associated with the metabolite, and e is the error.

Table 4.3. Linear regression between methane intensity (g/kg of fat- and protein-corrected milk; FPCM[1]) and milk non-volatile metabolite (relative area[2]) and between methane intensity (g/kg of FPCM) and milk non-volatile metabolites, including FPCM as a covariate[3]

Non-volatile metabolite (relative peak area)	Linear regression between methane intensity and milk non-volatile metabolites[4]				Linear regression between methane intensity and milk volatile metabolites, including FPCM as a covariate[5]						
	Slope	SE	Slope *P*	R^2	Slope (non-volatile)	SE	Slope *P* (non-volatile)	Slope (FPCM)	SE	Slope *P* (FPCM)	R^2
Acetate	36.09	40.55	0.381	0.028	12.34	28.51	0.666	-0.438	0.079	<0.001	0.547
Acetoacetate	193.99	72.20	0.012	0.205	75.30	59.55	0.217	-0.399	0.083	<0.001	0.569
Acetone	-33.41	24.00	0.175	0.065	-17.41	17.00	0.315	-0.429	0.078	<0.001	0.561
Acetylcarnitine	-6.00	26.23	0.847	0.001	-6.77	18.01	0.710	-0.443	0.078	<0.001	0.546
Betaine	-1.33	6.30	0.834	0.002	-2.90	4.31	0.506	-0.446	0.078	<0.001	0.551
Butyrate	3.59	2.05	0.091	0.099	1.27	1.53	0.416	-0.424	0.081	<0.001	0.555
β-hydroxybutyrate	42.80	35.95	0.244	0.048	14.59	25.72	0.575	-0.434	0.079	<0.001	0.549
Carnitine	-2.84	16.43	0.864	0.001	-14.38	11.14	0.208	-0.460	0.077	<0.001	0.570
Choline	2.25	1.40	0.119	0.085	0.48	1.06	0.656	-0.431	0.082	<0.001	0.547
Citrate	-2.04	0.87	0.026	0.166	-1.43	0.60	0.025	-0.412	0.072	<0.001	0.622
Creatine	-3.11	6.39	0.631	0.008	-2.88	2.10	0.516	-0.442	0.077	<0.001	0.551
Creatinine	130.24	45.10	0.007	0.229	58.60	36.98	0.125	-0.390	0.082	<0.001	0.583
Ethanol	328.82	88.71	0.001	0.329	167.66	115.62	0.159	-0.367	0.092	<0.001	0.577
Formate	225.08	98.76	0.030	0.156	63.71	80.17	0.434	-0.415	0.085	<0.001	0.554
Galactose-1-phosphate	-0.31	130.12	0.998	<0.001	42.23	89.45	0.641	-0.446	0.078	<0.001	0.547
Glycerophospho-choline	2.10	1.37	0.137	0.077	1.242	0.97	0.209	-0.427	0.077	<0.001	0.570
Hippurate	59.46	32.56	0.079	0.106	43.00	22.41	0.066	-0.425	0.074	<0.001	0.598
Lactate	6.73	13.13	0.612	0.009	5.83	9.01	0.523	-0.442	0.077	<0.001	0.551
Lactose	-0.72	0.39	0.074	0.110	-0.49	0.27	0.084	-0.422	0.075	<0.001	0.592
Malonate	-6.60	39.22	0.868	0.001	7.25	27.07	0.791	-0.445	0.078	<0.001	0.545
Methylmalonate	101.03	33.51	0.005	0.245	34.28	29.93	0.262	-0.392	0.088	<0.001	0.565
N-acetylsugar A	43.12	16.05	0.012	0.205	5.31	14.94	0.724	-0.424	0.094	<0.001	0.546
N-acetylsugar B	0.95	2.87	0.743	0.004	-3.75	2.00	0.072	-0.498	0.079	<0.001	0.596

Table 4.3. Continued

Non-volatile metabolite (relative peak area)	Slope	SE	Slope *P*	R^2	Slope (non-volatile)	SE	Slope *P* (non-volatile)	Slope (FPCM)	SE	Slope *P* (FPCM)	R^2
N-acetylsugar C	1.31	3.72	0.728	0.004	4.01	2.49	0.119	-0.464	0.076	<0.001	0.584
N-acetylsugar D	2.36	10.32	0.821	0.002	11.23	6.93	0.117	-0.469	0.076	<0.001	0.584
N-acetylsugar E	24.76	28.47	0.392	0.026	32.81	18.87	0.093	-0.452	0.074	<0.001	0.590
Orotate	3.89	25.59	0.880	0.001	-4.55	17.65	0.799	-0.455	0.078	<0.001	0.545
Oxaloacetate	86.94	94.55	0.366	0.029	53.89	65.46	0.418	-0.437	0.077	<0.001	0.555
Oxoglutarate	29.74	32.47	0.367	0.029	-9.77	23.69	0.683	-0.453	0.082	<0.001	0.547
Phosphocreatine	1.67	32.15	0.959	<0.001	28.48	21.92	0.205	-0.463	0.077	<0.001	0.571
Phosphorylcholine	-2.38	4.01	0.558	0.012	3.21	2.88	0.274	-0.473	0.081	<0.001	0.564
Proline	-37.24	19.84	0.071	0.112	3.24	16.53	0.846	-0.451	0.089	<0.001	0.544
Pyruvate	4.67	28.60	0.871	0.001	17.83	19.51	0.369	-0.451	0.077	<0.001	0.557
Succinate	55.40	37.84	0.154	0.071	-20.62	27.49	0.460	-0.429	0.079	<0.001	0.553
Sugar A (derivative phosphate)	4.69	55.66	0.933	<0.001	-5.65	38.32	0.884	-0.443	0.078	<0.001	0.544
Sugar B (derivative phosphate)	-13.96	31.11	0.657	0.007	33.85	22.05	0.136	-0.487	0.080	<0.001	0.580
Sugar C (derivative phosphate)	-12.93	22.77	0.575	0.011	19.06	16.31	0.253	-0.474	0.081	<0.001	0.566
UDP-hexose A	-282.90	303.41	0.359	0.030	-12.78	217.50	0.954	-0.442	0.080	<0.001	0.544
UDP-hexose B	-347.39	88.66	0.001	0.354	-185.00	78.55	0.026	-0.353	0.081	<0.001	0.621
UDP-hexose C	144.61	76.05	0.068	0.114	87.48	54.05	0.116	-0.419	0.076	<0.001	0.584
UDP-hexose D	-169.83	210.77	0.427	0.023	-17.70	149.15	0.906	-0.441	0.079	<0.001	0.544

[1] Fat- and protein-corrected milk (FPCM; kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d).

[2] Numbers are relative peak area values in relation to the peak area of internal standard (arbitrary units).

[3] Milk non-volatile metabolites are ordered alphabetically, n = 30.

[4] Parameters were extracted from the equation: CH_4 intensity = a + b × non-volatile metabolite + e, where a is the intercept of the regression line, b is the slope of the regression line associated with the metabolite, and e is the error.

[5] Parameters were extracted from the equation: CH_4 intensity = a + b ×non-volatile metabolite + c × FPCM + e, where a is the intercept of the regression line, b is the slope of the regression line associated with the non-volatile metabolite, c is the slope of the regression line associated with FPCM, and e is the error.

Table 4.4. Linear regression between methane yield (g/kg of DMI) and milk non-volatile metabolites (relative area[1])[2]

Non-volatile metabolite[3] (relative peak area)	Intercept	SE	Slope	SE	Slope *P*	R^2
Acetate	25.6	1.75	-32.91	36.288	0.372	0.029
Acetoacetate	25.5	2.07	-50.55	71.864	0.488	0.017
Acetone	25.6	0.79	-43.20	20.664	0.046	0.135
Acetylcarnitine	25.1	1.99	-12.38	23.388	0.601	0.010
Betaine	27.0	1.70	-9.32	5.358	0.093	0.098
Butyrate	24.0	0.56	0.35	1.931	0.858	0.001
β-hydroxybutyrate	26.2	1.85	-37.62	32.213	0.253	0.046
Carnitine	29.2	2.67	-26.95	13.806	0.061	0.120
Choline	23.1	1.15	1.11	1.290	0.397	0.026
Citrate	25.9	2.28	-0.70	0.838	0.412	0.024
Creatine	25.7	2.28	-4.10	5.691	0.477	0.018
Creatinine	23.1	2.97	15.41	45.908	0.740	0.004
Ethanol	25.4	2.17	-59.55	96.318	0.541	0.013
Formate	25.9	1.77	-99.20	94.431	0.302	0.038
Galactose-1-phosphate	24.7	0.95	-83.17	115.441	0.477	0.018
Glycerophosphocholine	23.8	1.07	0.32	1.277	0.804	0.002
Hippurate	22.3	1.57	34.50	30.141	0.262	0.045
Lactate	25.1	0.99	-13.12	11.546	0.265	0.044
Lactose	30.2	7.66	-0.29	0.363	0.430	0.022
Malonate	25.8	1.81	-33.90	34.543	0.335	0.033
Methylmalonate	24.3	1.34	-6.30	34.512	0.856	0.001
N-acetylsugar A	28.7	2.54	-27.76	15.241	0.079	0.106
N-acetylsugar B	27.0	1.51	-4.75	2.411	0.059	0.122
N-acetylsugar C	23.7	0.92	1.33	3.326	0.693	0.006
N-acetylsugar D	23.9	0.99	2.00	9.239	0.830	0.002
N-acetylsugar E	24.1	1.24	-0.79	25.834	0.976	<0.001
Orotate	23.8	1.29	5.15	22.903	0.824	0.002
Oxaloacetate	25.0	1.95	-41.34	85.567	0.633	0.008
Oxoglutarate	24.5	1.61	-8.39	29.458	0.778	0.003
Phosphocreatine	23.0	1.15	27.73	28.309	0.336	0.033
Phosphorylcholine	23.5	0.66	3.83	3.539	0.288	0.04
Proline	25.0	1.64	-10.39	18.745	0.584	0.011
Pyruvate	25.7	2.27	-18.05	25.387	0.483	0.018
Succinate	24.0	1.92	3.18	35.144	0.929	<0.001
Sugar A (derivative phosphate)	25.6	1.33	-56.85	48.672	0.253	0.046
Sugar B (derivative phosphate)	23.5	1.08	15.27	27.805	0.587	0.011
Sugar C (derivative phosphate)	23.6	0.67	17.01	20.251	0.408	0.025
Uridine diphosphate (UDP)-hexose A	24.5	0.88	-150.60	274.370	0.587	0.011
UDP-hexose B	25.2	1.31	-88.20	97.360	0.373	0.028
UDP-hexose C	25.3	2.21	-39.70	71.965	0.586	0.011
UDP-hexose D	24.4	1.15	-54.77	190.607	0.776	0.003

[1] Numbers are relative peak area values in relation to the peak area of internal standard (arbitrary units).

[2] Milk non-volatile metabolites are ordered alphabetically, n = 30.

[3] Parameters were extracted from the equation: CH_4 yield = a + b × non-volatile metabolite + e, where a is the intercept of the regression line, b is the slope of the regression line associated with the non-volatile metabolite, and e is the error.

Changes in methane intensity are related to lactose synthesis

In our data set, CH_4 intensity is negatively related to UDP-hexose B ($P = 0.001$, Table 4.3). This negative relation may be due to the fact that UDP-sugars, including UDP-hexoses, are intermediates in lactose synthesis (Cant et al., 2002). For the production of lactose, glucose is transported from the bloodstream into the cytosol of epithelial cells, where part of it is converted into UDP-sugars. These are precursors of galactose that together with glucose will form lactose (Cant et al., 2002). This may explain the positive correlation between UDP-hexose B and lactose yield (g/d; Figure 4.1A). Milk yield is mainly controlled by the synthesis of lactose due to the contribution of this disaccharide to the osmolality of milk (Linzell and Peaker, 1971), and as discussed previously, an increase in milk yield (as FPCM) per animal reduces CH_4 intensity. Although in our data set UDP-hexose B is positively related ($P = 0.008$, data not shown) to FPCM, even when including FPCM in the regression model the UDP-hexose B is still significantly related to CH_4 intensity. This suggests that the lower amount of UDP-hexose B in milk might be related to the involvement of glucose in different metabolic pathways in the mammary gland. The large majority of glucose that is taken up by the epithelial cells is used in the biosynthesis of lactose (Cant et al., 2002). Glucose is also involved in NADPH production, which is paramount in the de novo MFA synthesis, and is required to synthesize glycerol, forming the backbone of triglycerides (Dijkstra et al., 1996). Thus, the negative relation between UDP-hexose B and CH_4 intensity may be explained by a decrease in metabolic rate in the mammary gland due to a lower milk yield.

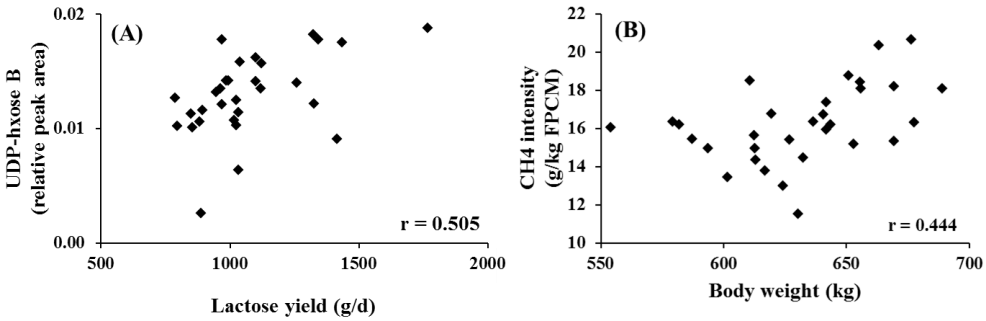

Figure 4.1 Scatter plots of (A) uridine diphosphate (UDP)-hexose B and lactose yield (g/d), and (B) CH_4 intensity (g/kg of fat- and protein-corrected milk; FPCM) and BW (kg). The Pearson correlation coefficient is indicated by r (n = 30).

In our data set, milk citrate is negatively related to CH_4 intensity ($P = 0.026$, Table 4.3) and is not related to FPCM ($P = 0.347$, data not shown). Therefore, when including FPCM in the regression model, citrate is still negatively related to CH_4 intensity ($P = 0.025$, Table 4.3). Milk citrate is regarded as a marker for the energy metabolism in the mammary gland because the mammary epithelium is impermeable to citrate (Linzell et al., 1976; Faulkner and Peaker, 1982). In our study, a decrease in milk citrate and associated increase in CH_4 intensity (Table 4.3) could indicate a disruption in the TCA cycle in the mammary gland because citrate is a regulatory compound of the acetyl-CoA metabolism in the

mitochondria (Bremer and Davis, 1974). Further, by providing NADPH, the citrate metabolism and the TCA cycle may be indirectly involved in de novo synthesis of MFA in the mammary gland, inducing a negative correlation between citrate and de novo synthesized MFA (Faulkner and Peaker, 1982). In our data set, C12:0 and C14:0 were the only de novo synthesized MFA negatively related to milk citrate (data not shown). This may be due to the contribution of NADPH to the elongation step during the de novo MFA synthesis. In each elongation step, rumen-derived acetate and NADPH are needed and longer de novo MFA require more elongation steps and therefore more NADPH; C6:0 requires 2 elongation steps in contrast to the 5 elongation steps needed for C12:0 (Garnsworthy et al., 2006).

Together with NADPH, each elongation step in the de novo synthesis of MFA requires rumen-derived acetate, which mainly originates from fermentation of complex carbohydrates in the rumen. Decreased rates of MFA synthesis in the mammary gland induced by dietary changes have been shown to increase citrate levels of milk (Garnsworthy et al., 2006). Increasing the level of rapidly fermentable carbohydrates by increasing concentrate proportion of the diet, or increasing dietary lipid content, are generally associated with reduced de novo MFA synthesis, and such diets in general are also associated with reduced CH_4 production (Ellis et al., 2008). This may further explain the observed negative relationship between citrate and CH_4 intensity in the present study.

Milk acetone relates to methane yield

Expressing CH_4 emission relative to feed intake avoids confounding effects of DMI on total CH_4 production (Dijkstra et al. (2011). In our data set, acetone measured both by ^{1}H-NMR and GC-MS was the only metabolite negatively related to CH_4 yield. Acetone is a ketone body that may be positively or negatively related to milk production. In general, milk acetone levels of energy deficient cows increase rapidly and may indicate subclinical ketosis (Miettinen, 1994), and subclinical ketosis may negatively affect milk production. Milk acetone is also influenced by parity and lactation stage, and a rise in milk production was accompanied by a rise in milk acetone levels (Heuer et al., 2001). In our study, CH_4 yield and acetone were not related to milk production (expressed as FPCM; $P < 0.675$, data not shown), and therefore the mechanisms explaining the relation between CH_4 yield and acetone are not totally clear. In general, levels of ketone bodies including acetone are not just related to energy balance of the cow, but also to glucose levels (Andersson, 1988). As discussed in a previous section, glucogenic diets are generally associated with reduced CH_4 production. Therefore, we assessed the effect of diet on the established relations between individual volatile metabolites or non-volatile metabolites, and CH_4 emission, by including dietary treatment as a covariate in the linear regressions. The majority of relations were not significantly changed (data not shown). Only hydrogen-azide became significantly related to CH_4 intensity ($P = 0.043$) and sugar A (derivative phosphate) became significantly related to CH_4 yield ($P = 0.031$). More specifically, including treatment as a covariate in the regression model did not significantly change the relation between acetone (non-volatile) and CH_4 yield ($P = 0.044$), but acetone (volatile) was no longer significantly related to CH_4 yield ($P = 0.229$). Our results suggest that although acetone may help to understand changes in the physiology of the dairy

cow associated with CH_4 yield, it may also be influenced by the diet.

Body weight relates to methane intensity, but not to methane yield

In the present study, body weight (**BW**) of cows was positively related to CH_4 intensity ($P = 0.012$, $r = 0.444$; Figure 4.1B), but was not related to CH_4 yield ($P = 0.671$, $r = 0.079$). The importance of BW in relation to CH_4 production has been previously acknowledge by other authors who have included BW in regression equations to predict CH_4 production (Kirchgebner et al., 1995; Moraes et al., 2014). In a meta-analysis using a Bayesian model selection procedure, Moraes et al. (2014) identified BW as a key explanatory variable in predicting CH_4 emissions, in addition to the key dietary variables energy intake, dietary fiber, and lipid proportions. The association between BW and CH_4 emission might be explained by the relation between BW and rumen capacity (Demment and Van Soest, 1985). A higher BW is proportional to a larger rumen capacity. When feed intake is kept constant, a higher rumen capacity results in a lower passage rate (Demment and Van Soest, 1985), resulting in a higher CH_4 production (Moraes et al., 2014).

Because feed intake, either as DMI or gross energy intake, are confounding factors of BW and enteric CH_4 production (Ellis et al., 2008), cows in the present experiment had a restricted feed intake, and no correlation between BW and CH_4 yield ($P = 0.671$, $r = 0.079$) or BW and FPCM ($P = 0.232$, $r = -0.221$) was observed. A higher BW requires more feed to be used for maintenance purposes, thus having less feed available for milk production, which is expected to increase CH_4 intensity. However, the absence of relation between BW and FPCM supports the idea that rumen size and passage rate may explain the positive relation between BW and CH_4 intensity.

CONCLUSIONS

The results of the present study suggest that milk is a suitable matrix to better understand the biological pathways involved in CH_4 emission. In general, milk non-volatile metabolites have a more pronounced relationship with CH_4 emission compared with milk volatile metabolites, especially when referring to CH_4 intensity (g/kg of FPCM) rather than CH_4 yield (g/ kg of DMI). However, relationships between several metabolites and CH_4 intensity are partly dependent on milk production (as FPCM). The relations between milk UDP-hexose B and citrate with CH_4 intensity (g/ kg of FPCM) remained significant when FPCM was included as a covariate in the regression models. The UDP-hexose B is an intermediate metabolite of lactose metabolism, whereas citrate is an important intermediate of Krebs cycle–related energy metabolic processes. This suggests that CH_4 intensity may be related to lactose synthesis and energy metabolism in the mammary gland. The negative relation between milk acetone and CH_4 yield may be related to glucogenic nutrient supply, and implies that acetone is important to monitor CH_4 emission related to feed intake. We observed a positive relationship between BW and CH_4 intensity, which may be related to differences in rumen capacity and rumen passage rate. Our results suggest that an integrative approach including milk production and composition, dietary and animal traits will help to explain the biological metabolism of dairy cows in relation to CH_4 emission.

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SUPPORTING INFORMATION

Supplementary Table S4.1. Summary statistics of milk volatile metabolites measured (peak area[1])

Volatile metabolite	Mean	SD	Minimum	Maximum
1-heptanol	3.88×10^{5}	2.657×10^{5}	1.22×10^{5}	1.53×10^{6}
1-heptanol-decanol	7.75×10^{5}	2.085×10^{5}	0.00	1.11×10^{6}
1-pentanol	2.46×10^{6}	9.270×10^{5}	9.19×10^{5}	4.27×10^{6}
2-butanone	8.37×10^{6}	6.378×10^{6}	2.00×10^{6}	3.13×10^{7}
2-heptanone	2.76×10^{6}	2.729×10^{6}	3.02×10^{5}	1.30×10^{7}
3-nonanone	1.78×10^{6}	4.428×10^{5}	0.00	2.41×10^{6}
Acetone	1.24×10^{8}	9.076×10^{7}	5.46×10^{7}	5.31×10^{8}
Alkane A	6.27×10^{5}	2.515×10^{5}	0.00	1.20×10^{6}
Alkane B	6.34×10^{5}	1.720×10^{5}	0.00	9.16×10^{5}
Alkane C	1.38×10^{6}	9.267×10^{5}	5.73×10^{5}	4.73×10^{6}
Benzene alkane	6.96×10^{5}	1.905×10^{5}	0.00	1.01×10^{6}
Benzene compound	4.30×10^{5}	5.438×10^{5}	5.95×10^{4}	3.14×10^{6}
Butanoic acid	3.93×10^{7}	4.272×10^{7}	6.46×10^{6}	2.32×10^{8}
Cyclohexane	1.52×10^{5}	4.747×10^{4}	0.00	2.32×10^{5}
Dimethyl sulfone	2.01×10^{6}	1.098×10^{6}	7.48×10^{5}	5.52×10^{6}
Ethanol	4.25×10^{6}	1.474×10^{6}	2.62×10^{6}	8.27×10^{6}
Ethyl acetate	4.90×10^{6}	1.551×10^{6}	2.29×10^{6}	8.44×10^{6}
Hexanal	1.46×10^{7}	1.291×10^{7}	2.53×10^{6}	6.13×10^{7}
Hexanoic acid	5.19×10^{7}	6.760×10^{7}	8.46×10^{6}	3.82×10^{8}
Hydrogen azide	4.89×10^{7}	1.491×10^{7}	0.00	7.47×10^{7}
Ketone A	3.89×10^{5}	3.986×10^{5}	8.69×10^{4}	2.21×10^{6}
Limonene	8.68×10^{4}	2.531×10^{4}	4.46×10^{4}	1.53×10^{5}
Octanoic acid	3.86×10^{7}	5.099×10^{7}	7.70×10^{6}	2.88×10^{8}
Tetrahydrofuran	4.48×10^{7}	2.511×10^{7}	6.72×10^{6}	1.21×10^{8}

[1] Numbers are peak area values (arbitrary units).

Supplementary Table S4.2. Summary statistics of milk non-volatile metabolites measured (relative area[1])

Non-volatile metabolite	Mean	SD	Minimum	Maximum
Acetate	0.047	0.0096	0.030	0.068
Acetoacetate	0.028	0.0049	0.020	0.039
Acetone	0.035	0.0159	0.023	0.102
Acetylcarnitine	0.084	0.0150	0.057	0.125
Betaine	0.311	0.0624	0.188	0.422
Butyrate	0.225	0.1823	0.084	1.018
β-hydroxybutyrate	0.056	0.0107	0.039	0.088
Carnitine	0.192	0.0239	0.149	0.246
Choline	0.849	0.2694	0.376	10.658
Citrate	20.690	0.4151	10.931	30.378
Creatine	0.396	0.0613	0.299	0.564
Creatinine	0.064	0.0077	0.048	0.081
Ethanol	0.022	0.0036	0.015	0.030
Formate	0.018	0.0037	0.011	0.027
Galactose-1-phosphate	0.008	0.0030	0.002	0.015
Glycerophosphocholine	0.789	0.2756	0.449	1.396
Hippurate	0.051	0.0114	0.038	0.084
Lactate	0.081	0.0298	0.051	0.170
Lactose	21.063	0.9583	18.065	22.765
Malonate	0.052	0.0100	0.030	0.073
Methylmalonate	0.023	0.0097	0.013	0.064
N-acetylsugar A	0.165	0.0219	0.124	0.224
N-acetylsugar B	0.611	0.1369	0.334	0.890
N-acetylsugar C	0.257	0.1056	0.089	0.516
N-acetylsugar D	0.101	0.0381	0.039	0.197
N-acetylsugar E	0.046	0.0136	0.022	0.087
Orotate	0.054	0.0154	0.028	0.100
Oxaloacetate	0.023	0.0041	0.016	0.034
Oxoglutarate	0.053	0.0119	0.032	0.082
Phosphocreatine	0.039	0.0122	0.023	0.071
Phosphorylcholine	0.161	0.0975	0.062	0.444
Proline	0.086	0.0187	0.059	0.142
Pyruvate	0.089	0.0138	0.066	0.122
Succinate	0.054	0.0100	0.040	0.092
Sugar A (derivative phosphate)	0.027	0.0071	0.012	0.039
Sugar B (derivative phosphate)	0.037	0.0126	0.016	0.061
Sugar C (derivative phosphate)	0.029	0.0172	0.006	0.072
Uridine disphosphate (UDP)-hexose A	0.003	0.0013	0.001	0.005
UDP-hexose B	0.013	0.0036	0.003	0.019
UDP-hexose C	0.030	0.0049	0.020	0.046
UDP-hexose D	0.006	0.0019	0.003	0.010

[1] Numbers are relative peak area values in relation to the peak area of internal standard (arbitrary units).

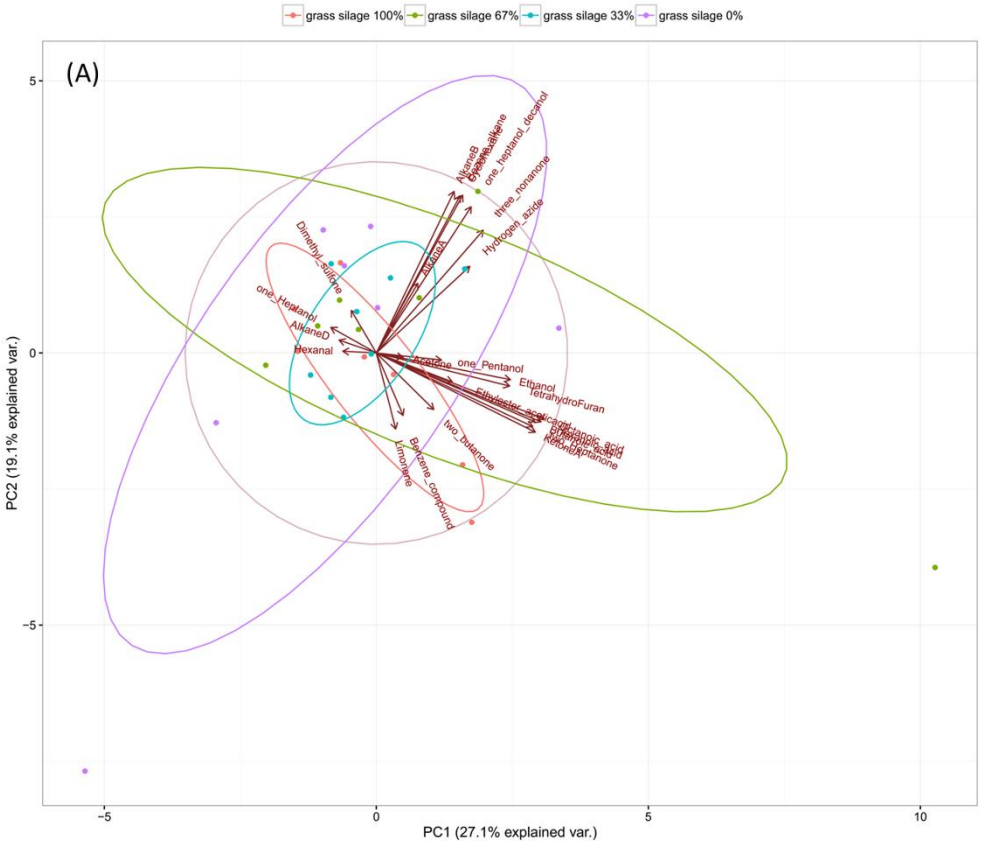
Supplementary Table S4.3. List of chemical shift values[1], proton assignments[2], and multiplicity[3] for metabolites identified by ^{1}H-NMR in milk samples of cows

Metabolite	Chemical shift (ppm)	Assignment	Multiplicity
Acetate	1.930	CH3	Singlet
Acetoacetate	2.264	CH3	Singlet
Acetone	2.230	CH3	Singlet
Acetylcarnitine	3.193	3×CH3	Singlet
Betaine	3.266	3×CH3	Singlet
Butyrate	2.172	CH2	Triplet
β-hydroxybutyrate	2.330	CH2	Multiplet
Carnitine	3.496	CH2	Multiplet
Choline	3.203	3×CH3	Singlet
Citrate	2.702	CH2	Doublet
Creatine	3.041	CH3	Singlet
Creatinine	3.060	CH3	Singlet
Ethanol	1.175	CH3	Triplet
Formate	8.457	CH	Singlet
Galactose-1-phosphate	5.477	CH	Doublet
Glycerophosphoholine	3.231	3×CH3	Singlet
Hippurate	7.557	CH2	Multiplet
Lactate	1.333	CH3	Doublet
Lactose	4.459	CH	Doublet
Malonate	3.114	CH2	Singlet
Methylmalonate	1.256	CH	Doublet
N-acetylsugar A	2.043	CH3	Singlet
N-acetylsugar B	2.059	CH3	Singlet
N-acetylsugar C	2.069	CH3	Singlet
N-acetylsugar D	5.404	CH2	Multiplet
N-acetylsugar E	8.144	CH2	Doublet
Orotate	6.198	CH	Singlet
Oxaloacetate	2.396	CH2	Singlet
Oxoglutarate	2.452	CH2	Triplet
Phosphocreatine	3.047	CH3	Singlet
Phosphorylcholine	3.222	3×CH3	Singlet
Proline	2.360	CH2	Multiplet
Pyruvate	2.377	CH3	Singlet
Succinate	2.426	2×CH2	Singlet
Sugar A (derivative phosphate)	5.192	CH	Doublet
Sugar B (derivative phosphate)	5.434	CH	Doublet
Sugar C (derivative phosphate)	5.517	CH	Doublet
Uridine disphosphate (UDP)-hexose A	5.903	CH	Doublet
UDP-hexose B	5.951	CH	Doublet
UDP-hexose C	8.072	CH	Doublet

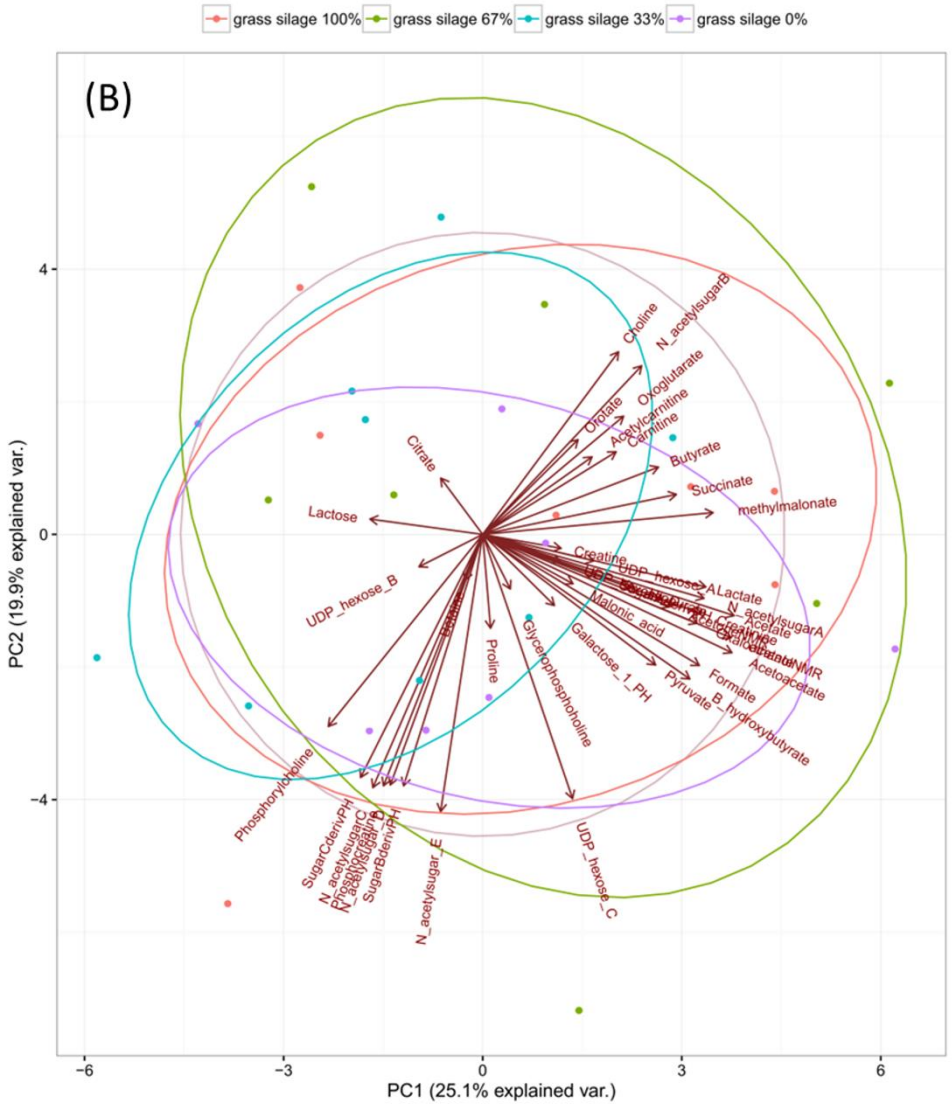
[1] Position of the signals.

[2] Type of proton(s).

[3] Number of peaks corresponding to detected proton(s).



Supplementary Figure S4.1A. PCA score plot derived from volatile metabolite profiles according to the different treatments used.



Supplementary Figure S4.1B. PCA score plot derived from non-volatile metabolite profiles according to the different treatments used.

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Chapter 5

Relationships between methane emission of Holstein Friesian dairy cows and fatty acids, volatile metabolites and non-volatile metabolites in milk

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ABSTRACT

This study investigated the relationships between methane (**CH_4**) emission and fatty acids, volatile metabolites (**V**) and non-volatile metabolites (**NV**) in milk of dairy cows. Data from an experiment with 32 multiparous dairy cows and 4 diets were used. All diets had a roughage:concentrate ratio of 80:20 based on dry matter (**DM**). Roughage consisted of either 1000 g/kg DM grass silage (**GS**), 1000 g/kg DM maize silage (**MS**), or a mixture of both silages (667 g/kg DM GS and 333 g/kg DM MS; 333 g/kg DM GS and 677 g/kg DM MS). Methane emission was measured in climate respiration chambers and expressed as production (gram per day), yield (gram per kg dry matter intake; **DMI**) and intensity (gram per kg fat- and protein-corrected milk; **FPCM**). Milk was sampled during the same days and analysed for fatty acids by gas chromatography, for V by gas chromatography-mass spectrometry, and for NV by nuclear magnetic resonance. Several models were obtained using a stepwise selection of (1) milk fatty acids (**MFA**), V, or NV alone, and (2) the combination of MFA, V and NV, based on the minimum Akaike's information criterion statistic. Dry matter intake was 16.8 ± 1.23 kg/d, FPCM yield was 25.0 ± 3.14 kg/d, CH_4 production was 406 ± 37.0 g/d, CH_4 yield was 24.1 ± 1.87 g/kg DMI, and CH_4 intensity was 16.4 ± 1.91 g/kg FPCM. The observed CH_4 emissions were compared with the CH_4 emissions predicted by the obtained models, based on concordance correlation coefficient (**CCC**) analysis. The best models with MFA alone predicted CH_4 production, yield, and intensity with a CCC of 0.80, 0.71, and 0.69, respectively. The best models combining the three types of metabolites included MFA and NV for CH_4 production and CH_4 yield, whereas for CH_4 intensity MFA, NV, and V were all included. These models predicted CH_4 production, yield, and intensity better with a higher CCC of 0.92, 0.78, and 0.93, respectively, and with increased accuracy (C_b) and precision (r). The results indicate that MFA alone have moderate to good potential to estimate CH_4 emission, and furthermore that including V (CH_4 intensity only) and NV increases the CH_4 emission prediction potential. This holds particularly for the prediction model for CH_4 intensity.

Keywords: methane emission, dairy cow, milk fatty acid, milk volatile metabolite, milk non-volatile metabolite

IMPLICATIONS

There is a need to quantify methane (**CH_4**) emission of dairy cows, given the importance of methane as a greenhouse gas. This study investigated the relationship between CH_4 emission and potential biomarkers in milk, viz. fatty acids (**FA**), volatile metabolites (**V**) and non-volatile metabolites (**NV**) of dairy cows. Results indicate that milk fatty acids (**MFA**) alone have moderate to good potential to predict methane emission, and furthermore that the prediction models become more accurate and precise when including V and, in particular, NV. These models can aid in the effort to understand and mitigate CH_4 emissions of dairy cows.

INTRODUCTION

Quantification of CH_4 emission of dairy cows is important to understand factors that contribute to the variation in CH_4 emission, and to identify effective mitigation strategies. Several CH_4 measurement techniques have been developed, but are not suitable for precise and accurate

large scale measurements (Hammond et al., 2016). Proxies (i.e., indirect traits related to CH_4 emission), such as milk composition, may be good alternatives. Milk contains a large number of metabolites, including MFA, that may give information on rumen metabolism (Fievez et al., 2012). Milk FA arise from two sources, viz. *de novo* synthesis within the mammary gland mainly from rumen acetate and β-hydroxybutyrate, and mammary uptake of FA that originate from intestinal absorption of dietary and microbial FA and FA from body fat mobilization (Chilliard et al., 2009). Common biochemical pathways between CH_4, acetate and butyrate in the rumen, and CH_4 reducing effects of dietary long chain FA (Chilliard et al., 2009), suggest a relationship between CH_4 emission and MFA profile, and several studies have predicted CH_4 emission of dairy cows from MFA concentrations (reviewed by Van Gastelen and Dijkstra, 2016). Milk also contains V and NV from different chemical families. Milk V have been used for tracing animal feeding systems (e.g., Villeneuve et al., 2013), because diet composition can influence V composition in milk, either by transferring odour-active molecules (Buchin et al., 1999) or by interacting with rumen metabolism (Urbach, 1990). Milk NV may be related to the health status of cows, and these metabolites are potential biomarkers for mastitis and subclinical ketosis (Enjalbert et al., 2001; Sundekilde et al., 2013). More recently, Antunes-Fernandes et al. (2016) investigated the relation between both milk V and NV and CH_4 emission of dairy cows. They showed that milk NV have a more pronounced relationship with CH_4 emission compared with milk V. Based on the relations found between NV and CH_4 emission, Antunes-Fernandes et al. (2016) concluded that CH_4 intensity (g/kg fat- and protein-corrected milk; **FPCM**) may be related to lactose synthesis and energy metabolism in the mammary gland, as reflected by the milk NV uridine diphosphate (**UDP**)-hexose B and citrate. Methane yield (g/kg dry matter intake; **DMI**) on the other hand, may be related to glucogenic nutrient supply, as reflected by the milk NV acetone. Acetone is a ketone body, related to blood glucose levels, which in turn relate to the supply of glucogenic nutrients, and glucogenic diets are generally associated with reduced CH_4 emissions (Antunes-Fernandes et al., 2016).

To the best of our knowledge, prediction models for CH_4 emission of dairy cows combining MFA and other milk metabolites have not been developed. Therefore, the objectives of this study were (i) to develop prediction models for CH_4 emission based on MFA, V or NV alone; (ii) to develop prediction models for CH_4 emission combining MFA with V and NV; (iii) to evaluate the improvement in prediction potential upon inclusion of V and NV in the prediction models, compared with the prediction models based on MFA alone.

MATERIALS AND METHODS

Experimental design and analyses

Data from a randomized block design experiment with 32 multiparous Holstein Friesian cows and 4 diets were used. The experiment has been described by Van Gastelen et al. (2015) and was conducted in accordance with Dutch law and approved by the Animal Care and Use Committee of Wageningen University & Research (Wageningen, The Netherlands). Briefly, diets had a roughage:concentrate ratio of 80:20 based on dry matter (**DM**). Roughage consisted of either 1000 g/kg DM grass silage (**GS**), 1000 g/kg DM maize silage (**MS**), or mixtures of both silages (667 g/kg DM GS and 333 g/kg DM MS; 333 g/kg DM GS and 333 g/kg DM MS).

Methane emission was measured in climate respiration chambers for a 5-d period and expressed as production (g/d), yield (g/kg DMI) and intensity (g/kg FPCM). Milk yield was recorded and milk samples collected according to Antunes-Fernandes et al. (2016). These milk samples were subsequently analysed for MFA composition (g/100 g FA) using gas chromatography according to Van Gastelen et al. (2015), for V (peak area in arbitrary units) using gas chromatography-mass spectrometry according to Antunes-Fernandes et al. (2016), and for NV (relative area in arbitrary units) using nuclear magnetic resonance according to Antunes-Fernandes et al. (2016).

Statistical analysis

As described by Antunes-Fernandes et al. (2016), one cow was excluded because of large feed residuals, the gas chromatography-mass spectrometry results of another cow were considered outliers and could not be used, and the nuclear magnetic resonance results of a third cow could not be used because of repeatedly high background interference. Therefore, a total of 29 observations were used to determine the relation between CH_4 emission and milk metabolites.

Data on DMI, CH_4 emission, milk production, milk composition, and MFA are described in Table 5.1. The PROC REG procedure (SAS Institute Inc., Cary, NC, USA, version 9.2) was used to determine the relationship between individual MFA and CH_4 production, CH_4 yield, and CH_4 intensity. The summary statistics of the V and NV and the relation between the individual V and NV and CH_4 emission are presented by Antunes-Fernandes et al. (2016).

The PROC GLMSELECT procedure (SAS Institute Inc., Cary, NC, USA, version 9.2) was used to develop prediction models, with CH_4 emission (i.e., production, yield, and intensity) as dependent variable, the milk metabolites (i.e., MFA, V, and NV) as independent variables, and stepwise selection as selection procedure. Milk fat and protein content, and milk production were also included as selection variables when developing overall prediction models (i.e., all types of metabolites combined) for CH_4 production and CH_4 yield, but not for CH_4 intensity because these parameters are part of the FPCM calculation. The significance level for a variable to enter or stay in the model was 0.05 and 0.10, respectively. The best models were selected based on the minimum Akaike's information criterion statistic. Selected models were evaluated with PROC REG procedure in terms of multicollinearity (variation inflation factor > 10), but no multicollinearity was observed in any of the prediction models (i.e., for all prediction models, the variation inflation factors of the variables was < 10).

The developed prediction models were evaluated with the coefficient of determination (adjusted R^2) and the concordance correlation coefficient analysis (**CCC**; Lin, 1989). A detailed calculation of CCC is described by Dijkstra et al. (2016). Briefly, CCC is calculated by multiplying r (i.e., a measure of precision) with C_b (i.e., a measure of accuracy). The calculation of the C_b variable involves v (i.e., a measure of scale shift, which indicates the change in standard deviation, if any, between predicted and observed values) and μ (i.e., a measure of location shift).

Table 5.1. Summary statistics of experimental data used for modelling (n = 29)

Item	Mean	SD	Minimum	Maximum
Dry matter intake (kg/d)	16.8	1.23	13.7	19.5
Milk production (kg/d)	23.1	3.52	17.8	30.5
FPCM[A] (kg/d)	25.0	3.14	19.8	31.7
Milk fat content (g/100 g milk)	4.72	0.527	3.94	6.20
Milk protein content (g/100 g milk)	3.52	0.418	2.61	4.53
Milk lactose content (g/100 g milk)	4.53	0.181	3.80	4.84
Methane production (g/d)	406	37.0	307	465
Methane yield (g/kg DMI[B])	24.1	1.87	18.8	28.0
Methane intensity (g/kg FPCM)	16.4	1.91	13.0	20.7
Fatty acid (g/100g fatty acids)				
C4:0	3.4	0.30	2.9	4.3
C6:0	2.2	0.13	2.0	2.5
C8:0	1.2	0.10	1.0	1.4
C10:0	2.7	0.34	2.0	3.4
C12:0	3.3	0.48	2.4	4.4
C14:0	11.3	0.78	9.6	12.6
iso C14:0	0.09	0.014	0.06	0.12
C14:1 *cis*-9	1.22	0.272	0.84	1.95
C15:0	1.04	0.205	0.77	1.47
iso C15:0	0.25	0.037	0.19	0.33
anteiso C15:0	0.41	0.049	0.31	0.51
C16:0	35.4	2.72	32.0	42.3
iso C16:0	0.17	0.023	0.13	0.21
C16:1 *trans*-9	0.21	0.034	0.14	0.27
C16:1 *cis*-9	2.0	0.30	1.5	3.0
C17:0	0.62	0.084	0.47	0.79
iso C17:0	0.37	0.032	0.32	0.46
anteiso C17:0	0.41	0.051	0.34	0.54
C17:1 *cis*-9	2.0	0.30	1.5	3.0
C18:0	7.5	1.03	5.0	9.3
C18:1 *cis*-9[C]	17.8	2.76	12.3	22.0
C18:1 *cis*-12	0.20	0.097	0.07	0.47
C18:1 *cis*-13	0.11	0.022	0.07	0.17
C18:1 *trans*-6	0.22	0.079	0.12	0.42
C18:1 *trans*-9	0.14	0.038	0.08	0.25
C18:1 *trans*-10	0.22	0.144	0.10	0.65
C18:1 *trans*-11	0.84	0.332	0.49	2.18
C18:1 *trans*-15 + C18:1 *cis*-11	0.59	0.127	0.33	0.78
C18:2 *cis*-9, *trans*-11	0.44	0.199	0.25	1.29
C18:2n-6	1.46	0.251	0.89	1.92
C18:3n-3	0.39	0.125	0.14	0.66
C18:3n-6	0.08	0.012	0.06	0.11
C20:0	0.12	0.017	0.08	0.15
C20:1 *cis*-11	0.05	0.009	0.03	0.07
C20:2n-6	0.05	0.007	0.03	0.06
C20:3n-6	0.09	0.018	0.05	0.12
C20:4n-3	0.07	0.04	0.00	0.13

Table 5.1. Continued

Item	Mean	SD	Minimum	Maximum
C20:4n-6	0.13	0.025	0.08	0.18
C20:5n-3	0.06	0.013	0.03	0.09
C22:0	0.06	0.02	0.00	0.10
C22:5n-6	0.09	0.015	0.07	0.12
C24:0	0.05	0.02	0.00	0.08

Data from Van Gastelen et al. (2015).

[A] Fat- and protein-corrected milk(kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

[B] Dry matter intake (kg/d).

[C] C18:1 cis-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12, as these 2 FA could not be separated in the analysis. The portion of C18:1 *trans*-12 is considered to be negligible, as this FA is always present in small amounts.

RESULTS

Relation between individual milk fatty acids and methane emission

The relationships between individual MFA and CH_4 production, CH_4 yield, and CH_4 intensity are shown in Table 5.2 and Supplementary Tables S5.1, S5.2, and S5.3. Short- and medium-chain MFA (defined here as straight, even chain MFA up to 16 carbon chain length; **SMCFA**) were not related to CH_4 emission, except for C14:1 *cis*-9 which was positively related to CH_4 production ($P = 0.03$) and to CH_4 intensity ($P = 0.04$). Of the odd- and branched-chain FA (**OBCFA**), *anteiso* FA were not related to CH_4 emission. No relation was found between the *iso* FA and CH_4 production, but *iso* C15:0 tended to be positively related ($P = 0.06$) whereas *iso* C14:0 was negatively related ($P = 0.03$) to CH_4 yield, and *iso* C15:0 was positively related ($P < 0.01$) to CH_4 intensity. Additionally, C15:0 and C17:0 were not related to CH_4 production, but C17:0 was positively related ($P = 0.01$) to CH_4 yield, and C15:0 was positively related ($P = 0.03$) to CH_4 intensity. Negative relations were found between CH_4 emission and several C18:1, C18:2, and C18:3 isomers in milk, with the exception of C18:3n-3 which was positively related ($P = 0.05$) to CH_4 yield. The long-chain saturated FA (**SFA**) C20:0, C22:0, and C24:0 were not related to CH_4 production, but positively related to CH_4 yield and CH_4 intensity ($P < 0.05$).

Regression analyses for methane emission

Four sets of test variables were used to develop CH_4 prediction models; (1) only MFA, (2) only V, (3) only NV, and (4) all three types of metabolites combined. In total, 11 prediction models were obtained; three for CH_4 production (no model was obtained with V only), and four for both CH_4 yield and CH_4 intensity (Table 5.3). The observed and residuals (observed minus predicted) versus predicted CH_4 production, CH_4 yield, and CH_4 intensity plots are shown in Figures 5.1, 5.2, and 5.3, respectively. The results of the CCC analysis of the 11 obtained CH_4 prediction models are shown in Table 5.4.

Table 5.2. Correlations between methane production (g/d), yield (g/kg dry matter intake; DMI), and intensity (g/kg fat- and protein-corrected milk; FPCM), and milk fatty acid concentrations

Fatty acid (g/100 g fatty acids)	CH_4 (g/d)		CH_4 (g/kg DMI)		CH_4 (g/kg FPCM[A])	
	r	*P* - value	*r*	*P* - value	*r*	*P* - value
C4:0	-0.05	0.80	-0.09	0.65	-0.28	0.15
C6:0	0.20	0.30	0.03	0.87	-0.07	0.72
C8:0	0.30	0.11	0.08	0.70	0.10	0.60
C10:0	0.21	0.27	0.04	0.82	0.18	0.36
C12:0	0.25	0.19	0.02	0.91	0.25	0.19
C14:0	0.13	0.50	-0.06	0.74	0.20	0.30
iso C14:0	-0.10	0.60	-0.40	0.03	0.13	0.49
C14:1 *cis*-9	0.40	0.03	-0.09	0.63	0.39	0.04
C15:0	0.29	0.13	0.22	0.24	0.40	0.03
iso C15:0	0.26	0.17	0.35	0.06	0.56	< 0.01
anteiso C15:0	0.05	0.78	-0.12	0.53	0.30	0.12
C16:0	0.31	0.10	0.15	0.44	0.23	0.23
iso C16:0	-0.19	0.33	-0.29	0.12	-0.23	0.23
C16:1 *trans*-9	-0.16	0.41	-0.21	0.27	-0.37	0.05
C16:1 *cis*-9	0.07	0.73	-0.08	0.67	0.22	0.26
C17:0	0.10	0.62	0.46	0.01	0.10	0.61
iso C17:0	-0.13	0.49	0.08	0.69	0.01	0.94
anteiso C17:0	-0.07	0.71	-0.02	0.93	-0.22	0.24
C17:1 *cis*-9	-0.12	0.53	0.19	0.32	-0.16	0.41
C18:0	-0.11	0.57	0.27	0.15	-0.21	0.26
C18:1 *cis*-9[B]	-0.25	0.18	0.04	0.85	-0.27	0.15
C18:1 *cis*-12	-0.47	< 0.01	-0.70	< 0.01	-0.33	0.08
C18:1 *cis*-13	-0.27	0.16	-0.31	0.11	-0.30	0.11
C18:1 *trans*-6	-0.34	0.07	-0.64	< 0.01	-0.32	0.09
C18:1 *trans*-9	-0.47	0.01	-0.65	< 0.01	-0.22	0.25
C18:1 *trans*-10	-0.48	< 0.01	-0.71	< 0.01	-0.33	0.08
C18:1 *trans*-11	-0.63	< 0.01	-0.72	< 0.01	-0.16	0.41
C18:1 *trans*-15 + C18:1 *cis*-11	-0.44	0.02	-0.32	0.09	-0.40	0.03
C18:2 *cis*-9, *trans*-11	-0.58	< 0.01	-0.74	< 0.01	-0.08	0.67
C18:2n-6	-0.48	< 0.01	-0.53	< 0.01	-0.31	0.11
C18:3n-3	0.09	0.62	0.36	0.05	0.28	0.15
C18:3n-6	-0.51	< 0.01	-0.63	< 0.01	-0.25	0.18
C20:0	0.15	0.45	0.58	< 0.01	0.37	0.05
C20:1 *cis*-11	-0.22	0.25	0.13	0.52	-0.26	0.18
C20:2n-6	-0.27	0.15	0.16	0.42	0.19	0.32
C20:3n-6	-0.41	0.03	-0.28	0.15	-0.02	0.90
C20:4n-3	0.45	0.01	0.19	0.32	0.01	0.94
C20:4n-6	-0.46	0.01	-0.52	< 0.01	0.16	0.41
C20:5n-3	0.06	0.76	0.18	0.36	0.33	0.08
C22:0	0.21	0.27	0.48	< 0.01	0.52	< 0.01
C22:5n-3	-0.16	0.42	-0.15	0.43	0.42	0.02
C24:0	0.24	0.20	0.43	0.02	0.53	< 0.01

[A] Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

[B] C18:1 cis-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12, as these 2 FA could not be separated in the analysis. The portion of C18:1 *trans*-12 is considered to be negligible, as this FA is always present in small amounts.

Table 5.3. The prediction models developed for methane production (g/d), yield (g/kg dry matter intake; DMI), and intensity (g/kg fat- and protein-corrected milk; FPCM) based on milk fatty acids (MFA), volatile (V) or non-volatile (NV) metabolites, or all metabolites (ALL) combined

Metabolite included	Methane production (g/d)				Methane yield (g/kg DMI)				Methane intensity (g/kg FPCM[A])			
	Metabolite	Estimate	SE	*P*-value	Metabolite	Estimate	SE	*P*-value	Metabolite	Estimate	SE	*P*-value
MFA[B]	Intercept	211.2	66.59	< 0.01	Intercept	27.2	0.58	< 0.01	Intercept	16.5	2.94	< 0.01
	C4:0	50.4	16.62	< 0.01	C18:2 *cis*-9, *trans*-11	-7.0	1.21	< 0.01	*iso* C15:0	24.6	12.38	< 0.01
	C14:1 *cis*-9	77.7	17.76	< 0.01					C17:0	-15.5	4.70	0.03
	C18:1 *trans*-11	-82.0	13.46	< 0.01					C22:0	52.4	23.02	0.03
V[C]	No best model available				Intercept	25.1	0.56	< 0.01	Intercept	12.0	1.21	< 0.01
					Acetone	-7.6×10^{-9}	3.64×10^{-9}	0.05	3-nonanone	2.51×10^{-6}	6.610×10^{-7}	< 0.01
NV[D]	Intercept	479.4	28.87	< 0.01	Intercept	17.4	2.49	< 0.01	Intercept	19.7	2.38	< 0.01
	N-acetylsugar B	-118.9	45.94	0.02	Oxaloacetate	245.8	95.70	< 0.01	UDP-hexose B[F]	-257.2	69.25	< 0.01
					Acetone	-138.9	40.38	0.05	Citrate	-1.62	0.594	< 0.01
					Ethanol	255.1	75.34	0.02	Acetoacetate	152.5	48.72	< 0.01
ALL[E]	Intercept	302.2	55.46	< 0.01	Intercept	23.3	1.58	< 0.01	Intercept	8.8	1.58	< 0.01
	C4:0	38.7	12.69	< 0.01	C18:2 *cis*-9, *trans*-11	-6.3	1.13	< 0.01	Hexanal	3.1×10^{-8}	1.44×10^{-8}	0.02
	C14:1 *cis*-9	77.1	12.72	< 0.01	Ethanol	157.6	61.46	0.02	3-nonanone	1.72×10^{-6}	3.830×10^{-7}	< 0.01
	C18:1 *trans*-11	-75.5	9.76	< 0.01					C24:0	45.8	10.02	< 0.01
	Choline	28.4	13.18	0.04					C20:4n-3	20.6	4.56	< 0.01
	N-acetylsugar B	-131.5	25.92	< 0.01					UDP-hexose B	-277.2	54.43	0.02
									Creatinine	63.3	22.76	0.01

[A] Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

[B] Only milk fatty acids as selection variables; in g/100g fatty acids.

[C] Only volatile metabolites as selection variables; peak area value (arbitrary unit of quantity).

[D] Only non-volatile metabolites as selection variable; peak area relative to calibration standard 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate.

[E] All metabolites combined as selection variables.

[F] Uridine diphosphate (UDP)-hexose B.

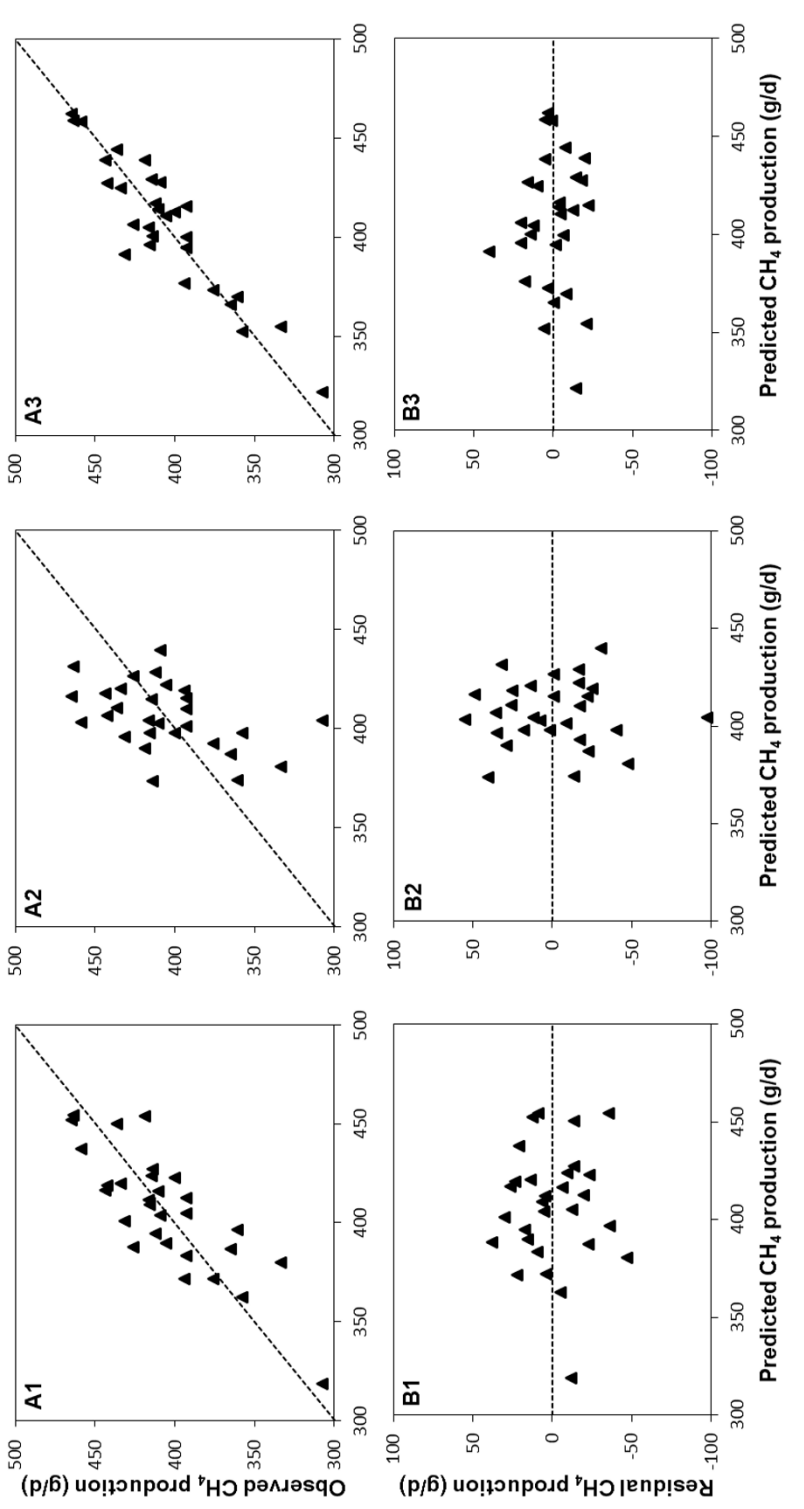

Figure 5.1. (**A**) Observed and predicted methane production (g/d), and (**B**) residual (i.e., observed − predicted) methane production (g/d), from the regression analyses based on milk fatty acids (g/100 g fatty acids) alone (**1**), non-volatile metabolites alone (**2**), and all metabolites combined (**3**). The slope of residuals regressed on predicted values did not differ significantly from zero.

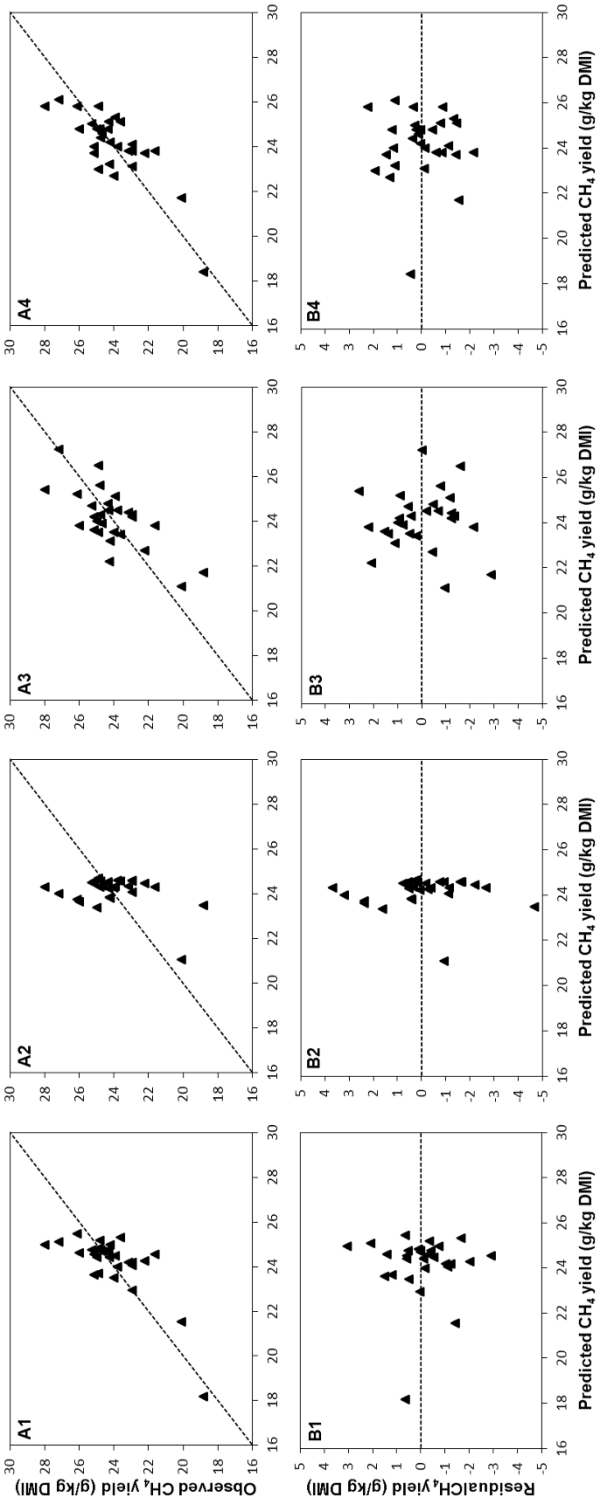

Figure 5.2. (**A**) Observed and predicted methane yield (g/kg DMI), and (**B**) residual (i.e., observed − predicted) methane yield (g/kg DMI), from the regression analyses based on milk fatty acids (g/100 g fatty acids) alone (**1**), volatile metabolites alone (**2**), non-volatile metabolites alone (**3**), and all metabolites combined (**4**). The slope of residuals regressed on predicted values did not differ significantly from zero.

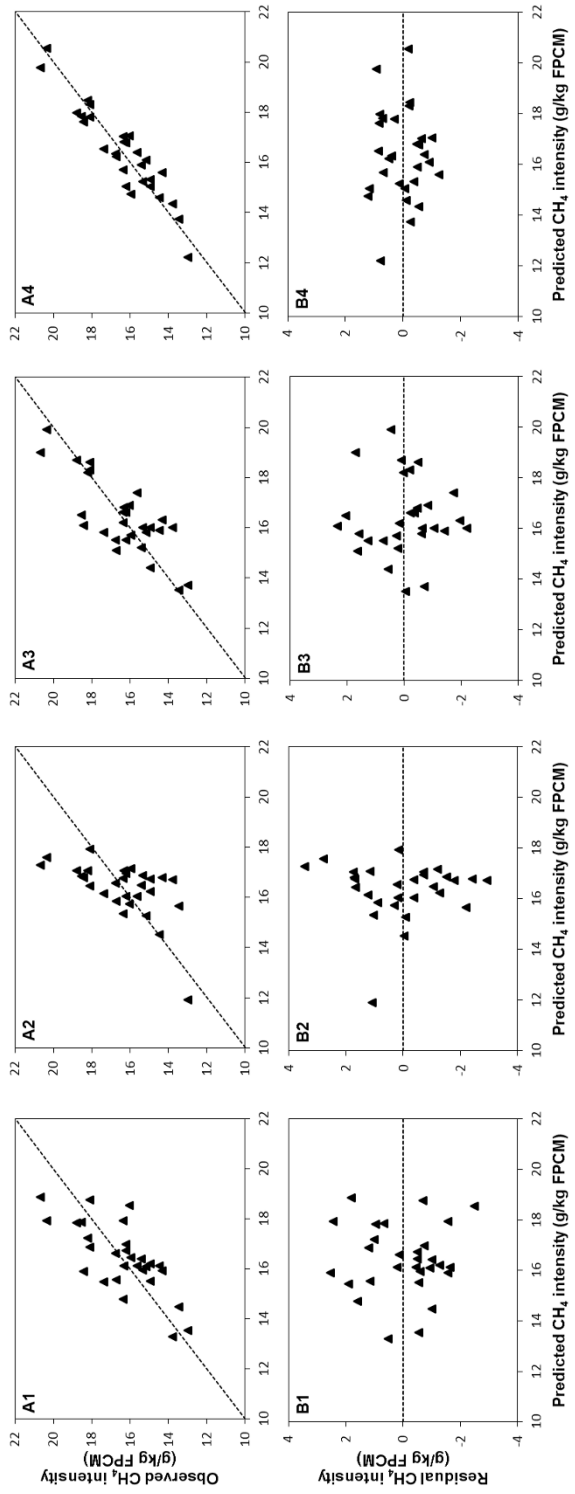

Figure 5.3. (**A**) Observed and predicted methane intensity (g/kg FPCM), and (**B**) residual (i.e., observed − predicted) methane intensity (g/kg FPCM), from the regression analyses based on milk fatty acids (g/100 g fatty acids) alone (**1**), volatile metabolites alone (**2**), non-volatile metabolites alone (**3**), and all metabolites combined (**4**). The slope of residuals regressed on predicted values did not differ significantly from zero.

Table 5.4. The coefficient of determination (R^2) and concordance correlation coefficient (CCC) of the prediction models

Model	Adjusted R^2	CCC[A]	r[B]	C_b[C]	v[D]	μ[E]
Methane production (g/d)						
MFA[F]	0.63	0.80	0.82	0.98	1.22	0.00
V[G]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
NV[H]	0.17	0.33	0.45	0.74	2.24	0.00
ALL[I]	0.81	0.92	0.92	1.00	1.09	0.00
Methane yield (g/kg DMI[J])						
MFA	0.54	0.71	0.74	0.96	1.34	-0.02
V	0.11	0.24	0.37	0.65	2.69	-0.02
NV	0.41	0.64	0.69	0.93	1.45	0.00
ALL	0.62	0.78	0.80	0.98	1.25	0.00
Methane intensity (g/kg FPCM[K])						
MFA	0.47	0.69	0.73	0.95	1.37	0.00
V	0.41	0.62	0.67	0.93	1.48	-0.02
NV	0.59	0.77	0.79	0.97	1.26	0.00
ALL	0.83	0.93	0.93	1.00	1.08	0.02

[A] Concordance correlation coefficient, where CCC = $r \times C_b$.

[B] Pearson correlation coefficient; a measure of precision.

[C] Bias correction factor; a measure of accuracy.

[D] Scale shift; change in standard deviation between predicted and observed methane emission.

[E] Location shift; if positive underprediction, if negative overprediction.

[F] Only milk fatty acids as selection variables; in g/100 g fatty acids.

[G] Only volatile metabolites as selection variables; peak area value (arbitrary unit of quantity).

[H] Only non-volatile metabolites as selection variable; peak area relative to calibration standard 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate.

[I] All metabolites combined as selection variables.

[J] Dry matter intake (kg/d).

[K] Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

The adjusted R^2 and the CCC varied between 0.47 and 0.63, and between 0.69 and 0.80, respectively, for the prediction models obtained using only MFA, with the prediction model for CH_4 production performing the best. No model was obtained for CH_4 production with V only. The significance level for a variable to enter the model was 0.05, whereas the significance level of the strongest correlation between a volatile metabolite (i.e., acetone) and CH_4 production was 0.08 (results not shown). The prediction models for CH_4 yield and intensity with only V performed worse than MFA alone, with an adjusted R^2 and CCC ranging from 0.11 to 0.41, and from 0.24 to 0.62, respectively, and the prediction model for CH_4 intensity performed the best.

The adjusted R^2 and the CCC varied between 0.17 and 0.59, and between 0.33 and 0.77, respectively, for the prediction models obtained with NV only, with the prediction model for CH_4 intensity performing the best. The prediction models for CH_4 yield and for CH_4 production with only NV performed worse than the prediction models with only MFA, whereas the opposite was observed for CH_4 intensity. Relative to the prediction models with only V, the prediction models with only NV performed considerably better for CH_4 production, yield, and intensity. The adjusted R^2 and the CCC varied between 0.62 and 0.83, and between 0.78 and 0.93,

respectively, for the prediction models obtained combining the three types of metabolites, with the prediction model for CH_4 intensity performing the best. Milk production, milk fat and milk protein content were not selected in the prediction models for CH_4 production and yield. The three prediction models using the combination of all milk metabolites performed better than the prediction models using the three types of metabolites separately, in particular for CH_4 intensity. Milk FA and NV were selected in all three models, whereas milk V were selected only in the CH_4 intensity prediction model.

DISCUSSION

Relation between individual milk fatty acids and methane emission

The lack of relation between SMCFA and CH_4 emission differs from most other studies (e.g., Chilliard et al. (2009) for CH_4 production; Dijkstra et al. (2011) for CH_4 yield; Van Lingen et al. (2014) for CH_4 intensity). The absence of a relation between SMCFA and CH_4 emission in this study is in line with the absence of a positive relation between CH_4 emission and both ruminal acetate and butyrate. Van Gastelen et al. (2015) observed a decrease in CH_4 emission upon replacement of GS with MS, whereas the molar proportion of acetate was unaffected and the molar proportions of butyrate increased; both are substrates for *de novo* synthesized SMCFA (Bauman and Griinari, 2003).

The negative relation of *iso* C14:0 with CH_4 yield was unexpected, because *iso* FA are generally more abundant in fibrolytic bacteria (Vlaeminck et al., 2006) and thus hypothesized to be positively associated with CH_4 emission (Castro-Montoya et al., 2011). The positive relation between *iso* C15:0 and CH_4 yield and intensity is in agreement with this hypothesis and with findings of Castro-Montoya et al. (2011) and Dijkstra et al. (2011). Both C15:0 and C17:0 are hypothesized to be negatively related to CH_4 emission (Vlaeminck and Fievez, 2005), which was also found by Castro-Montoya et al. (2011) and Rico et al. (2016). In the present study, however, a positive association was found. Similarly, Chilliard et al. (2009) reported a positive relation between these linear odd-chain FA and CH_4 production, Van Lingen et al. (2014) found a positive relation between C15:0 and CH_4 intensity, and Dijkstra et al. (2011) found a tendency for a positive relation between C17:0 and CH_4 yield.

The generally negative relations between C18:1, C18:2, and C18:3 isomers in milk and CH_4 emission, is in agreement with the findings of Chilliard et al. (2009), Dijkstra et al. (2011), Van Lingen et al. (2014), and Rico et al. (2016). Possible explanations for these relations, such as dietary unsaturated FA, and their biohydrogenation products, have been described by Van Gastelen and Dijkstra (2016). The positive relation between the long-chain SFA and CH_4 emission (both yield and intensity) has not been reported in any other study investigating the relation between MFA and CH_4 emission (Van Gastelen and Dijkstra, 2016). The individual relationships found in the present study, in combination with the inclusion of a long-chain SFA in the prediction model for CH_4 intensity, suggest that these MFA are important in terms of CH_4 prediction.

Overall, the relation between individual MFA and CH_4 emission depends on the unit in which CH_4 emission is expressed (i.e., production, yield, or intensity). For example, similar to Van Lingen et al. (2014), a reduced correlation strength for C18:1 *trans*-10 and C18:1 *trans*-11

with CH_4 intensity compared with CH_4 production and yield was observed. These MFA are associated with milk fat depression, causing a decline in FPCM yield and, therefore, a reduction in correlation strength (Van Lingen et al., 2014). In general, the differences found for the various CH_4 emission units were expected when considering the discrepancies between other studies. For example, several studies (Chilliard et al., 2009; Dijkstra et al., 2011; Mohammed et al., 2011) found a positive relation between C8:0 and both CH_4 production and yield, whereas Van Lingen et al. (2014), Williams et al. (2014), Rico et al. (2016), and Dijkstra et al. (2016) did not find a relation between C8:0 and both CH_4 production and intensity. These discrepancies have been reviewed in more detail by Van Gastelen and Dijkstra (2016).

Regression analyses for methane emission

The prediction model with only MFA for CH_4 production performed better than the prediction models with only MFA for CH_4 yield and CH_4 intensity. This is evident by the higher adjusted R^2 and CCC values. The higher CCC value is mainly caused by the considerably improvement in precision (r) and only to a minor extent by the improvement in accuracy (C_b). In general, the prediction potential of the CH_4 prediction models with MFA only appears to be moderate, and the adjusted R^2 reported in this study are lower compared to Chilliard et al. (2009), Dijkstra et al. (2011), Mohammed et al. (2011), and Rico et al. (2016), but of similar magnitude as Van Lingen et al. (2014). This could be the result of the dietary treatments used in the present experiment, namely replacing GS with MS, and its moderate effect on CH_4 emission and MFA composition. Van Gastelen et al. (2015) reported a reduction of 7% in CH_4 production upon completely replacing GS with MS (800 g/kg silage diets on DM basis), and Kliem et al. (2008) reported minor changes in MFA composition upon replacing GS with MS (500 g/kg silage diets on DM basis). This decline is, for example, much smaller than the decline reported by Chilliard et al. (2009) who observed a reduction of 64% in CH_4 production and large effects on the MFA composition for linseed oil supplementation compared to a control diet.

The prediction potential of V appears low and considerably less promising compared with MFA, especially for CH_4 production (no prediction model could be derived) and CH_4 yield (low adjusted R^2 and CCC values). Additionally, the variation in predicted CH_4 yield was considerably smaller than that in observed CH_4 yield, and also smaller than that in predicted CH_4 yield based on only MFA, as evidenced by the large scale shift (v = 2.69). This indicates the inability of only V to predict the range of observed CH_4 yield. The potential of V to predict CH_4 intensity is greater than their potential to predict CH_4 production and CH_4 yield, which is evident by the higher adjusted R^2 and CCC values and lower scale shift, and of almost the same magnitude as the prediction potential of MFA only.

These results suggest that V have low potential to predict CH_4 emission, except when CH_4 emission is expressed as intensity. Antunes-Fernandes et al. (2016) already reported weak correlations between individual V and CH_4 emissions and demonstrated that 3-nonanone (i.e., the volatile metabolite in the prediction model for CH_4 intensity) is no longer associated with CH_4 intensity when including FPCM as a covariate. This suggests that V hold potential to predict CH_4 intensity only, which can be explained by the relationship between the V in milk and FPCM yield. In other words, V in the milk which originate from odour-active molecules from the diet,

have no clear relations with the ruminal CH_4 emission metabolism, but rather are suggested to be related to milk synthesis.

The prediction potential of NV is also low and less promising than MFA alone for CH_4 production (low adjusted R^2 and CCC values), and the scale shift is large (v = 2.24), indicating inability of only NV to predict the range of observed CH_4 production. Although the prediction potential of NV for CH_4 yield is lower than that of MFA alone, the differences in CCC and v were rather small. The prediction model for CH_4 intensity with only NV, however, performed better than the prediction model with only MFA, which is evident by the higher adjusted R^2 and CCC values, and the smaller scale shift. The higher CCC value is caused by the considerable improvement in precision (r) and to a lesser extent by the improvement in accuracy (C_b). These results suggest that NV have a good potential for predicting CH_4 intensity, which can be explained to a significant extent by the relation between the NV in milk and FPCM yield (Antunes-Fernandes et al., 2016).

To the best of our knowledge, the present study is the first to combine MFA with other milk metabolites to predict CH_4 emissions. The prediction potential improved when combining all three types of metabolites. This is evident by the increased adjusted R^2 and CCC values, including r and C_b, and the smaller scale shift relative to the prediction models using only MFA, only V, and only NV. For all CH_4 emission units, the prediction model combining the three types of metabolites performed the best. Additionally, the scale shift (v < 1.25) was minor, indicating the ability of these models to describe most of the observed variation. The improved prediction potential when combining the three types of metabolites relative to only MFA for both CH_4 production and CH_4 yield, is relatively small (i.e., the increase in adjusted R^2 and CCC is smaller than 0.18 and 0.12, respectively) and caused only by NV as no V were included in both prediction models. For CH_4 intensity, however, combining the three types of metabolites resulted in significant improvement of the prediction potential; the adjusted R^2 and CCC increase with 0.36 and 0.24, respectively, relative to the prediction model with MFA only. This improvement can be equally assigned to V and NV.

As illustrated, combining MFA with V (CH_4 intensity only) and NV helps to improve the prediction potential. Unfortunately, the techniques used for identifying V and NV are not suitable for large-scale measurements. Analyses of these metabolites, however, contribute to our understanding of factors that influence the variation in CH_4 emission, and thereby give a better understanding of the relation between milk composition and CH_4 emission. Although the present study focussed largely on the statistical relationship between the milk metabolites and CH_4 emissions of dairy cattle, the physiological interpretations of relationships between NV or V and CH_4 emissions are described by Antunes-Fernandes et al. (2016). It should be noted that the area of validity of the relations that have been established in this study, is limited to roughage-based diets varying in GS and MS content, and the robustness of the reported relationships have not yet been evaluated within this area of validity. As shown by Dijkstra et al. (2016), quantitative relationships between MFA and CH_4 yield in cattle fed grass- or grass silage-based diets differ from those determined for other types of diets. This might also be valid for the relation between CH_4 emission and both V and NV. Therefore, the promising results of this study, need to be validated in further work.

CONCLUSIONS

This study demonstrated for the first time that the potential to predict CH_4 production, CH_4 yield, and CH_4 intensity in dairy cattle increased, both in terms of precision and accuracy, when combining MFA with V and, in particular, with NV in milk. The combination of the three metabolites also has the ability to describe more of the observed variation in CH_4 emission relative to MFA alone. The improved prediction potential was relatively small for CH_4 production and CH_4 yield, suggesting that it may not be worthwhile to perform complex analyses to determine the V and NV in milk in order to estimate CH_4 production or CH_4 yield of dairy cows. For CH_4 intensity, the prediction potential increased considerably when combining the three types of metabolites compared with MFA alone. Therefore, analysing milk for these types of metabolites may be worthwhile to estimate CH_4 intensity of dairy cattle.

SUPPORTING INFORMATION

Supplementary Table S5.1. Linear regressions between methane production (g/d) and milk fatty acid concentrations

Fatty acid (g/100 g fatty acids)	Intercept	SE	Slope	SE	Slope *P*	R^2
C4:0	427	79.5	-6.2	23.57	0.80	< 0.01
C6:0	280	118.7	56.8	53.40	0.30	0.04
C8:0	271	82.4	111.7	67.97	0.11	0.09
C10:0	343	56.6	23.3	20.55	0.27	0.05
C12:0	344	47.0	19.1	14.30	0.19	0.06
C14:0	337	102.5	6.2	9.05	0.50	0.02
iso C14:0	430	45.0	-274.2	516.17	0.60	0.01
C14:1 *cis*-9	341	30.1	53.8	24.08	0.03	0.16
C15:0	351	35.4	52.7	33.26	0.13	0.09
iso C15:0	341	46.9	258.1	184.36	0.17	0.07
anteiso C15:0	390	60.0	40.5	144.18	0.78	< 0.01
C16:0	255	88.3	4.3	2.48	0.10	0.10
iso C16:0	459	53.1	-305.1	306.38	0.33	0.04
C16:1 *trans*-9	442	43.2	-172.1	205.65	0.41	0.03
C16:1 *cis*-9	390	47.1	8.2	23.41	0.73	< 0.01
C17:0	380	52.4	42.7	84.50	0.62	< 0.01
iso C17:0	464	82.5	-156.4	221.47	0.49	0.02
anteiso C17:0	428	57.2	-52.4	139.28	0.71	< 0.01
C17:1 *cis*-9	438	50.1	-114.0	178.58	0.53	0.01
C18:0	436	51.5	-4.0	6.85	0.57	0.01
C18:1 *cis*-9[A]	467	45.0	-3.4	2.50	0.18	0.06
C18:1 *cis*-12	442	14.2	-181.2	65.05	< 0.01	0.22
C18:1 *cis*-13	458	35.7	-455.4	312.13	0.16	0.07
C18:1 *trans*-6	442	19.9	-160.6	84.48	0.07	0.12
C18:1 *trans*-9	469	23.8	-449.4	163.93	0.01	0.22
C18:1 *trans*-10	434	11.4	-122.9	43.35	< 0.01	0.23
C18:1 *trans*-11	466	15.1	-70.5	16.65	< 0.01	0.40
C18:1 *trans*-15 + C18:1 *cis*-11	482	30.4	-128.5	50.36	0.02	0.19
C18:2 *cis*-9, *trans*-11	454	13.9	-108.9	29.13	< 0.01	0.34
C18:2n-6	511	36.8	-71.4	24.84	< 0.01	0.23
C18:3n-3	395	23.4	28.1	56.75	0.62	< 0.01
C18:3n-6	529	39.7	-1611.9	517.80	< 0.01	0.26
C20:0	367	51.8	325.1	420.31	0.45	0.02
C20:1 *cis*-11	445	34.1	-854.3	733.46	0.25	0.05
C20:2n-6	478	49.2	-1535.2	1038.17	0.15	0.07
C20:3n-6	482	33.3	-843.9	362.83	0.03	0.17
C20:4n-3	376	13.0	426.9	162.50	0.01	0.20
C20:4n-6	496	34.2	-683.4	265.95	0.01	0.21
C20:5n-3	397	32.2	165.8	527.29	0.76	< 0.01
C22:0	385	20.0	343.0	302.07	0.27	0.05
C22:5n-3	443	44.7	-398.6	483.07	0.42	0.02
C24:0	385	18.0	471.2	360.68	0.20	0.06

[A] C18:1 cis-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12, as these 2 FA could not be separated in the analysis. The portion of C18:1 *trans*-12 is considered to be negligible, as this FA is always present in small amounts.

Supplementary Table S5.2. Linear regressions between methane yield (g/kg DMI[A]) and milk fatty acid concentrations

Fatty acid (g/100 g fatty acids)	Intercept	SE	Slope	SE	Slope *P*	R^2
C4:0	25.9	4.00	-0.54	1.185	0.65	< 0.01
C6:0	23.1	6.10	0.45	2.746	0.87	< 0.01
C8:0	22.4	4.34	1.41	3.583	0.70	< 0.01
C10:0	23.4	2.92	0.24	1.059	0.82	< 0.01
C12:0	23.8	2.45	0.08	0.744	0.91	< 0.01
C14:0	25.9	5.20	-0.15	0.459	0.74	< 0.01
iso C14:0	28.8	2.09	-54.64	23.944	0.03	0.16
C14:1 *cis*-9	24.9	1.65	-0.65	1.315	0.63	< 0.01
C15:0	22.0	1.82	2.04	1.708	0.24	0.05
iso C15:0	19.7	2.29	17.68	9.001	0.06	0.13
anteiso C15:0	26.0	3.00	-4.57	7.224	0.53	0.01
C16:0	20.5	4.64	0.10	0.130	0.44	0.02
iso C16:0	28.2	2.60	-24.05	15.027	0.12	0.09
C16:1 *trans*-9	26.5	2.15	-11.50	10.262	0.27	0.04
C16:1 *cis*-9	25.1	2.37	-0.51	1.179	0.67	< 0.01
C17:0	17.8	2.35	10.31	3.791	0.01	0.22
iso C17:0	22.4	4.18	4.60	11.230	0.69	< 0.01
anteiso C17:0	24.4	2.89	-0.64	7.037	0.93	< 0.01
C17:1 *cis*-9	21.6	2.49	9.04	8.900	0.32	0.04
C18:0	20.4	2.51	0.50	0.340	0.15	0.08
C18:1 *cis*-9[B]	23.7	2.34	0.02	0.130	0.85	< 0.01
C18:1 *cis*-12	26.8	0.58	-13.62	2.640	< 0.01	0.50
C18:1 *cis*-13	27.0	1.78	-26.00	15.555	0.11	0.09
C18:1 *trans*-6	27.5	0.82	-15.19	3.466	< 0.01	0.42
C18:1 *trans*-9	28.6	1.02	-31.77	7.064	< 0.01	0.43
C18:1 *trans*-10	26.1	0.46	-9.16	1.757	< 0.01	0.50
C18:1 *trans*-11	27.5	0.68	-4.05	0.751	< 0.01	0.52
C18:1 *trans*-15 + C18:1 *cis*-11	26.9	1.62	-4.68	2.681	0.09	0.10
C18:2 *cis*-9, *trans*-11	27.2	0.58	-6.99	1.208	< 0.01	0.55
C18:2n-6	29.8	1.80	-3.91	1.217	< 0.01	0.28
C18:3n-3	22.0	1.10	5.38	2.680	0.05	0.13
C18:3n-6	31.6	1.82	-98.92	23.731	< 0.01	0.39
C20:0	16.2	2.15	64.77	17.417	< 0.01	0.34
C20:1 *cis*-11	23.0	1.75	24.64	37.587	0.52	0.02
C20:2n-6	22.1	2.54	43.86	53.744	0.42	0.02
C20:3n-6	26.7	1.77	-28.64	19.262	0.15	0.08
C20:4n-3	23.5	0.72	9.08	9.010	0.32	0.04
C20:4n-6	29.3	1.66	-39.30	14.429	< 0.01	0.27
C20:5n-3	22.7	1.60	24.30	26.211	0.36	0.03
C22:0	21.7	0.90	39.23	13.633	0.01	0.23
C22:5n-3	25.9	2.25	-19.60	24.362	0.43	0.02
C24:0	22.2	0.85	41.43	16.964	0.02	0.18

[A] Dry matter intake (kg/d).

[B] C18:1 cis-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12, as these 2 FA could not be separated in the analysis. The portion of C18:1 *trans*-12 is considered to be negligible, as this FA is always present in small amounts.

Supplementary Table S5.3. Linear regressions between methane intensity (g/kg FPCM[A]) and milk fatty acid concentrations

Fatty acid (g/100 g fatty acids)	Intercept	SE	Slope	SE	Slope *P*	R^2
C4:0	22.3	3.95	-1.75	1.171	0.15	0.08
C6:0	18.7	6.24	-1.03	2.809	0.72	< 0.01
C8:0	14.1	4.44	1.93	3.664	0.60	0.01
C10:0	13.7	2.95	0.99	1.069	0.36	0.03
C12:0	13.2	2.43	1.00	0.738	0.19	0.06
C14:0	10.9	5.23	0.49	0.462	0.30	0.04
iso C14:0	14.8	2.32	18.78	26.558	0.49	0.02
C14:1 *cis*-9	13.1	1.56	2.74	1.247	0.04	0.15
C15:0	12.5	1.75	3.74	1.646	0.03	0.16
iso C15:0	9.2	2.08	28.75	8.165	< 0.01	0.31
anteiso C15:0	11.6	2.96	11.56	7.119	0.12	0.09
C16:0	10.6	4.67	0.16	0.132	0.23	0.05
iso C16:0	19.7	2.72	-19.38	15.677	0.23	0.05
C16:1 *trans*-9	20.7	2.10	-20.77	9.990	0.05	0.14
C16:1 *cis*-9	13.7	2.38	1.37	1.183	0.26	0.05
C17:0	15.0	2.71	2.24	4.364	0.61	< 0.01
iso C17:0	16.1	4.30	0.82	11.545	0.94	< 0.01
anteiso C17:0	19.8	2.89	-8.37	7.032	0.24	0.05
C17:1 *cis*-9	18.5	2.57	-7.67	9.176	0.41	0.03
C18:0	19.4	2.62	-0.40	0.348	0.26	0.05
C18:1 *cis*-9[B]	19.8	2.31	-0.19	0.128	0.15	0.08
C18:1 *cis*-12	17.7	0.79	-6.57	3.597	0.08	0.11
C18:1 *cis*-13	19.4	1.83	-26.28	15.965	0.11	0.09
C18:1 *trans*-6	18.1	1.04	-7.69	4.405	0.09	0.10
C18:1 *trans*-9	17.9	1.35	-10.92	9.341	0.25	0.05
C18:1 *trans*-10	17.4	0.63	-4.34	2.411	0.08	0.11
C18:1 *trans*-11	17.2	0.99	-0.91	1.096	0.41	0.03
C18:1 *trans*-15 + C18:1 *cis*-11	20.0	1.61	-6.03	2.656	0.03	0.16
C18:2 *cis*-9, *trans*-11	16.8	0.88	-0.79	1.847	0.67	< 0.01
C18:2n-6	19.8	2.07	-2.33	1.396	0.11	0.09
C18:3n-3	14.7	1.17	4.24	2.829	0.15	0.08
C18:3n-6	19.5	2.31	-41.23	30.154	0.18	0.06
C20:0	11.3	2.51	42.16	20.397	0.05	0.14
C20:1 *cis*-11	18.8	1.74	-51.95	37.519	0.18	0.07
C20:2n-6	13.8	2.59	55.87	54.712	0.32	0.04
C20:3n-6	16.6	1.88	-2.60	20.529	0.90	< 0.01
C20:4n-3	16.4	0.75	0.76	9.405	0.94	< 0.01
C20:4n-6	14.8	1.96	12.36	14.720	0.41	0.03
C20:5n-3	13.6	1.59	46.71	25.765	0.08	0.11
C22:0	13.7	0.90	43.02	13.659	< 0.01	0.27
C22:5n-3	11.4	2.12	55.20	22.924	0.02	0.18
C24:0	14.0	0.81	53.23	16.253	< 0.01	0.28

[A] Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

[B] C18:1 cis-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12, as these 2 FA could not be separated in the analysis. The portion of C18:1 *trans*-12 is considered to be negligible, as this FA is always present in small amounts.

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Chapter 6

The relationship between milk metabolome and methane emission of Holstein Friesian dairy cows – metabolic interpretation and prediction potential

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ABSTRACT

This study aimed to quantify the relationship between methane (**CH_4**) emission and fatty acids, volatile metabolites, and non-volatile metabolites in milk of dairy cows fed forage-based diets. Data from six studies was used, including 27 dietary treatments and 123 individual observations from lactating Holstein-Friesian cows. These dietary treatments covered a large range of forage-based diets, with different qualities and proportions of grass silage and corn silage. Methane emission was measured in climate respiration chambers and expressed as production (g per day), yield (g per unit dry matter intake; **DMI**), and intensity (g per unit fat- and protein-corrected milk; **FPCM**). Milk samples were analyzed for fatty acids by gas chromatography, for volatile metabolites by gas chromatography-mass spectrometry, and for non-volatile metabolites by nuclear magnetic resonance. Dry matter intake was 15.9 ± 1.90 kg/d, FPCM yield was 25.2 ± 4.57 kg/d, CH_4 production was 359 ± 51.1 g/d, CH_4 yield was 22.6 ± 2.31 g/kg DMI, and CH_4 intensity was 14.5 ± 2.59 g/kg FPCM. The results show that changes in individual milk metabolite concentrations can be related to the ruminal CH_4 production pathways. Several of these relationships were diet driven, whereas some were partly dependent on FPCM yield. Next, prediction models were developed and subsequently evaluated based on root mean square error of prediction (**RMSEP**), concordance correlation coefficient (**CCC**) analysis, and on random 10-fold cross validation. The best models with milk fatty acids (in g/100 g fatty acids; **MFA**) alone predicted CH_4 production, yield, and intensity with a RMSEP of 34 g/d, 2.0 g/kg DMI, and 1.7 g/kg FPCM, and with a CCC of 0.67, 0.44, and 0.75, respectively. The CH_4 prediction potential of both volatile metabolites alone and non-volatile metabolites alone was low, regardless of the unit of CH_4 emission, as evidenced by the low CCC values (< 0.35). The best models combining the three types of metabolites as selection variables, resulted in the inclusion of only MFA for CH_4 production and CH_4 yield. For CH_4 intensity, MFA, volatile metabolites, and non-volatile metabolites were included in the prediction model. This resulted in a small improvement in prediction potential (CCC of 0.80; RMSEP of 1.5 g/kg FPCM) relative to MFA alone. These results indicate that volatile and non-volatile metabolites in milk contain some information to increase our understanding of enteric CH_4 production of dairy cows, but that it is not worthwhile to determine the volatile and non-volatile metabolites in milk in order to estimate CH_4 emission of dairy cows. We conclude that MFA have moderate potential to predict CH_4 emission of dairy cattle fed forage-based diets, and the models can aid in the effort to understand and mitigate CH_4 emissions of dairy cows.

Keywords: dairy cow, enteric methane production, milk metabolome

INTRODUCTION

Enteric methane (**CH_4**) production is one of the main targets of greenhouse gas mitigation practices for the dairy industry (Hristov et al., 2013). Quantification of enteric CH_4 production is therefore important. Several CH_4 measuring techniques have been developed, but these are not yet suitable for large scale measurements (Hammond et al., 2016). Proxies (i.e., indirect traits or indicators correlated to CH_4 emission) might, therefore, serve as a good alternative.

Milk fatty acid (**MFA**) concentrations have been suggested as proxy to estimate CH_4 emission in dairy cattle, and many studies have evaluated this proposed relationship between MFA concentrations and CH_4 emission (Chilliard et al., 2009; Mohammed et al., 2011; Rico et al., 2016). However, the results of these studies are inconsistent, with some studies finding a clear and strong relationship between MFA and CH_4 emission (Chilliard et al., 2009, Rico et al., 2016), whereas other studies conclude that MFA alone might not be suitable to develop universal CH_4 prediction models (Mohammed et al., 2011). Recently, Castro-Montoya et al. (2017) concluded that MFA are no reliable predictors for specific amounts of CH_4 emitted by a cow. Furthermore, individual MFA selected in optimal models to predict CH_4 emission largely differ between studies, further hampering the applicability of MFA to predict CH_4 emission in various circumstances. Some of these inconsistencies can be explained by dietary composition and lactation stage, both being factors that can influence the relationship between MFA and CH_4 emission (Mohammed et al., 2011; Dijkstra et al., 2016; Vanrobays et al., 2016).

These findings warrant further investigation of other proxies in milk to estimate CH_4 emission of dairy cattle, including volatile metabolites and non-volatile metabolites. Antunes-Fernandes et al. (2016) and Van Gastelen et al. (2017) evaluated the relationship between CH_4 emission and both volatile and non-volatile metabolites in milk to better understand the biological pathways involved in CH_4 emission in dairy cattle as well as to determine the prediction potential of these milk metabolites. Antunes-Fernandes et al. (2016) concluded that CH_4 intensity (g/kg fat- and protein-corrected milk; **FPCM**) may be related to lactose synthesis and energy metabolism in the mammary gland, as reflected by the significant relationship between both milk non-volatile metabolites citrate and uridine diphosphate (**UDP**)-hexose B and CH_4 intensity. Methane yield (g/kg DMI), on the other hand, may be related to glucogenic nutrient supply, as reflected by the milk non-volatile metabolite acetone. In a recent review of CH_4 proxies, Negussie et al. (2017) concluded that no single proxy accurately predicts CH_4 emission, and that combining two or more proxies is the best way forward for the prediction of CH_4 emission. Van Gastelen et al. (2017) concluded that volatile metabolites and, in particular, non-volatile metabolites in combination with MFA hold potential to predict CH_4 emission of dairy cows more precisely and accurately compared with MFA alone. The improved prediction potential was relatively small (i.e., the increase in adjusted R^2 and CCC is <0.18 and <0.12, respectively) for CH_4 production (g/d) and CH_4 yield (g/kg DMI), whereas the prediction potential for CH_4 intensity (g/kg FPCM) increased considerably (i.e., the adjusted R^2 and CCC increased with 0.36 and 0.24, respectively).

The analysis of both Antunes-Fernandes et al. (2016) and Van Gastelen et al. (2017) was based upon a small range of diets (i.e., four forage-based diets in which grass silage was replaced partly or fully by corn silage) in one experiment. Therefore, the present study aims to quantify the relationship between CH_4 emission and the milk metabolome in dairy cattle fed a range of forage-based diets with different qualities and proportions of grass silage and corn silage.

MATERIALS AND METHODS

Experiments and data

Data on individual cows from six experiments, all designed as randomized block experiments, from Wageningen University & Research (Wageningen, the Netherlands) were used. These experiments were conducted in accordance with Dutch law, and approved by the Animal Care and Use Committee of Wageningen University & Research. Experiment 1 (Warner et al., 2015) involved 25 Holstein-Friesian dairy cows and four grass herbage diets (forage to concentrate ratio of 85:15 based on DM basis. The grass herbage was cut after 3 or 5 weeks of regrowth, after receiving either a low (20 kg of nitrogen (**N**)/ha) or a high (90 kg of N/ha) fertilization rate after initial cut. Experiment 2 (Van Gastelen et al., 2015) involved 29 Holstein-Friesian dairy cows and four diets (forage to concentrate ratio of 80:20 on DM basis). The forage consisted of 1000 g/kg DM grass silage, 1000 g/kg DM corn silage, or a mixture of both silages (667 g/kg DM grass silage and 333 g/kg DM corn silage; 333 g/kg DM grass silage and 667 g/kg DM corn silage). Experiment 3 (Warner et al., 2016) involved 52 Holstein-Friesian dairy cows and six grass silage-based diets (forage to concentrate ratio of 80:20 on DM basis). The grass silage received low (65 kg of N/ha) or high (150 kg of N/ha) N fertilization level preceding its growth period, and there were three regrowth periods (28 days, 41 days, and 62 days of regrowth). Experiment 4 (Warner et al., 2017) involved 55 Holstein-Friesian dairy cows and eight grass silage based diets (grass silage, corn silage and concentrate at a ratio of 70:10:20 on DM basis). The grass silage was cut at four growth stages (leafy, boot, early heading, and late heading) and fed at two levels of DMI (15.5 and 16.6 kg DM/d). Experiment 5 (Hatew et al., 2016) involved 25 Holstein-Friesian dairy cows and four corn silage based diets with whole-plant corn harvested at a very early (25% DM), early (28% DM), medium (32% DM), and late (40% DM) stage of maturity, and with corn silage, concentrate and wheat straw at a ratio of 75:20:5 (DM basis). Experiment 6 (Klop et al., 2017) involved eight Holstein-Friesian dairy cows and three diets containing corn silage, grass silage, and concentrate at a ratio of 40:30:30 (DM basis). The concentrate was either a basal concentrate or contained a blend of essential oils or lauric acid. Repeated measures resulted in 32 observations.

The experimental setup of these experiments was similar. After an adaptation period of 12 d, cows were housed individually in open circuit, indirect climate respiration chambers for a 5 d period to determine CH_4 emission (expressed as production in g/d, as yield in g/kg DMI, and as intensity in g/kg FPCM). The climate respiration chambers are described by van Gastelen et al. (2015) and Heetkamp et al. (2015). Cows were milked twice daily and water was freely available, both during the adaptation period and in the climate respiration chambers. Diets were fed as a total mixed ration twice daily and intake was restricted to 95% of the voluntarily DMI of the cow consuming the least within a block.

Sample collection and analysis

Milk yield was recorded and 10 mL milk samples were collected at each milking in the climate respiration chambers. These milk samples were analyzed for fat, protein, and lactose content, and for milk urea nitrogen as described by the respective studies. In addition, a representative milk sample (i.e., 5 g/kg of milk production at each milking from each cow) was

collected according to Antunes-Fernandes et al. (2016). We selected all observations from experiment 2 (n = 29), and we randomly selected another 94 observations from the other experiments based on complete blocks (i.e., all cows within the same block were selected; incomplete blocks, due to observations being removed from the experiment or statistical analysis, were excluded from the selection). The selected observations, a total of 123 observations, represented all dietary treatments without feed additives (i.e., 27 in total), and resulted in no repeated measurements from the same cows. The representative milk samples matching the selected observations were subsequently analyzed for MFA composition (g/100 g total fatty acids) using gas chromatography (**GC**) according to Van Gastelen et al. (2015), for volatile metabolites (peak area in arbitrary units) using gas chromatography-mass spectrometry (**GC-MS**) according to Antunes-Fernandes et al. (2016), and for non-volatile metabolites (relative area in arbitrary units) using proton nuclear magnetic resonance (**^{1}H-NMR**) according to Antunes-Fernandes et al. (2016).

Statistical analysis

Linear regression. The descriptive statistics of the feed intake, dietary composition, animal performance, and CH_4 emission are presented in Table 6.1. Descriptive statistics of the MFA, volatile metabolites, and non-volatile metabolites used for modelling are presented in Supplementary Table S6.1. To determine the relationship between CH_4 emission (i.e., production, yield and intensity) and individual MFA, volatile metabolites, and non-volatile metabolites, mixed model univariate regression procedures (PROC MIXED of SAS; SAS Institute Inc., Cary, NC, USA, version 9.2) were applied. These included a random experiment effect and individual MFA, volatile metabolites, and non-volatile metabolites as fixed effects. Having the experiment effect as a random effect resulted in the equation parameter estimates to be estimated first within study, and then averaged to obtain overall estimates. To evaluate the influence of FPCM on the established relationships between individual MFA, volatile metabolites or non-volatile metabolites and CH_4 intensity, FPCM was included as a covariate in the linear regressions.

Model development. The PROC GLMSELECT procedure of SAS was used to develop multivariate models retaining the experiment effect in every step, with CH_4 emission (i.e., production, yield, and intensity) as the dependent variable, the milk metabolites (i.e., MFA, volatile metabolites and non-volatiles metabolites) as independent variables, and stepwise selection as selection procedure. The significance level for milk metabolites to enter or stay in the model was 0.01 and 0.05, respectively. The best models were selected based on the minimum Akaike's information criterion statistic. Adjusted independent variable values were calculated based on regression parameters of the final model to determine the adjusted R^2 corrected for experiment effect, as described by St-Pierre (2001). The selected models were evaluated with the PROC REG procedure in terms of multicollinearity (variation inflation factor > 10), but no multicollinearity was observed for any of the CH_4 prediction models.

Table 6.1. Descriptive statistics of dry matter intake, dietary composition, animal characteristics, and methane emissions (n=123) [data from Van Gastelen et al. (2015), Warner et al. (2015, 2016, 2017), Hatew et al. (2016), Klop et al. (2017)]

Item	Mean	Median	SD	Minimum	Maximum
Dry matter intake (kg/d)	15.9	15.9	1.90	10.8	19.8
Forage content diet (g/100 g DM)	80	80	3.0	70	85
Dietary characteristics (in g/kg DM, unless stated otherwise)					
Dry matter (g/kg)	507	519	113.4	306	797
Ash	76	76	14.7	53	103
Crude protein	170	158	43.2	82	251
NDF	386	386	53.4	242	501
ADF	225	219	26.9	183	291
ADL	14	15	4.7	6	26
Crude fat	31	28	7.2	21	46
Starch	111	79	92.6	5	326
Sugar	95	76	66.4	21	265
GE (MJ/kg DM)	18.6	18.7	0.41	17.6	19.3
NDF to starch ratio	8.8	5.0	16.03	1.0	86.2
Lactation characteristics					
Milk production (kg/d)	22.6	22.5	3.87	14.6	33.7
FPCM[(1)] (kg/d)	25.2	24.7	4.57	15.0	38.4
Milk fat content (g/100 g milk)	4.64	4.65	0.635	2.94	6.44
Milk protein content (g/100 g milk)	3.37	3.33	0.359	2.62	4.53
Milk lactose content g/100 g milk)	4.58	4.59	0.219	3.80	5.03
Urea (mg/dl)	19.6	18.8	6.79	8.4	41.4
Days in milk	176	191	70.9	70	403
Parity	2.6	2.0	1.29	1.0	7.0
Methane emission					
Methane production (g/d)	359	358	51.1	234	469
Methane yield (g/kg DMI[(2)])	22.6	22.9	2.31	17.2	28.0
Methane intensity (g/kg FPCM)	14.5	14.6	2.59	8.5	24.0

[(1)] Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

[(2)] Dry matter intake (kg/d).

Model evaluation. The CH_4 prediction models were evaluated using two methods. Firstly, the mean square prediction error (**MSEP**), calculated as

$$\text{MSEP} = \sum_{i=1}^{n} (O_i - P_i)^2/n,$$

where n is the total number of observations, O_i is the observed value and P_i is the predicted value. The square root of the MSEP (**RMSEP**), expressed in the same unit as the observed mean or as percentage of the observed mean, gives an estimate of the overall prediction error. Secondly, concordance correlation coefficient analysis (**CCC**; Lin, 1989) was performed, where CCC is calculated as

$$\text{CCC} = r \times C_b,$$

where r is the correlation coefficient providing a measure of precision, and C_b is a bias correction factor providing a measure of accuracy. The C_b variable is calculated as

$$C_b = \frac{2}{[v + 1 / v + \mu^2]},$$

where

$$v = \frac{S_o}{S_p},$$

$$\mu = \frac{\bar{O} - \bar{P}}{(S_o \times S_p)^{0.5}},$$

where v provides a measure of scale shift, while μ provides a measure of location shift, S_o and S_p are the observed and predicted standard deviations, and $\bar{O}$ and $\bar{P}$ are the observed and predicted means.

Cross validation. We performed a random cross validation with 10 splits and 10 iterations as recommended by Rodriguez et al. (2010) for all prediction models in order to calculate the performance parameters of the models (i.e., root mean square error of cross validation; **RMSECV**, and the coefficient of determination of cross validation; **R^2CV**). For each iteration, a model was developed as described above using nine splits of the dataset, and the selected model was subsequently evaluated as described above on the remaining part of the dataset (i.e., one split). The cross validation performance values represent the average of the 10-fold cross validation.

RESULTS

The relationship between individual milk metabolites and methane emission

Milk fatty acids. In the present study, 43 milk fatty acids were identified. The relationships between each individual MFA and CH_4 production, CH_4 yield, and CH_4 intensity are shown in Supplementary Tables S6.2, S6.3, and S6.4, respectively. Several short- and medium-straight even-chain MFA (**SMCFA**; ≤ 16 carbon fatty acids), some odd- and branched-chain fatty acids (**OBCFA**; C13:0, C15:0, *iso* C15:0, and *iso* C17:0), and C20:4n-3 were positively related with CH_4 production, whereas another OBCFA (i.e., C17:0), all C18:1 and C18:2 isomers, and other long-chain fatty acids (> 16 carbon fatty acids) were negatively related to CH_4 production. The SMCFA C16:0 was positively related with CH_4 yield, similar to some OBCFA and several long-chain fatty acids, whereas the SMCFA C4:0, and all C18:1, C18:2, and C18:3 isomers were negatively related to CH_4 yield. Furthermore, many SMCFA, OBCFA, and long-chain fatty acids were positively related to CH_4 intensity, whereas mostly C18:1, C18:2, and C18:3 isomers were negatively related to CH_4 intensity. The relationships between each MFA and CH_4 intensity including FPCM as a covariate are shown in Supplementary Table S6.4. Including FPCM as a covariate in the regression model resulted in several changes. Many MFA remained significantly related to CH_4 intensity, whereas the relationship between CH_4 intensity and two MFA (i.e., C4:0 and C22:5n-3) disappeared. In total, 8 relationships appeared or strengthened, including C6:0, C8:0, C17:1 *cis*-9, several C18:1 isomers, and C18:2 *cis*-9, *trans*-11, that became significantly related upon including FPCM as covariate.

Volatile metabolites. In the present study, a total of 14 volatile metabolites were identified, including ketones, aldehydes, organic acids, alcohols, esters, and sulfur compounds. The relationships between each individual volatile metabolite and CH_4 production, CH_4 yield, and CH_4 intensity are shown in Supplementary Tables S6.5, S6.6, and S6.7, respectively. The volatile metabolites 1-pentanol, acetone, and hexanal were negatively related to CH_4 production, whereas no positive relationship between volatile metabolites and CH_4 production were observed. Ethyl butanoate and two free fatty acids (butanoic and hexanoic acid) were positively related, whereas hexanal was negatively related to CH_4 yield. Many volatile metabolites were related to CH_4 intensity, with 2-heptanone, ethyl butanoate, and all free fatty acids being positively related to CH_4 intensity, and 1-pentanol, acetone, and dimethyl sulfone being negatively related to CH_4 intensity. The relationships between each volatile metabolite and CH_4 intensity including FPCM as a covariate are shown in Supplementary Table S6.7. Including FPCM as a covariate in the regression model resulted in most volatile metabolites remaining to be related to CH_4 intensity, with a few exceptions. Some relationships disappeared or weakened, such as acetic acid ethyl ester which no longer showed a tendency to be positively related with CH_4 intensity, and the significant positive relationship of ethyl butanoate that became a tendency upon including FPCM as covariate. Another relationship strengthened; the tendency for a negative relationship of hexanal became significant upon including FPCM as covariate.

Non-volatile metabolites. In the present study, 41 ^{1}H-NMR resonances could be assigned either to a non-volatile compound or to a member of a class of non-volatile compounds. The relationships between each individual non-volatile metabolite and CH_4 production, CH_4 yield, and CH_4 intensity are shown in Supplementary Tables S6.8, S6.9, and S6.10, respectively. The non-volatile metabolites acetylcarnitine and UDP-hexose D were negatively related, whereas 11 non-volatile metabolites, including acetate, three *N*-acetylsugars, and succinate, were positively related to CH_4 production. Only one non-volatile metabolite, UDP-hexose C, was negatively related, and no single non-volatile metabolite was positively related to CH_4 yield. Similarly, with respect to CH_4 intensity, only citrate was negative related. In contrast, 14 non-volatile metabolites, including acetate, methylmalonate, and succinate were positively related to CH_4 intensity. The relationships between each non-volatile metabolite and CH_4 intensity including FPCM as a covariate are shown in Supplementary Table S6.10. Including FPCM as a covariate in the regression model resulted in several changes. Many non-volatile metabolites remained significantly related to CH_4 intensity, whereas the relationship between CH_4 intensity and five non-volatile metabolites (including citrate and ethanol) disappeared. Other relationships, however, appeared or strengthened, including acetylcarnitine and the three *N*-acetylsugars C, D, and E that became significantly positively related upon including FPCM as covariate.

Table 6.2. The prediction equations developed for methane production (g/d), yield (g/kg DMI[1]), and intensity (g/kg FPCM[2]) based on milk fatty acids, volatile or non-volatile metabolites alone, or all metabolites combined

	Methane production (g/d)				Methane yield (g/kg DMI)				Methane intensity (g/kg FPCM)			
Metabolites included	Item	Esti-mate	SE	*P*-value	Item	Esti-mate	SE	*P*-value	Item	Esti-mate	SE	*P*-value
MFA[3]	Intercept	522	19.2	< 0.001	Intercept	28.4	0.99	< 0.001	Intercept	13.7	2.46	0.003
	C18:1 *trans*-15 + C18:1 *cis*-11	-98.0	18.91	< 0.001	C18:1 *cis*-12	-18.79	2.586	< 0.001	C4:0	-1.33	0.465	0.005
	C18:2 *cis*-9, *trans*-11	-116.7	21.31	< 0.001	C18:3n-3	-5.44	1.296	< 0.001	*iso* C15:0	32.00	4.183	< 0.001
	C18:3n-3	-80.9	23.59	0.008					C18:3n-3	-4.12	1.108	< 0.001
									C20:1 *cis*-11	-35.44	9.579	< 0.001
									C22:5n-3	27.62	9.292	0.004
V[4]	Intercept	372	15.0	< 0.001	Intercept	22.2	0.53	< 0.001	Intercept	14.4	0.726	< 0.001
	Hexanal	-5.4 $\times 10^{-7}$	1.76 $\times 10^{-7}$	0.003	Ethyl butanoate	1.56 $\times 10^{-7}$	5.250 $\times 10^{-8}$	0.004	2-Heptanone	1.08 $\times 10^{-7}$	2.070 $\times 10^{-8}$	< 0.001
									1-Pentanol	-1.38 $\times 10^{-7}$	3.320 $\times 10^{-8}$	< 0.001
NV[5]	Intercept	420	26.6	< 0.001	No model obtained				Intercept	12.9	0.76	< 0.001
	UDP-hexose D	-1438.8	495.01	0.005					Butyrate	3.52	0.708	< 0.001
	Acetyl-carnitine	-442.4	99.94	0.002								
	Oxalo-acetate	535.7	158.14	< 0.001								
ALL[6]	Intercept	522	19.2	< 0.001	Intercept	28.4	0.99	< 0.001	Intercept	7.7	1.38	< 0.001
	C18:1 *trans*-15 + C18:1 *cis*-11	-98.0	18.91	< 0.001	C18:1 *cis*-12	-18.79	2.586	< 0.001	Acetyl-carnitine	9.93	3.788	0.008
	C18:2 *cis*-9, *trans*-11	-116.7	21.31	< 0.001	C18:3n-3	-5.44	1.296	< 0.001	*iso* C15:0	23.63	4.946	< 0.001
	C18:3n-3	-80.9	23.59	0.008					C18:3n-3	-4.95	1.093	< 0.001
									C20:1 *cis*-11	-34.11	8.999	0.004
									C22:5n-3	31.93	8.768	0.003
									C24:0	37.27	12.425	0.008

Table 6.2. Continued

Metabolites included	Item	Esti-mate	SE	*P*-value	Item	Esti-mate	SE	*P*-value	Item	Esti-mate	SE	*P*-value
									2-Heptanone	5.14 $\times 10^{-8}$	1.500 $\times 10^{-8}$	0.004

(1) Dry matter intake (kg/d).

(2) Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

(3) Milk fatty acids alone as selection variables; in g/100 g FA.

(4) Volatile metabolites alone as selection variables; peak area value (arbitrary unit of quantity).

(5) Non-volatile metabolites alone as selection variables; peak area relative to calibration standard 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP).

(6) All metabolites combined as selection variables.

Table 6.3. The coefficient of determination (R^2) and concordance correlation coefficient (CCC) analysis of the prediction equations and the 10-fold cross validation results

	Overall prediction equations								Cross validation		
Model	Adjusted R^2	RMSEP[1]	RMSEP %[2]	CCC[3]	r[4]	C_b[5]	v[6]	μ[7]	R^2CV	RMSECV[8]	RMSECV (%)[9]
Methane production (g/d)											
MFA[10]	0.51	34	9.6	0.67	0.76	0.88	1.67	-0.019	0.47	41.0	11.5
V[11]	0.08	49	13.6	0.13	0.30	0.42	4.56	-0.148	0.09	51.5	14.3
NV[12]	0.30	45	12.7	0.35	0.48	0.73	2.26	-0.206	0.32	52.6	14.7
ALL[13]	0.51	34	9.6	0.67	0.76	0.88	1.67	-0.019	0.48	40.6	11.4
Methane yield (g/kg DMI[14])											
MFA	0.38	2.0	8.7	0.44	0.53	0.84	1.83	0.000	0.38	2.1	9.2
V	0.07	2.3	10.1	0.07	0.16	0.46	4.08	0.038	0.08	2.3	10.3
NV	n.a.[15]	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
ALL	0.38	2.0	8.7	0.44	0.53	0.84	1.83	0.000	0.39	2.1	9.2
Methane intensity (g/kg FPCM[16])											
MFA	0.75	1.7	11.7	0.75	0.76	0.99	1.14	0.052	0.70	2.0	13.7
V	0.27	2.4	16.3	0.29	0.39	0.73	2.28	0.043	0.29	2.4	16.8
NV	0.20	2.6	17.7	0.14	0.21	0.66	2.65	0.054	0.32	2.8	19.3
ALL	0.74	1.5	10.4	0.80	0.82	0.97	1.24	0.094	0.69	2.0	13.8

[1] Root mean squared error of prediction expressed in the same unit as the observed mean.

[2] Root mean squared error of prediction expressed as a percentage of the observed mean.

[3] Concordance correlation coefficient, where CCC = $r \times C_b$.

[4] Pearson correlation coefficient; a measure of precision.

[5] Bias correction factor; a measure of accuracy.

[6] Scale shift; change in standard deviation between predicted and observed methane emission.

[7] Location shift; if positive under prediction, if negative over prediction.

[8] Root mean squared error of cross validation expressed in the same unit as the observed mean.

[9] Root mean squared error of cross validation expressed as a percentage of the observed mean.

[10] Milk fatty acids alone as selection variables; in g/100 g FA.

[11] Volatile metabolites alone as selection variables; peak area value (arbitrary unit of quantity).

[12] Non-volatile metabolites alone as selection variables; peak area relative to calibration standard 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP).

[13] All metabolites combined as selection variables.

[14] Dry matter intake (kg/d).

Table 6.3. Continued

[15] Not applicable, because no model was obtained.

[16] Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

Prediction models for methane emission

Four sets of test variables were used to develop CH_4 prediction models; (1) MFA alone, (2) volatile metabolites alone, (3) non-volatile metabolites alone, and (4) all three types of metabolites combined. In total, 11 prediction models were obtained; four for CH_4 production, three for CH_4 yield (no model was obtained with non-volatile metabolites only), and four for CH_4 intensity (Table 6.2). The observed and residual (observed minus predicted) versus predicted CH_4 production, CH_4 yield, and CH_4 intensity plots are shown in Figures 6.1, 6.2, and 6.3, respectively. The evaluation results (i.e., adjusted R^2, RMSEP, and CCC analysis) of the 11 obtained CH_4 prediction models are shown in Table 6.3.

Milk fatty acids alone. The RMSEP of the MFA-based CH_4 prediction models was 34 g CH_4/d, 2.0 g CH_4/kg DMI, and 1.7 g CH_4/kg FPCM, respectively. Additionally, the adjusted R^2 and CCC of the MFA-based CH_4 prediction models ranged from 0.38 to 0.75, and from 0.44 to 0.75, respectively, with the prediction model for CH_4 yield performing the worst and the prediction model for CH_4 intensity performing the best. This is also evident by the lower r and C_b values for the MFA-based prediction model for CH_4 yield relatively to the MFA-based prediction model for CH_4 intensity. Although the MFA-based prediction models for CH_4 production and CH_4 intensity were equally precise (i.e., both having a r value of 0.76), the MFA-based prediction model for CH_4 intensity was more accurate (C_b of 0.99 for CH_4 intensity and C_b of 0.88 for CH_4 production). The MFA-based prediction model for CH_4 intensity had the ability to describe more of the observed variation in CH_4 emissions compared with MFA-based prediction models for CH_4 production and CH_4 yield. This is evident by the scale shift values, with the MFA-based prediction models for CH_4 production and CH_4 yield having a scale shift which was clearly higher than 1 ($v > 1.67$), whereas the scale shift of the MFA-based prediction for CH_4 intensity was close to 1 ($v = 1.14$).

Volatile metabolites alone. The RMSEP of the volatile metabolite-based CH_4 prediction models was 49 g CH_4/d, 2.3 g CH_4/kg DMI, and 2.4 g CH_4/kg FPCM. Additionally, the adjusted R^2 and CCC of the volatile metabolite-based CH_4 prediction models ranged from 0.07 to 0.27, and from 0.07 to 0.29, respectively, with the prediction model for CH_4 yield performing the worst and the prediction model for CH_4 intensity performing the best. The precision of these models (i.e., r) followed the same pattern, which was not the case for the accuracy of these models (i.e., C_b). The C_b value was lowest for the volatile metabolite-based prediction model for CH_4 production (0.42) and highest for the volatile metabolite-based prediction model for CH_4 intensity (0.73). Further, all volatile metabolite-based CH_4 prediction models had a scale shift which was clearly higher than 1 ($v > 2.28$), indicating the inability of volatile metabolites alone to predict the range of observed CH_4 emissions.

Non-volatile metabolites alone. No model was obtained for CH_4 yield with non-volatiles metabolites alone. The significance level for a variable to enter the model was 0.01, whereas the significance level of the strongest correlation between a non-volatile metabolite (i.e., UDP-hexose C) and CH_4 yield was 0.035. The RMSEP of the non-volatile metabolite-based CH_4 prediction models was 45 g CH_4/d and 2.6 g CH_4/kg FPCM. Additionally, the adjusted R^2 was 0.30 and 0.20, and the CCC was 0.35 and 0.14 for the non-volatile metabolite-based prediction models for CH_4 production and CH_4 intensity, respectively. Contrary to what was observed for

MFA and volatile metabolites alone, the non-volatile metabolite-based prediction model for CH_4 production performed better than the non-volatile metabolite-based prediction model for CH_4 intensity. Both non-volatile metabolite-based CH_4 prediction models had a scale shift which was clearly higher than 1 ($v > 2.26$), indicating the inability of non-volatile metabolites alone to predict the range of observed CH_4 emissions.

All metabolites combined. When combining the three types of milk metabolites, the RMSEP of the CH_4 prediction models was 34 g CH_4/d, 2.0 g CH_4/kg DMI, and 1.5 g CH_4/kg FPCM. Additionally, the adjusted R^2 and CCC of the CH_4 prediction models including all three types of metabolites ranged from 0.38 to 0.74, and from 0.44 to 0.80, respectively, with the prediction model for CH_4 yield performing the worst and the prediction model for CH_4 intensity performing the best. A similar pattern was observed for the precision (r), accuracy (C_b), and the scale shift (v) of the CH_4 prediction models combining the three types of metabolites. The prediction models using the combination of all three types of milk metabolites performed better than the prediction models with volatile metabolites alone and non-volatile metabolites alone. For both CH_4 production and CH_4 yield, only MFA were selected in the prediction models, resulting in CH_4 prediction models identical to the MFA-based CH_4 prediction models. For CH_4 intensity all three types of milk metabolites were selected in the prediction model; five MFA, one volatile metabolite, and one non-volatile metabolite.

Cross validation. The results of the internal cross validation of all CH_4 prediction models are also shown in Table 6.3. Additionally, Supplementary Table S6.11 shows the MFA, volatile metabolites, and non-volatile metabolites that were included in the prediction models in the cross validation, and whether or not these milk metabolites were also part of the best overall prediction models (Table 6.2). The R^2CV and the RMSECV (%) of the MFA-based CH_4 prediction models ranged from 0.38 to 0.70 and from 9.2 to 13.7, respectively. Further, the R^2CV and the RMSECV (%) of the volatile metabolite-based CH_4 prediction models ranged from 0.08 to 0.29 and from 10.3 to 16.8, respectively. The R^2CV was 0.32 and 0.32, and the RMSECV (%) was 14.7 and 19.3 for the non-volatile metabolite-based prediction models for CH_4 production and CH_4 intensity, respectively. For the CH_4 prediction models combining all three types of milk metabolites, the R^2CV and the RMSECV (%) ranged from 0.39 to 0.69 and from 9.2 to 13.8, respectively.

DISCUSSION

The relationship between individual milk metabolites and methane emission

Milk fatty acids. Van Gastelen and Dijkstra (2016) reviewed studies that investigated the predictive power of MFA composition for CH_4 emission. In line with this review, in the present study several SMCFA were positively associated with CH_4 emission. In general these SMCFA remained related to CH_4 intensity, or the relationship appeared or strengthened, upon including FPCM as covariate. These positive relationships are the result of the *de novo* synthesis of these MFA in the mammary gland mainly from acetate and butyrate produced in the rumen (Bauman and Griinari, 2003), which are both positively associated with CH_4 emission (Ellis et al., 2008). The main exception is C4:0. The observed negative relationship between C4:0 and CH_4 emission in the present study was also observed by Dijkstra et al. (2011) and Van Lingen et

al. (2014). The significant negative relationship between C4:0 and CH_4 intensity disappeared ($P > 0.10$) upon including FPCM as covariate, which may indicate a dilution effect with C4:0 increasing with decreasing FPCM.

The *iso* OBCFA were often positively associated with CH_4 emission in the present study and remained positively related with CH_4 intensity upon including FPCM as covariate, which is in agreement with *iso* OBCFA being generally more abundant in fibrolytic bacteria (Vlaeminck et al., 2006). The *anteiso* OBCFA are generally more abundant in amylolytic bacteria and thus expected to be negatively associated with CH_4 emission (Vlaeminck et al., 2006). This was observed for *anteiso* C17:0 and CH_4 production, but *anteiso* C15:0 was positively associated with CH_4 intensity. The latter MFA remained positively related to CH_4 intensity upon including FPCM as covariate. A high level of ruminal propionate is associated with low CH_4 production, and propionate is a substrate for *de novo* synthesis of C15:0 and C17:0. Hence, both C15:0 and C17:0 are hypothesized to be negatively associated with CH_4 emissions (Vlaeminck and Fievez, 2005). In the present study, only C17:0 was negatively associated with CH_4 production, whereas C15:0 was positively associated with all units of CH_4 emission and C17:0 was positively associated with CH_4 yield. The reason behind the positive associations is not completely clear to us, but they are in agreement with other studies, such as Chilliard et al. (2009), Dijkstra et al. (2011), and Van Lingen et al. (2014), although inconsistent with the findings of Rico et al. (2016). Additionally, it is unclear why C15:0 and C17:0 are differently related to CH_4 emission, despite their similar synthesis pathways.

The negative relationships found in the present study between C18:1, C18:2, and C18:3 isomers in milk and CH_4 emissions are in general agreement with others (Van Lingen et al., 2014; Rico et al., 2016), and can be explained by dietary unsaturated fatty acids and their biohydrogenation products (Van Gastelen and Dijkstra, 2016). The relationship between CH_4 intensity and several C18:1 isomers as well as C18:2 *cis*-9, *trans*-11 strengthened or appeared after correcting for FPCM yield. This suggests that the relationship between these MFA and FPCM can hamper the direct association between MFA and CH_4 intensity. The associations found in the present study between CH_4 emissions and long-chain fatty acids, which derive from absorption from the digestive tract and body fat mobilization (Bauman and Griinari, 2003), have been reported before (i.e., Chilliard et al., 2009; Rico et al., 2016; Van Gastelen et al., 2017). The individual relationships found in the present study between CH_4 emission and the long-chain fatty acids with more than 20 carbons were generally unaffected when including FPCM as a covariate. Additionally, these long-chain fatty acids were also included in the CH_4 prediction models. This together suggests that these MFA are important in terms of CH_4 prediction.

Volatile metabolites. In contrast to Antunes-Fernandes et al. (2016), in the present study, many volatile metabolites in milk were related to CH_4 emission and the relationships found between the volatile metabolites and CH_4 intensity were not only the result of the relationship between the volatile metabolites in milk and FPCM. The lack of relationships found by Antunes-Fernandes might be the result of the limited variation in dietary treatments used, which was the exchange of fiber-rich grass silage with starch-rich corn silage. As shown by Hettinga et al. (2008), the volatile composition of milk was affected by supplementing diets with specific byproducts (including onions and cabbage) but was not affected by variation in the

starch to fiber content of the diet, even though the latter manipulation has an influence on ruminal fermentation and CH_4 emission (Hassanat et al., 2013; Van Gastelen et al., 2015). The present study involved a wide variety of dietary treatments, including different qualities of forage, which are known to effect the volatile composition of milk (Thomson et al., 2005), ruminal fermentation, and CH_4 emission (Warner et al., 2016).

In the present study, acetone was negatively related with both CH_4 production and CH_4 intensity. Acetone is a ketone body which can be used to identify cows with negative energy balance and subclinical ketosis (Andersson and Emanuelson, 1985). Gravert et al. (1991) reported that the quality of grass or maize silage was negatively related to milk acetone, and it has been suggested that, in general, silage feeding or the content of butyric acid in silage may affect milk acetone (Andersson and Emanuelson, 1985; Andersson and Lundström, 1985). This suggests that milk acetone can be affected by the same dietary factors that can impact CH_4 emissions, explaining the negative relationship found in the present study.

Kalač (2011) reported that the occurrence of both acids and alcohols in silage result in the formation of various ethyl esters. There is limited information available on the transfer efficiency of both acids and esters from silage to milk, but the dietary treatments could have affected the relationships found. However, esters can also be formed within the mammary gland, from esterification of short-chain alcohols and free fatty acids (Toso et al., 2002), and they can indicate bacterial action (Hettinga et al., 2009). In the present study, both volatile esters (ethyl acetate and ethyl butanoate) tended to be or were positively related with CH_4 intensity, but including FPCM as a covariate resulted in the disappearance or weakening of these relationships. This suggests that the positive relationships with CH_4 intensity were due to a relationship between FPCM and both volatile esters.

In the present study, 1-pentanol was negatively associated with CH_4 production and CH_4 intensity. According to Moio et al. (1993), primary alcohols are formed by reduction of their respective aldehyde. Consequently, 1-pentanol is formed by the reduction of pentanal, which was not identified in the present study. Based on Villeneuve et al. (2013), it seems likely that the content of 1-pentanol in milk reflects the content of its respective aldehyde, pentanal. Straight-chain aldehydes, such as pentanal and hexanal, can derive from lipid degradation (Moio et al., 1993). Dietary lipids are negatively associated with CH_4 emissions (Grainger and Beauchemin, 2011), potentially explaining the negative relationships found between CH_4 emission and both 1-pentanol and hexanal in the present study. Further, dimethyl sulfone was negatively associated with CH_4 intensity. In the rumen, dimethyl sulfide is derived from the catabolism of sulfur amino acids, particularly methionine (Taylor and Kiene, 1989). Dimethyl sulfide is subsequently oxidized to dimethyl sulfone, which can be transferred to milk (Villeneuve et al., 2013). This suggests that the relationship between CH_4 intensity and dimethyl sulfone could be the result of the dietary protein content, although the effect of dietary protein content on CH_4 emissions is variable in the literature (Ellis et al., 2009, Reynolds et al., 2010).

We also identified four volatile free fatty acids (**FFA**) in the present study, all positively associated with CH_4 yield and CH_4 intensity, but not with CH_4 production. The concentration of FFA in milk is generally low and can be the result of incomplete esterification in the mammary gland before lipid secretion (Marsili et al., 2003) or spontaneous lipolysis (Chazal et al., 1987).

The latter study also reported higher FFA concentrations in milk from cows fully fed on good quality grass silage compared with cows fully fed on good quality hay. Additionally, Chazal and Chilliard (1986) observed that milk from cows fed poor quality grass silage had higher FFA levels than milk from cows supplemented with corn silage. Moreover, Thomson et al. (2005) reported that FFA concentrations in milk were highest in summer when the quality of the pasture declines. This together suggests that the positive association between volatile FFA in milk and both CH_4 yield and CH_4 intensity might be the results of dietary composition and quality, which affect both the composition of volatile metabolites in milk and enteric CH_4 production.

Non-volatile metabolites. Similar to Antunes-Fernandes et al. (2016), non-volatile metabolites were generally better correlated with CH_4 intensity than with CH_4 yield. Antunes-Fernandes et al. (2016) reported that the positive relationship between CH_4 intensity and the non-volatile metabolites acetoacetate, creatinine, ethanol, formate, methylmalonate, and *N*-acetylsugar A were due to the relationship between these non-volatile metabolites and FPCM. This was also observed for creatinine and ethanol in the present study, as well as for the metabolites betaine, citrate, *N*-acetylsugar B, and sugar A. This suggest that these metabolites have no clear relationships with the ruminal CH_4 emission metabolism, but rather are related to changes in milk yield or metabolic processes involved in milk synthesis. In contrast to Antunes-Fernandes et al. (2016), the three non-volatile metabolites acetoacetate, methylmalonate, and *N*-acetylsugar A remained related to CH_4 intensity upon including FPCM as a covariate. Acetoacetate is a ketone body and, as described above, can be positively associated with fiber-rich diets and subsequently ketogenic VFA (Van Knegsel et al., 2007), explaining the positive relationship found between acetoacetate and CH_4 intensity. The concentration of methylmalonate in milk has been associated with dietary composition (Bauman and Griinari, 2001). When high grain or low forage diets are fed, the ruminal production of vitamin B_{12} decreases, whereas the production of propionate increases. This results in the accumulation of methylmalonate in the liver and subsequently, by transport via the circulatory system, in elevated methylmalonate supply to the mammary gland and increased concentration in milk (Bauman and Griinari, 2001). High grain or low forage diets are also associated with decreased CH_4 emissions, and hence a negative relationship between methylmalonate and CH_4 emission would be expected. In the present study, however, methylmalonate was positively related with CH_4 intensity. The latter may be explained by the absence of a negative relationship between ruminal propionate and CH_4 emissions in some of the studies of which the data was used for the present analysis (e.g., Hatew et al., 2016, Van Gastelen et al., 2015).

N-acetylsugars are intermediates of biological pathways that occur in cell cytosol (Lu et al., 2013). *N*-acetylsugars C, D and E were significantly and positively related with CH_4 intensity only when including FPCM as a covariate. The results of the present study suggest that *N*-acetylsugars are related to the ruminal CH_4 production pathway. According to Lu et al. (2015), a higher concentration of *N*-acetylsugars could indicate leakage of cellular components to milk or higher permeability of the cell membrane in the epithelial cells in the mammary gland. Both can subsequently be associated with the differences in the epithelial cell membrane stability. Tian et al. (2016) found lower concentrations of *N*-acetylsugars in milk of cows experiencing heat stress, and Antunes-Fernandes et al. (2016) found some *N*-acetylsugars tending to be negatively

associated with CH_4 yield. However, it is unclear how differences in dietary composition could have changed the epithelial cell membrane stability in the mammary gland and how this relates to CH_4 emissions, warranting more research.

Antunes-Fernandes et al. (2016) showed that citrate and UDP-hexose B were both negatively related to CH_4 intensity, potentially reflecting decreased metabolic activity in the mammary gland with increased CH_4 intensity as citrate is an intermediate of the Kreb-cycle (Bremer and Davis, 1974) and UDP-hexose B is an intermediate of lactose metabolism (Cant et al., 2002). In the present study, citrate was also negatively associated with CH_4 intensity. However, this relationship disappeared upon inclusion of FPCM as a covariate. This suggests that changes in milk citrate may be related to the energy metabolism of the mammary gland, as previously suggested by Faulkner and Peaker (1982), which is not necessarily related to changes in CH_4 intensity. Furthermore, UDP-hexose B was not related to CH_4 intensity in the present study, which is in disagreement with Antunes-Fernandes et al. (2016). The other UDP-hexoses were negatively related to CH_4 production (i.e., UDP-hexose D), CH_4 yield (i.e., UDP-hexose C) and CH_4 intensity (i.e., UDP-hexose D upon including FPCM as a covariate). The negative relationship with CH_4 production is surprising, because UDP-hexoses are intermediates of lactose synthesis (Cant et al., 2002), and milk yield is controlled by the synthesis of lactose. Increased milk yield is often associated with increased feed intake and it is consistently reported in literature that increased feed intake is positively associated with CH_4 production (Hristov et al., 2013). Subsequently, a positive association between UDP-hexoses and CH_4 production would be expected. The negative association between UDP-hexoses and CH_4 yield can probably be explained by increased feed intake and the associated decreased ruminal retention time of starch, which may result in increased post-ruminal digestion and subsequently glucose absorption (Rius et al., 2010) and conversion to UDP-hexoses in the mammary gland.

Acetate was positively associated with CH_4 emission, and remained related to CH_4 intensity upon including FPCM as a covariate. This is in agreement with ruminal acetate production being positively associated with CH_4 emission (Ellis et al., 2008). Furthermore, Krause and Oetzel (2005) demonstrated that ruminal succinate is usually only found at very low levels in the rumen, but that the concentration increased during a subacute ruminal acidosis. In general, diets with a high starch content and a low fiber content are associated with decreased ruminal pH and CH_4 emission (Beauchemin et al., 2008). Hence, a negative relationship between ruminal succinate and CH_4 emission would be expected. However, we found a positive relationship between succinate in milk and CH_4 emission. This suggests that succinate in milk does probably not directly reflect ruminal succinate levels.

The decrease of acetylcarnitine in milk with increasing CH_4 production and CH_4 intensity (acetylcarnitine became negative related to CH_4 intensity upon including FPCM as a covariate) is in agreement with the results of the SMCFA in milk. It has been shown that acetylcarnitine reflects the inhibition of *de novo* fatty acid synthesis from acetate in the mammary gland (Erfle et al., 1970). *De novo* fatty acid synthesis in the mammary gland is inhibited by specific *trans* unsaturated fatty acids, which are formed during ruminal biohydrogenation of dietary unsaturated fatty acids (Bauman and Griinari, 2003). Certain dietary strategies (including low-fiber diets and high-concentrate diets) alter the rumen environment, lowering the ruminal pH

and subsequently increasing the *trans* unsaturated fatty acids being formed from ruminal biohydrogenation. Both dietary unsaturated fatty acids and lower ruminal pH are also associated with reduction in CH_4 emission, explaining the negative relationship between acetylcarnitine and CH_4 emission.

Hippurate was positively associated with CH_4 production and intensity in the present study. Boudonck et al. (2009) showed that the concentration of hippurate was lower in milk of cows receiving organic diets (mainly forage-based) than in milk of cows receiving conventional diets (mainly concentrate-based). This suggest that increased forage content in the diet, which is accompanied by increased dietary fiber, increases hippurate content in milk. This might explain the relationship found in the present study, because dietary forage content, as well as dietary fiber content, is positively associated with CH_4 emission (Beauchemin et al., 2008).

Overall, these results indicate that, next to MFA, both volatile and non-volatile metabolites in milk are often associated with CH_4 emissions. These relationships are most likely the result of changes in dietary composition that affect not only enteric CH_4 production, but also the profile of volatile and non-volatile metabolites in milk. This illustrates that these milk metabolites might provide useful information increasing our understanding of CH_4 emission of dairy cows.

Prediction models for methane emission

Milk fatty acids. The prediction potential of MFA varies between studies. The adjusted R^2 values for the MFA-based prediction models for both CH_4 production and CH_4 yield from the present study are lower than the ones reported by other studies (Chilliard et al. 2009; Dijkstra et al., 2011; Rico et al. 2016). In contrast, the adjusted R^2 values for the MFA-based prediction models for CH_4 intensity of the present study are higher than those reported in literature (i.e., Van Lingen et al. 2014). The scale shift results of the present study indicate that MFA are able to describe more of the observed variation in CH_4 intensity than of the observed variation in CH_4 production and CH_4 yield. It is known that MFA are related to ruminal CH_4 production pathways (Chilliard et al., 2009; Ellis et al., 2008). The MFA can predict CH_4 intensity better than CH_4 production and CH_4 yield. According to Dehareng et al. (2012), this might be due to CH_4 intensity taking milk yield into account, which is directly associated with enteric CH_4 production by cows and reflected by the MFA profile because of possible dilution effects. The results of the present study indeed show that some of the MFA are associated with CH_4 intensity due to their relationship with FPCM (e.g., C4:0). However, this is not the case for all MFA that are important for the prediction of CH_4 intensity (such as *iso* C15:0).

Volatile metabolites. The CH_4 prediction potential of volatile metabolites alone appears low and is considerably less promising compared with MFA. Although the prediction potential of the volatile metabolites for CH_4 production in this study is higher than Van Gastelen et al. (2017) in which no model could be obtained for CH_4 production, the adjusted R^2 and CCC values of the volatile metabolite-based prediction models for CH_4 yield and CH_4 intensity are lower than the ones reported by Van Gastelen et al. (2017).

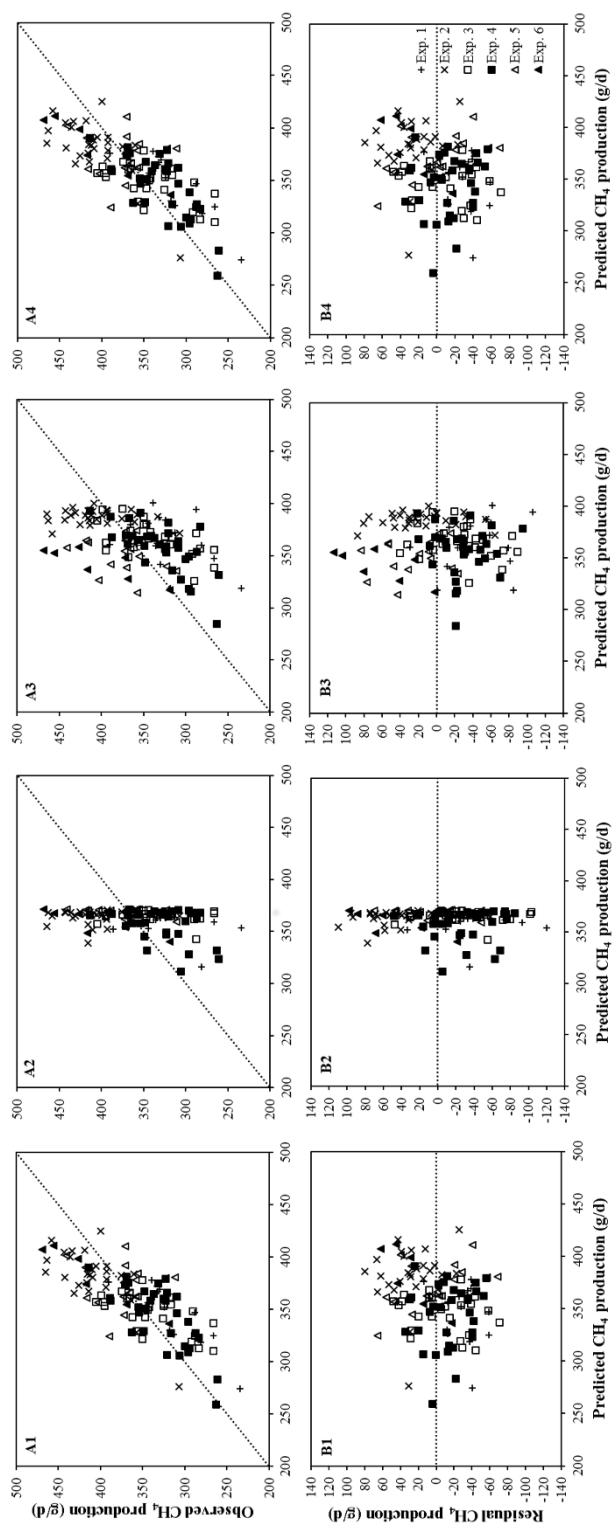

Figure 6.1. (A) Observed and predicted methane production (g/d), and (B) residual (i.e., observed − predicted) methane production (g/d), from the regression analyses based on milk fatty acids (g/100 g fatty acids) alone (1), volatile metabolites alone (2), non-volatile metabolites alone (3), and all metabolites combined (4). The slope of residuals regressed on predicted values did not differ significantly from zero. The different symbols identify the six individual experiments described in the materials and methods.

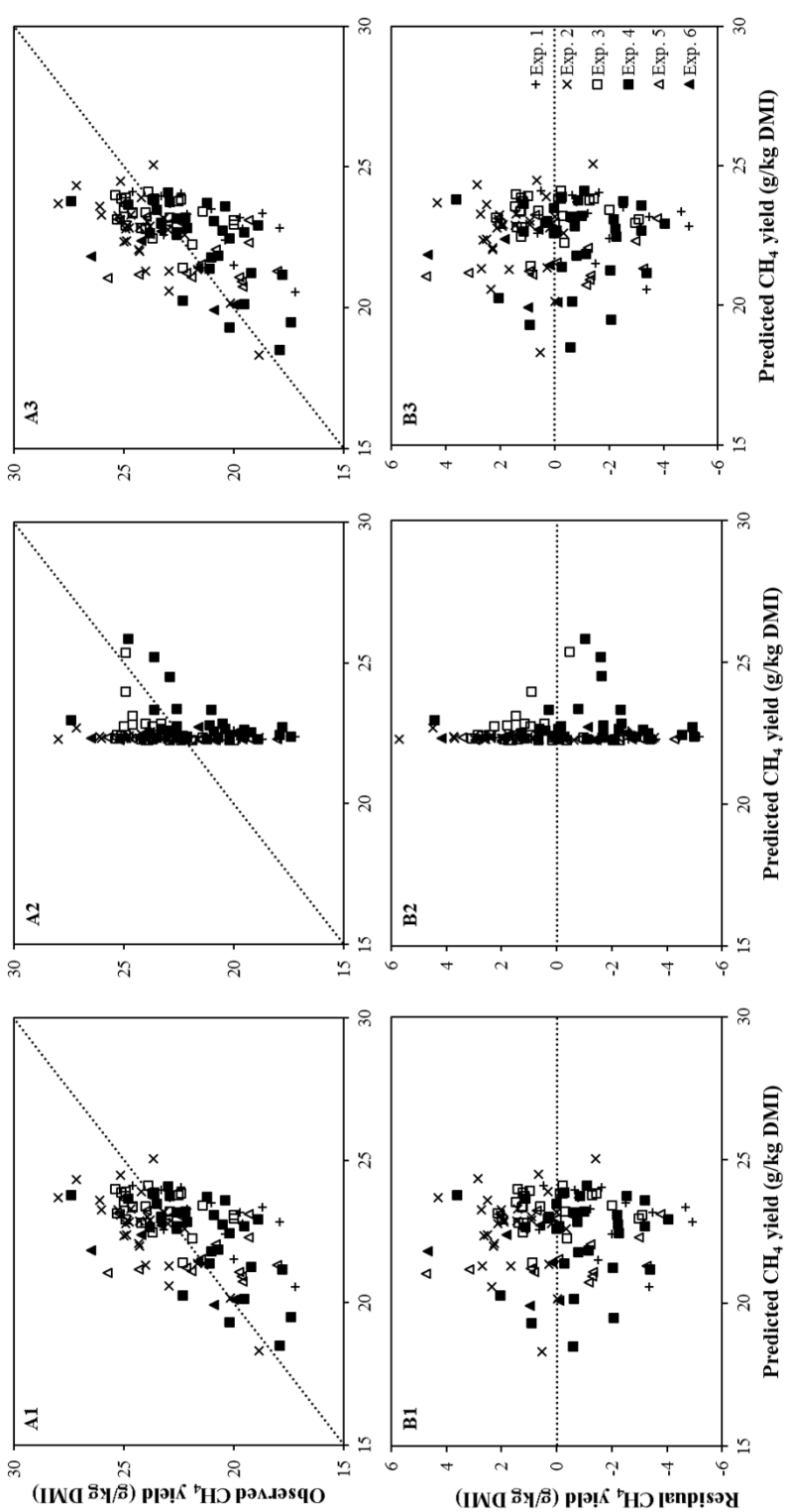

Figure 6.2. (A) Observed and predicted methane yield (g/kg DMI), and (B) residual (i.e., observed − predicted) methane yield (g/kg DMI), from the regression analyses based on milk fatty acids (g/100 g fatty acids) alone (1), volatile metabolites alone (2), and all metabolites combined (3). The slope of residuals regressed on predicted values did not differ significantly from zero. The different symbols identify the six individual experiments described in the materials and methods.

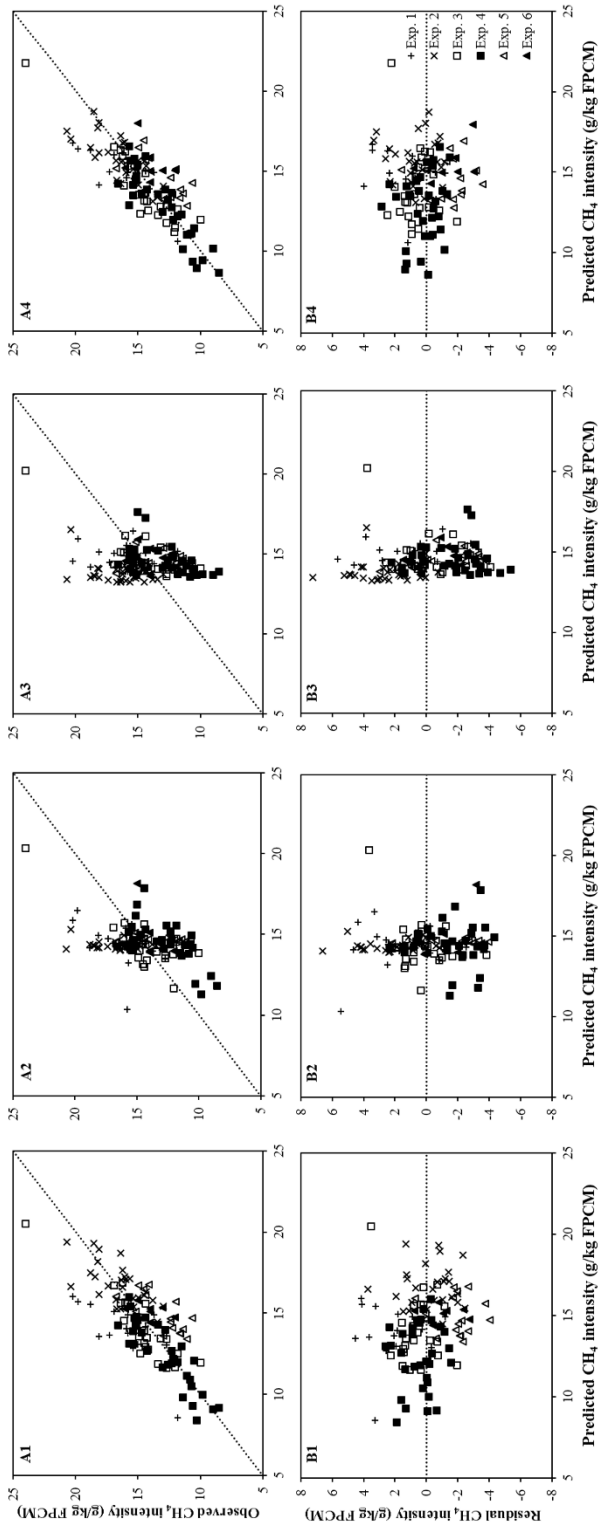

Figure 6.3. (A) Observed and predicted methane intensity (g/kg FPCM), and (B) residual (i.e., observed − predicted) methane intensity (g/kg FPCM), from the regression analyses based on milk fatty acids (g/100 g fatty acids) alone (1), volatile metabolites alone (2), non-volatile metabolites alone (3), and all metabolites combined (4). The slope of residuals regressed on predicted values did not differ significantly from zero. The different symbols identify the six individual experiments described in the materials and methods.

Similar to Van Gastelen et al. (2017), the potential of volatile metabolites to predict CH_4 intensity in the present study was greater than their potential to predict CH_4 production and CH_4 yield, which is evidenced by the higher adjusted R^2 and CCC values and lower scale shift. However, even for CH_4 intensity, the large scale shift (> 2.28) shows that the variation in predicted CH_4 emissions was considerably smaller than that in observed CH_4 emission. This is illustrated in Figures 6.1, 6.2, and 6.3, and indicates the inability of volatile metabolites alone to predict the range of observed CH_4 emissions.

Based on 1 experiment with 4 dietary treatments, Van Gastelen et al. (2107) concluded that volatile metabolites alone hold potential to predict CH_4 intensity, which might be the result of the relationship between volatile metabolites in milk and FPCM yield (Antunes-Fernandes et al., 2016). This is not supported by the results of the present study. First, the prediction potential of volatile metabolites for CH_4 intensity is low. Second, most volatile metabolites remained significantly related to CH_4 intensity upon including FPCM as a covariate. We therefore propose that, with a wide range of forage-based diets with a variety of quantity and quality of grass, grass silage, and corn silage, volatile metabolites in milk hold little potential to predict CH_4 emissions, despite the relationships found between individual volatile metabolites and CH_4 emissions.

Non-volatiles metabolites. The adjusted R^2 and CCC values reported in this study for non-volatile metabolite-based CH_4 prediction models were considerably lower than those reported by Van Gastelen et al. (2017). Our results suggest that non-volatile metabolites in milk have low CH_4 prediction potential. For CH_4 yield no model could be derived at all, which was to be expected given the low number of relationships (i.e., significant and tendency) between individual non-volatile metabolites and CH_4 yield. The variation in the predicted CH_4 emissions was considerably smaller than that in observed CH_4 emissions, as evidenced by the large scale shift (> 2.26). This is visualized in Figures 6.1 and 6.3, and suggests that non-volatile metabolites lack the ability to predict the range of CH_4 emissions observed.

Van Gastelen et al. (2017) concluded, based on 1 experiment with 4 dietary treatments, that non-volatile metabolites hold potential to predict CH_4 intensity, which could largely be explained by the relationship between the non-volatile metabolites in milk and FPCM yield as observed by Antunes-Fernandes et al. (2016). This is not supported by the results of the present study. We therefore propose that, with a wide range of forage-based diets with a variety of quantity and quality of grass, grass silage, and corn silage, non-volatile metabolites in milk hold little potential to predict CH_4 emissions despite the significant relationships found between individual non-volatile metabolites and CH_4 emissions.

All metabolites combined. No single proxy accurately predicts CH_4 emission, and combinations of two or more proxies are likely to be a better solution to predict CH_4 emission (reviewed by Negussie et al., 2017). In comparison with MFA alone, combining the three types of milk metabolites did not improve the potential to predict CH_4 production and CH_4 yield. The prediction models were actually identical to the ones obtained when selecting MFA alone. Also in the 10-fold cross-validation, volatile and non-volatile metabolites were rarely included in the prediction models (Supplementary Table S6.11). These results clearly show that combining MFA with both volatile and non-volatile metabolites has no added value in terms of CH_4 production and CH_4 yield prediction potential relative to MFA alone.

Similarly, for CH_4 intensity, combining MFA with volatile and non-volatile metabolites hardly improved prediction potential. Five MFA were included in the CH_4 intensity prediction model, of which four were identical to the MFA included in the MFA-based prediction model for CH_4 intensity. Despite the inclusion of one volatile metabolite (2-heptanone) and one non-volatile metabolite (acetylcarnitine) in the combined model, the adjusted R^2 was marginally lower (0.74) than that of the MFA-based prediction model for CH_4 intensity (0.75). The CCC value of the prediction model for CH_4 intensity combining the three metabolites was slightly higher than the one reported for the MFA-based CH_4 intensity prediction model (0.80 and 0.75, respectively), which was mainly the result of increased precision (*r*).

In terms of the 10-fold cross validation, MFA alone and the combination of the three types of milk metabolites performed equally well, having similar R^2CV and RMSECV values. When considering the metabolites that were included in the prediction models of the 10-fold cross validation (Supplementary Table S6.11), it appears that the CH_4 prediction models combining the three types of milk metabolites are less robust than the MFA-based CH_4 prediction models, especially for CH_4 intensity. Variation in the metabolites that were included in the cross validation was smaller for the MFA alone model (12 metabolites) than for the three types of metabolites combined (22 metabolites). Moreover, the MFA included in the best overall MFA-based prediction model for CH_4 intensity were included at least two times in the 10-fold cross validation. Contrary, two of the metabolites in the best overall prediction model for CH_4 intensity combining the three types of milk metabolites were included only once (i.e., acetylcarnitine) or not at all (i.e., C24:0) in the cross validation. The cross-validation results motivated us to develop a second best overall prediction model for CH_4 intensity, but without acetylcarnitine and C24:0. This resulted in a CH_4 intensity prediction model with an adjusted R^2 of 0.72, a RMSEP of 1.6 g CH_4/kg FPCM, a CCC of 0.78, and a scale shift of 1.23. This illustrates that the prediction potential for CH_4 intensity hardly differed when excluding acetylcarnitine and C24:0, which suggests that the prediction model for CH_4 intensity combining the three types of metabolites is not robust.

Overall, our results indicate that combining MFA with milk volatile metabolites and non-volatile metabolites does not improve the CH_4 prediction potential relative to MFA alone. This is in agreement with Van Gastelen et al. (2017) for CH_4 production and CH_4 yield. However, Van Gastelen et al. (2017) reported a considerable improvement in the prediction potential when combining MFA with volatile and non-volatile metabolites. The difference between this study and Van Gastelen et al. (2017) for CH_4 intensity might be explained by the differences in the dataset used by both studies. Although both studies only involved forage-based diets, the present study involved more observations (123 vs 29, respectively) and a larger variation in dietary treatments, CH_4 emissions, DMI, and FPCM.

Our analyses suggest that the relationship between CH_4 emission and both volatile and non-volatile metabolites is largely driven by dietary composition and rumen fermentation. When investigating this relationship within a single experiment, with a small range of dietary treatments, these individual relationships assure sufficient prediction potential, as evidenced by Van Gastelen et al. (2017) for CH_4 intensity. However, as illustrated in the present study, upon combining data of multiple experiments that represent a wide range of dietary treatments, the

diet driven individual relationships between CH_4 emission and both volatile and non-volatile metabolites are still present, but the prediction potential decreases considerably. This suggests that the wider range of dietary treatments hampers the CH_4 prediction potential of volatile and non-volatile metabolites. In contrast to volatile and non-volatile metabolites, MFA are more directly related to the ruminal CH_4 pathways (rumen microbial origin) and retain their prediction potential despite a wider range of dietary treatments, suggesting that MFA profile represents a more robust indicator for CH_4 emission of dairy cows.

It is important to note though, that this study was based upon 6 experiments with forage-based diets only (forage varied between 70 and 85 g/100 g diet DM). Hence, the diets represent only a relative narrow range of forage to concentrate ratios. Additionally, milk production of the cows did not exceed 35 kg/d, and all cows were restricted in their feed intake to ensure similar feed intake between treatments, thus avoiding confounding effects of DMI on CH_4 production. The results of the relationships between CH_4 emission and the three types of milk metabolites might be different, when more experiments would be included, involving more individual observations, and with more variety in dietary composition, feed intake, and milk yield.

Potential limitations aside, based on the results of the present study, it can be concluded that MFA have the greatest potential to predict CH_4 emission of dairy cows compared to milk volatile and non-volatile metabolites. Negussie et al. (2017) assessed several existing potential proxies for CH_4 emissions of dairy cows, including proxies related to (1) feed intake and feeding behavior, (2) rumen function, metabolites, and microbiome, (3) milk production and composition, (4) hindgut and feces, and (5) measurements at the level of the whole animal (e.g., body condition score, body weight, and lactation stage). The authors of that review indicated that the accuracy of MFA to estimate CH_4 emission was moderate to high, which is considerably higher than that of rumen-related variables, major milk components such as fat, lactose, and protein, and most variables of the whole animal, but similarly accurate as body weight, digestibility, and milk yield (Negussie et al., 2017). Only feed intake (both alone and in combination with dietary composition) and milk infrared spectroscopy scored higher in terms of accuracy to estimate CH_4 emission (Negussie et al., 2017). Because it is still a major challenge to measure feed intake in practice and because the current number of studies relating milk infrared spectroscopy with CH_4 emission is limited, MFA remain an interesting proxy for CH_4 emission of dairy cows.

CONCLUSIONS

Changes in concentrations of individual milk metabolites (i.e., MFA, volatile metabolites, and non-volatile metabolites) can be related to the ruminal CH_4 production pathway. These relationships are largely diet-driven, i.e., diet composition, intake, and passage affect both ruminal CH_4 production and the milk metabolites. Some of the relationships between individual milk metabolites and CH_4 intensity, however, were partly dependent on milk production (FPCM). Furthermore, the CH_4 prediction potential of both volatile metabolites alone and non-volatile metabolites alone is low, independent of the unit of CH_4 emission. The CH_4 prediction potential of MFA alone depended greatly on the unit in which CH_4 emissions was expressed. The potential was lowest for CH_4 yield, intermediate for CH_4 production, and highest for CH_4

intensity. This study also demonstrates that, relative to MFA alone, CH_4 prediction potential does not increase when combining MFA with volatile and non-volatile metabolites, in particular for CH_4 production and CH_4 yield. Volatile and non-volatile metabolites in milk contain information that may increase our understanding of enteric CH_4 production of dairy cows, but it is not worthwhile to determine the volatile and non-volatile metabolites in milk in order to estimate CH_4 emission of dairy cows. Milk fatty acids have moderate potential to predict CH_4 emission of dairy cattle fed forage-based diets, and the models can aid in the effort to understand and mitigate CH_4 emissions of dairy cows.

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SUPPORTING INFORMATION

Supplementary Table S6.1. Descriptive statistics of milk fatty acids, volatile metabolites, and non-volatile metabolites (n=123)

Item	Mean	Median	SD	Minimum	Maximum
Fatty acid (g/100 g FA)					
C4:0	3.5	3.5	0.30	2.5	4.3
C6:0	2.1	2.1	0.20	1.5	2.5
C8:0	1.1	1.1	0.16	0.6	1.4
C10:0	2.4	2.4	0.48	1.1	3.4
C12:0	2.8	2.7	0.62	1.3	4.4
C13:0	0.07	0.06	0.001	0.00	0.16
C14:0	10.3	10.4	1.29	6.7	13.2
iso C14:0	0.08	0.08	0.015	0.05	0.13
C14:1 *cis*-9	1.00	0.96	0.249	0.54	1.95
C15:0	0.97	0.97	0.159	0.53	1.47
iso C15:0	0.23	0.22	0.038	0.13	0.37
anteiso C15:0	0.41	0.40	0.062	0.28	0.62
C16:0	31.9	31.8	3.38	24.6	42.3
iso C16:0	0.18	0.18	0.032	0.13	0.34
C16:1 *trans*-9	0.22	0.21	0.037	0.14	0.35
C16:1 *cis*-9	1.9	1.9	0.36	1.0	3.0
C17:0	0.66	0.66	0.100	0.47	0.95
iso C17:0	0.40	0.39	0.063	0.25	0.63
anteiso C17:0	0.43	0.42	0.059	0.32	0.61
C17:1 *cis*-9	0.32	0.31	0.091	0.15	0.69
C18:0	9.6	9.8	1.63	5.0	13.1
C18:1 *cis*-9[(1)]	21.1	20.7	3.77	12.3	29.9
C18:1 *cis*-12	0.18	0.14	0.080	0.07	0.47
C18:1 *cis*-13	0.13	0.13	0.033	0.07	0.25
C18:1 *trans*-6	0.20	0.19	0.057	0.08	0.42
C18:1 *trans*-9	0.15	0.14	0.030	0.08	0.25
C18:1 *trans*-10	0.19	0.15	0.102	0.10	0.65
C18:1 *trans*-11	0.91	0.88	0.253	0.30	2.18
C18:1 *trans*-15 + C18:1 *cis*-11	0.76	0.76	0.181	0.33	1.23
C18:2 *cis*-9, *trans*-11	0.43	0.40	0.132	0.22	1.29
C18:2n-6	1.4	1.4	0.20	0.9	2.0
C18:3n-3	0.47	0.48	0.164	0.14	0.98
C18:3n-6	0.07	0.07	0.014	0.05	0.12
C20:0	0.13	0.12	0.019	0.08	0.17
C20:1 *cis*-11	0.06	0.06	0.019	0.03	0.12
C20:2n-6	0.04	0.04	0.007	0.03	0.06
C20:3n-6	0.07	0.07	0.019	0.04	0.13
C20:4n-3	0.04	0.03	0.028	0.00	0.13
C20:4n-6	0.12	0.11	0.024	0.06	0.18
C20:5n-3	0.06	0.06	0.013	0.03	0.09
C22:0	0.06	0.06	0.014	0.00	0.10
C22:5n-3	0.08	0.08	0.017	0.05	0.14

Supplementary Table S6.1. Continued

Item	Mean	Median	SD	Minimum	Maximum
C24:0	0.04	0.04	0.014	0.00	0.08
Volatile metabolite (peak area[2])					
1-Pentanol	6.16×10^6	4.14×10^6	6.139×10^6	1.92×10^5	3.35×10^7
2-Butanone	8.87×10^6	5.95×10^6	9.939×10^6	1.80×10^6	8.15×10^7
2-Heptanone	8.54×10^6	5.19×10^6	9.196×10^6	3.02×10^5	5.77×10^7
2-Pentanone	8.49×10^6	6.73×10^6	7.188×10^6	9.71×10^5	4.02×10^7
Acetone	1.34×10^8	8.39×10^7	2.207×10^8	2.13×10^7	2.25×10^9
Benzaldehyde	4.48×10^5	3.85×10^5	4.376×10^5	0.00	2.73×10^6
Butanoic acid	1.04×10^8	5.24×10^7	1.621×10^8	6.46×10^6	1.13×10^9
Dimethyl sulfone	5.43×10^6	3.17×10^6	5.602×10^6	7.48×10^5	2.81×10^7
Ethyl acetate	2.19×10^6	1.55×10^6	1.904×10^6	0.00	8.44×10^6
Ethyl butanoate	1.84×10^6	6.43×10^5	3.623×10^6	0.00	2.30×10^7
Hexanal	1.84×10^7	1.06×10^7	2.084×10^7	7.59×10^5	1.13×10^8
Hexanoic acid	1.19×10^8	5.94×10^7	1.948×10^9	8.46×10^6	1.21×10^9
Octanoic acid	9.96×10^7	4.64×10^7	1.626×10^8	7.71×10^6	1.05×10^9
Pentanoic acid	9.54×10^5	5.13×10^5	1.316×10^6	5.43×10^4	8.00×10^6
Non-volatile metabolite (relative peak area[3])					
Acetate	0.18	0.18	0.087	0.03	0.40
Acetoacetate	0.09	0.09	0.045	0.02	0.25
Acetone	0.06	0.06	0.039	0.02	0.43
Acetylcarnitine	0.14	0.13	0.044	0.06	0.30
Betaine	0.28	0.27	0.076	0.11	0.54
β-hydroxybutyrate	0.12	0.12	0.048	0.04	0.32
Butyrate	0.43	0.36	0.279	0.08	2.07
Carnitine	0.20	0.20	0.033	0.12	0.29
Choline	0.80	0.77	0.254	0.30	1.66
Citrate	2.6	2.6	0.51	1.7	4.8
Creatine	0.36	0.36	0.065	0.24	0.56
Creatinine	0.08	0.08	0.016	0.05	0.13
Ethanol	0.07	0.08	0.003	0.02	0.15
Formate	0.02	0.02	0.011	0.01	0.08
Galactose-1-phosphate	0.01	0.01	0.007	0.00	0.04
Glycerophosphocholine	0.82	0.77	0.256	0.43	1.55
Hippurate	0.07	0.06	0.025	0.03	0.17
Lactate	0.18	0.18	0.084	0.05	0.60
Lactose	16.0	14.6	3.28	11.4	22.8
Malonate	0.09	0.10	0.032	0.03	0.21
Methylmalonate	0.12	0.13	0.066	0.01	0.27
N-acetylsugar A	0.28	0.27	0.091	0.12	0.61
N-acetylsugar B	0.63	0.61	0.145	0.33	1.02
N-acetylsugar C	0.39	0.39	0.142	0.09	0.81
N-acetylsugar D	0.05	0.04	0.035	0.02	0.20
N-acetylsugar E	0.10	0.11	0.046	0.02	0.23
Orotate	0.06	0.05	0.017	0.02	0.14
Oxaloacetate	0.07	0.07	0.033	0.02	0.18
Oxoglutarate	0.06	0.06	0.017	0.04	0.12
Phosphocreatine	0.05	0.05	0.016	0.02	0.14

Supplementary Table S6.1. Continued

Item	Mean	Median	SD	Minimum	Maximum
Phosphorylcholine	0.15	0.13	0.091	0.06	0.62
Proline	0.12	0.12	0.036	0.06	0.31
Pyruvate	0.14	0.13	0.045	0.07	0.35
Succinate	0.07	0.06	0.021	0.04	0.16
Sugar A	0.02	0.02	0.005	0.01	0.04
Sugar B	0.03	0.03	0.012	0.01	0.06
Sugar C	0.02	0.02	0.017	0.00	0.09
UDP[4]-hexose A	0.00	0.00	0.001	0.00	0.01
UDP-hexose B	0.01	0.00	0.004	0.00	0.02
UDP-hexose C	0.07	0.07	0.032	0.02	0.18
UDP-hexose D	0.02	0.02	0.011	0.00	0.05

[1] C18:1 *cis*-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12, as these 2 FA could not be separated in the analysis. The portion of C18:1 *trans*-12 is considered to be negligible, as this FA is always present in small amounts.

[2] Peak area values (arbitrary unit of quantity).

[3] Peak area relative to calibration standard 3-trimethylsilyl-2,2,3,3-tetraduteropropionate (TSP).

[4] Uridine diphosphate.

Supplementary Table S6.2. Linear regression between methane production (g/d) and milk fatty acid concentration (g/100 g total fatty acids)

Fatty acid (g/100 g fatty acids)	Intercept	SE	Slope	SE	Slope *P*	R^2
C4:0	335	47.1	7.5	12.73	0.557	< 0.01
C6:0	175	46.9	86.4	20.92	< 0.001	0.19
C8:0	245	32.4	103.7	26.84	< 0.001	0.17
C10:0	272	25.1	36.2	9.10	< 0.001	0.18
C12:0	273	24.0	30.7	7.37	< 0.001	0.21
C13:0	331	16.2	423.4	171.40	0.014	0.04
C14:0	209	37.9	14.5	3.46	< 0.001	0.21
iso C14:0	339	24.5	276.7	241.27	0.254	0.01
C14:1 *cis*-9	314	20.9	48.3	15.89	0.003	0.09
C15:0	303	26.7	61.7	23.70	0.011	0.06
iso C15:0	309	26.9	233.8	101.74	0.023	0.05
anteiso C15:0	346	29.1	36.4	60.82	0.551	< 0.01
C16:0	167	39.8	6.1	1.21	< 0.001	0.25
iso C16:0	376	28.5	-79.1	129.12	0.542	< 0.01
C16:1 *trans*-9	427	25.6	-304.5	103.58	0.004	0.08
C16:1 *cis*-9	401	24.5	-21.9	11.03	0.050	0.04
C17:0	433	31.3	-110.8	44.16	0.014	0.08
iso C17:0	463	28.4	-254.5	65.37	< 0.001	0.15
anteiso C17:0	409	30.3	-109.7	62.14	0.080	0.03
C17:1 *cis*-9	438	17.7	-245.3	43.50	< 0.001	0.28
C18:0	399	33.7	-3.8	3.13	0.224	0.03
C18:1 *cis*-9[(1)]	483	25.4	-5.9	1.12	< 0.001	0.28
C18:1 *cis*-12	402	22.8	-218.8	59.27	< 0.001	0.18
C18:1 *cis*-13	414	20.3	-400.7	117.94	< 0.001	0.11
C18:1 *trans*-6	394	22.5	-158.0	75.48	0.039	0.05
C18:1 *trans*-9	452	25.8	-607.4	131.89	< 0.001	0.20
C18:1 *trans*-10	388	19.5	-131.5	43.56	0.003	0.11
C18:1 *trans*-11	425	17.7	-70.4	13.44	< 0.001	0.20
C18:1 *trans*-15 + C18:1 *cis*-11	462	20.3	-132.5	20.89	< 0.001	0.18
C18:2 *cis*-9, *trans*-11	417	16.9	-133.6	24.89	< 0.001	0.20
C18:2n-6	501	27.0	-96.9	15.47	< 0.001	0.26
C18:3n-3	415	17.2	-116.4	28.80	< 0.001	0.21
C18:3n-6	382	28.7	-278.9	321.33	0.387	< 0.01
C20:0	329	30.7	249.1	210.51	0.239	0.01
C20:1 *cis*-11	419	18.7	-927.9	238.69	< 0.001	0.18
C20:2n-6	395	27.9	-792.0	556.74	0.158	0.02
C20:3n-6	353	22.0	110.4	223.66	0.623	< 0.01
C20:4n-3	348	15.7	388.9	164.08	0.019	0.08
C20:4n-6	367	24.1	-51.2	166.26	0.759	< 0.01
C20:5n-3	376	23.6	-255.9	327.36	0.436	< 0.01
C22:0	345	20.9	299.3	261.91	0.255	0.01
C22:5n-3	407	26.2	-547.6	250.16	0.031	0.06
C24:0	346	18.5	403.6	286.62	0.162	0.02

[(1)] C18:1 *cis*-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12, as these 2 FA could not be separated in the analysis. The portion of C18:1 *trans*-12 is considered to be negligible, as this FA is always present in small amounts.

Supplementary Table S6.3. Linear regression between methane yield (g/kg DMI[1]) and milk fatty acid concentration (g/100 g total fatty acids)

Fatty acid (g/100 g fatty acids)	Intercept	SE	Slope	SE	Slope *P*	R^2
C4:0	28.4	2.24	-1.69	0.627	0.008	0.07
C6:0	23.1	2.44	-0.28	1.111	0.800	< 0.01
C8:0	21.8	1.66	0.59	1.414	0.678	< 0.01
C10:0	21.4	1.27	0.46	0.479	0.344	0.01
C12:0	21.2	1.21	0.44	0.388	0.259	0.02
C13:0	22.0	0.78	1.17	8.144	0.886	< 0.01
C14:0	18.9	1.96	0.34	0.181	0.062	0.05
iso C14:0	20.1	1.10	29.15	12.057	0.017	0.05
C14:1 *cis*-9	21.2	0.93	1.33	0.822	0.107	0.03
C15:0	19.0	1.22	3.66	1.188	0.003	0.08
iso C15:0	17.5	1.17	21.94	4.885	< 0.001	0.17
anteiso C15:0	21.0	1.36	3.72	3.069	0.228	0.01
C16:0	14.2	2.01	0.26	0.062	< 0.001	0.18
iso C16:0	20.6	1.30	9.90	6.418	0.126	0.03
C16:1 *trans*-9	26.2	1.22	-17.14	5.147	0.001	0.10
C16:1 *cis*-9	22.3	1.14	0.09	0.562	0.877	< 0.01
C17:0	19.3	1.52	4.85	2.210	0.030	0.06
iso C17:0	24.2	1.45	-4.28	3.422	0.214	0.02
anteiso C17:0	22.2	1.46	0.57	3.195	0.858	< 0.01
C17:1 *cis*-9	23.0	0.91	-1.61	2.428	0.509	< 0.01
C18:0	25.0	1.53	-0.26	0.151	0.089	0.04
C18:1 *cis*-9[2]	25.5	1.35	-0.15	0.060	0.018	0.07
C18:1 *cis*-12	25.7	0.87	-17.41	2.719	< 0.001	0.40
C18:1 *cis*-13	25.1	0.90	-19.57	5.960	0.001	0.10
C18:1 *trans*-6	25.1	0.91	-12.80	3.662	< 0.001	0.13
C18:1 *trans*-9	27.5	1.10	-34.05	6.494	< 0.001	0.24
C18:1 *trans*-10	24.3	0.70	-8.81	2.111	< 0.001	0.19
C18:1 *trans*-11	24.5	0.79	-2.22	0.728	0.003	0.08
C18:1 *trans*-15 + C18:1 *cis*-11	26.6	0.95	-5.35	1.108	< 0.001	0.22
C18:2 *cis*-9, *trans*-11	24.2	0.75	-4.16	1.353	0.003	0.08
C18:2n-6	28.8	1.29	-4.38	0.813	< 0.001	0.20
C18:3n-3	24.3	0.86	-3.94	1.480	0.009	0.10
C18:3n-6	25.4	1.29	-38.93	15.713	0.015	0.07
C20:0	18.7	1.43	29.07	10.303	0.006	0.07
C20:1 *cis*-11	24.9	0.88	-38.93	12.058	0.002	0.13
C20:2n-6	20.1	1.26	57.44	27.957	0.042	0.04
C20:3n-6	23.2	0.99	-8.99	11.238	0.425	< 0.01
C20:4n-3	22.3	0.56	6.24	8.282	0.453	< 0.01
C20:4n-6	22.7	1.09	-1.97	8.398	0.815	< 0.01
C20:5n-3	23.3	1.07	-14.16	16.289	0.387	< 0.01
C22:0	19.8	0.84	47.28	12.681	< 0.001	0.12
C22:5n-3	23.7	1.17	-15.35	12.728	0.230	0.02
C24:0	20.4	0.69	54.65	13.771	< 0.001	0.13

[1] Dry matter intake (kg/d).

[2] C18:1 *cis*-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12, as these 2 FA could not be separated in the analysis. The portion of C18:1 *trans*-12 is considered to be negligible, as this FA is always present in small amounts.

Supplementary Table S6.4. Linear regression between methane intensity (g/kg FPCM[1]) and milk fatty acid concentration (g/100 g total fatty acids) and between methane intensity (g/kg FPCM) and milk fatty acid concentration, including FPCM as a covariate

	Linear regression between methane intensity (g/kg FPCM) and MFA[2]						Linear regression between methane intensity (g/kg FPCM) and MFA, including FPCM as a covariate[3]								
Fatty acid (g/100 g fatty acids)	Inter-cept	SE	Slope	SE	Slope *P*	R^2	Inter-cept	SE	Slope MFA	SE	Slope *P* MFA	Slope FPCM	SE	Slope *P* FPCM	R^2
C4:0	24.7	2.37	-2.91	0.653	< 0.001	0.165	27.0	1.82	-0.88	0.535	0.104	-0.368	0.038	< 0.001	0.578
C6:0	15.7	2.70	-0.57	1.220	0.639	0.003	20.5	1.90	2.14	0.882	0.017	-0.415	0.036	< 0.001	0.606
C8:0	13.6	1.84	0.83	1.551	0.594	0.004	21.6	1.42	2.95	1.096	0.008	-0.408	0.035	< 0.001	0.604
C10:0	12.3	1.40	0.88	0.522	0.096	0.036	21.7	1.22	1.24	0.364	0.001	-0.403	0.034	< 0.001	0.613
C12:0	11.2	1.30	1.15	0.414	0.006	0.103	21.2	1.19	1.20	0.286	< 0.001	-0.395	0.033	< 0.001	0.637
C13:0	11.2	0.88	46.05	9.033	< 0.001	0.151	22.9	1.14	19.81	7.739	0.012	-0.382	0.035	< 0.001	0.575
C14:0	7.4	2.10	0.67	0.192	< 0.001	0.152	17.9	1.65	0.62	0.133	< 0.001	-0.390	0.033	< 0.001	0.640
iso C14:0	8.4	1.13	74.20	11.599	< 0.001	0.264	20.1	1.52	36.62	9.897	< 0.001	-0.337	0.037	< 0.001	0.597
C14:1 *cis*-9	10.4	0.94	4.18	0.829	< 0.001	0.215	21.0	1.17	2.86	0.618	< 0.001	-0.365	0.033	< 0.001	0.645
C15:0	7.5	1.30	7.26	1.170	< 0.001	0.274	19.4	1.51	4.07	0.940	< 0.001	-0.344	0.035	< 0.001	0.616
iso C15:0	4.2	0.99	45.46	4.059	< 0.001	0.561	14.4	1.56	29.60	3.966	< 0.001	-0.260	0.034	< 0.001	0.705
anteiso C15:0	6.6	1.33	19.11	2.891	< 0.001	0.290	19.3	1.72	9.04	2.511	0.001	-0.335	0.037	< 0.001	0.605
C16:0	2.3	2.13	0.38	0.064	< 0.001	0.317	14.7	1.78	0.29	0.046	< 0.001	-0.363	0.031	< 0.001	0.666
iso C16:0	12.2	1.48	11.96	7.026	0.091	0.031	24.3	1.51	0.79	5.093	0.878	-0.390	0.036	< 0.001	0.574
C16:1 *trans*-9	19.1	1.40	-21.64	5.550	< 0.001	0.138	27.0	1.23	-13.39	4.096	0.001	-0.376	0.034	< 0.001	0.582
C16:1 *cis*-9	13.1	1.26	0.77	0.612	0.210	0.016	25.0	1.41	-0.24	0.445	0.588	-0.394	0.036	< 0.001	0.575
C17:0	12.9	1.71	2.49	2.464	0.313	0.013	26.5	1.81	-2.54	1.868	0.177	-0.406	0.037	< 0.001	0.578
iso C17:0	12.9	1.61	3.98	3.765	0.293	0.013	26.6	1.70	-4.24	2.766	0.128	-0.407	0.037	< 0.001	0.572
anteiso C17:0	13.1	1.61	3.34	3.472	0.338	0.008	25.1	1.60	-1.30	2.490	0.603	-0.394	0.036	< 0.001	0.582
C17:1 *cis*-9	15.8	1.02	-4.15	2.641	0.119	0.029	27.7	1.16	-8.10	1.772	< 0.001	-0.420	0.033	< 0.001	0.645
C18:0	20.7	1.66	-0.63	0.161	< 0.001	0.203	26.9	1.40	-0.31	0.125	0.016	-0.371	0.035	< 0.001	0.592
C18:1 *cis*-9[4]	21.1	1.43	-0.32	0.062	< 0.001	0.275	29.5	1.20	-0.26	0.043	< 0.001	-0.378	0.031	< 0.001	0.668
C18:1 *cis*-12	16.8	0.79	-12.40	3.136	< 0.001	0.187	25.9	1.09	-10.20	2.271	< 0.001	-0.373	0.033	< 0.001	0.705
C18:1 *cis*-13	17.1	1.01	-19.76	6.565	0.003	0.086	26.4	1.08	-16.44	4.564	0.001	-0.381	0.034	< 0.001	0.621
C18:1 *trans*-6	15.8	1.05	-6.15	4.167	0.142	0.026	26.0	1.20	-7.08	2.926	0.017	-0.393	0.035	< 0.001	0.622

Supplementary Table S6.4. Continued

Fatty acid (g/100 g fatty acids)	Inter -cept	SE	Slope	SE	Slope *P*	R^2	Inter -cept	SE	Slope MFA	SE	Slope *P* MFA	Slope FPCM	SE	Slope *P* FPCM	R^2
C18:1 *trans*-9	18.9	1.19	-29.47	7.356	< 0.001	0.150	27.7	1.19	-23.39	5.124	< 0.001	-0.380	0.033	< 0.001	0.679
C18:1 *trans*-10	15.4	0.76	-4.60	2.419	0.060	0.045	25.5	1.08	-5.15	1.699	0.003	-0.391	0.034	< 0.001	0.645
C18:1 *trans*-11	14.9	0.96	-0.43	0.820	0.600	0.003	27.6	1.20	-2.39	0.559	< 0.001	-0.431	0.034	< 0.001	0.642
C18:1 *trans*-15 + C18:1 *cis*-11	19.7	0.98	-6.84	1.170	< 0.001	0.283	28.1	0.96	-5.61	0.791	< 0.001	-0.365	0.030	< 0.001	0.714
C18:2 *cis*-9, *trans*-11	14.5	0.88	0.04	1.531	0.979	< 0.001	27.5	1.20	-4.43	1.062	< 0.001	-0.436	0.035	< 0.001	0.639
C18:2n-6	19.9	1.46	-3.79	0.927	< 0.001	0.129	30.1	1.30	-3.79	0.620	< 0.001	-0.398	0.031	< 0.001	0.693
C18:3n-3	17.1	1.09	-5.66	1.629	< 0.001	0.169	26.4	1.07	-4.49	1.120	< 0.001	-0.387	0.033	< 0.001	0.591
C18:3n-6	17.0	1.41	-33.69	17.322	0.054	0.043	25.3	1.31	-13.37	12.495	0.287	-0.386	0.036	< 0.001	0.592
C20:0	6.6	1.53	60.73	10.292	< 0.001	0.260	18.8	1.69	33.65	8.243	< 0.001	-0.339	0.036	< 0.001	0.603
C20:1 *cis*-11	18.8	0.93	-69.48	12.305	< 0.001	0.313	26.8	0.92	-52.39	8.581	< 0.001	-0.354	0.032	< 0.001	0.669
C20:2n-6	6.8	1.20	183.25	26.446	< 0.001	0.330	20.5	1.97	60.54	26.152	0.022	-0.334	0.042	< 0.001	0.586
C20:3n-6	13.3	1.09	16.08	12.241	0.191	0.019	23.7	1.21	9.47	8.857	0.287	-0.390	0.035	< 0.001	0.575
C20:4n-3	14.6	0.72	-3.90	9.162	0.671	0.003	24.3	1.00	8.64	6.508	0.187	-0.398	0.036	< 0.001	0.592
C20:4n-6	9.5	1.07	43.84	8.316	< 0.001	0.222	21.8	1.52	16.13	6.999	0.023	-0.357	0.038	< 0.001	0.591
C20:5n-3	13.7	1.18	14.17	17.853	0.429	0.008	24.6	1.30	-2.76	13.022	0.832	-0.391	0.036	< 0.001	0.573
C22:0	9.7	0.84	84.71	12.495	< 0.001	0.301	20.1	1.34	46.76	10.481	< 0.001	-0.324	0.036	< 0.001	0.638
C22:5n-3	10.1	1.16	53.44	13.119	< 0.001	0.164	23.9	1.65	4.69	10.926	0.669	-0.384	0.039	< 0.001	0.574
C24:0	10.8	0.67	95.30	13.560	< 0.001	0.326	20.8	1.24	51.52	11.508	< 0.001	-0.323	0.036	< 0.001	0.641

(1) Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

(2) Milk fatty acids in g/100 g total fatty acids. Parameters were extracted from the equation: methane intensity = a + b × MFA + e, where a is the intercept of the regression line, b is the slope of the regression line associated with the MFA, and e is the error.

(3) Parameters were extracted from the equation: methane intensity = a + b × MFA + c × FPCM + e, where a is the intercept of the regression line, b is the slope of the regression line associated with the MFA, c is the slope of the regression line associated with FPCM, and e is the error.

(4) C18:1 *cis*-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12, as these 2 FA could not be separated in the analysis. The portion of C18:1 *trans*-12 is considered to be negligible, as this FA is always present in small amounts.

Supplementary Table S6.5. Linear regression between methane production (g/d) and volatile metabolites (peak area[1])

Volatile metabolite (peak area)	Intercept	SE	Slope	SE	Slope *P*	R^2
1-Pentanol	371	14.0	-1.5×10^{-6}	6.92×10^{-7}	0.035	0.05
2-Butanone	368	15.7	-6.7×10^{-7}	3.70×10^{-7}	0.075	0.03
2-Heptanone	356	16.5	6.5×10^{-7}	4.33×10^{-7}	0.136	0.02
2-Pentanone	372	14.9	-1.1×10^{-6}	6.21×10^{-7}	0.077	0.04
Acetone	367	14.8	-3.5×10^{-8}	1.67×10^{-8}	0.038	0.04
Benzaldehyde	361	15.6	2.2×10^{-6}	8.40×10^{-6}	0.791	< 0.01
Butanoic acid	360	15.5	2.5×10^{-8}	2.30×10^{-8}	0.281	0.01
Dimethyl sulfone	369	16.3	-1.2×10^{-6}	9.18×10^{-7}	0.188	0.03
Ethyl acetate	362	16.4	2.7×10^{-7}	3.19×10^{-6}	0.933	< 0.01
Ethyl butanoate	361	15.5	8.1×10^{-7}	1.05×10^{-6}	0.445	< 0.01
Hexanal	372	15.0	-5.4×10^{-7}	1.76×10^{-7}	0.003	0.08
Hexanoic acid	359	15.5	2.6×10^{-8}	1.89×10^{-8}	0.179	0.02
Octanoic acid	358	15.4	3.6×10^{-8}	2.29×10^{-8}	0.116	0.02
Pentanoic acid	359	15.5	3.6×10^{-6}	2.99×10^{-6}	0.237	0.02

[1] Peak area values (arbitrary unit of quantity).

Supplementary Table S6.6. Linear regression between methane yield (g/kg DMI[1]) and volatile metabolites (peak area[2])

Volatile metabolite (peak area)	Intercept	SE	Slope	SE	Slope *P*	R^2
1-Pentanol	22.8	0.53	-5.54×10^{-8}	3.540×10^{-8}	0.120	0.03
2-Butanone	22.5	0.54	-4.91×10^{-9}	1.940×10^{-8}	0.800	< 0.01
2-Heptanone	22.2	0.58	3.16×10^{-8}	2.230×10^{-8}	0.160	0.02
2-Pentanone	22.7	0.56	-2.40×10^{-8}	3.170×10^{-8}	0.451	< 0.01
Acetone	22.7	0.50	-1.10×10^{-9}	8.747×10^{-10}	0.210	0.02
Benzaldehyde	22.8	0.55	-6.19×10^{-7}	4.310×10^{-7}	0.154	0.02
Butanoic acid	22.2	0.52	2.41×10^{-9}	1.200×10^{-9}	0.043	0.04
Dimethyl sulfone	22.8	0.55	-6.15×10^{-8}	4.540×10^{-8}	0.178	0.03
Ethyl acetate	22.1	0.52	2.16×10^{-7}	1.496×10^{-7}	0.152	0.04
Ethyl butanoate	22.2	0.53	1.56×10^{-7}	5.250×10^{-8}	0.004	0.08
Hexanal	22.8	0.52	-1.85×10^{-8}	9.300×10^{-9}	0.049	0.04
Hexanoic acid	22.2	0.52	2.12×10^{-9}	9.664×10^{-10}	0.030	0.04
Octanoic acid	22.3	0.52	2.32×10^{-9}	1.200×10^{-9}	0.051	0.04
Pentanoic acid	22.2	0.53	2.61×10^{-7}	1.528×10^{-7}	0.091	0.03

[1] Dry matter intake (kg/d).

[2] Peak area values (arbitrary unit of quantity).

Supplementary Table S6.7. Linear regression between methane intensity (g/kg FPCM[1]) and volatile metabolites (peak area[2]) and between methane intensity (g/kg FPCM) and volatile metabolites, including FPCM as a covariate

	Linear regression between methane intensity (g/kg FPCM) and volatile metabolites[3]						Linear regression between methane intensity (g/kg FPCM) and volatile metabolites, including FPCM as a covariate[4]								
Volatile metabolite (peak area)	Inter-cept	SE	Slope	SE	Slope *P*	R^2	Inter-cept	SE	Slope volatile	SE	Slope *P* volatile	Slope FPCM	SE	Slope *P* FPCM	R^2
1-Pentanol	15.4	0.74	-1.40 × 10^{-7}	3.670 × 10^{-8}	< 0.001	0.16	24.4	0.96	-8.20 × 10^{-8}	2.700 × 10^{-8}	0.003	-0.37	0.035	< 0.001	0.57
2-Butanone	14.4	0.66	8.27 × 10^{-9}	2.080 × 10^{-8}	0.692	< 0.01	24.8	1.05	-1.70 × 10^{-8}	1.490 × 10^{-8}	0.263	-0.40	0.036	< 0.001	0.58
2-Heptanone	13.5	0.69	1.09 × 10^{-7}	2.210 × 10^{-8}	< 0.001	0.21	23.0	1.07	6.92 × 10^{-8}	1.650 × 10^{-8}	< 0.001	-0.36	0.034	< 0.001	0.57
2-Pentanone	15.0	0.71	-5.44 × 10^{-8}	3.410 × 10^{-8}	0.113	0.03	24.8	1.01	-4.40 × 10^{-8}	2.420 × 10^{-8}	0.071	-0.39	0.035	< 0.001	0.57
Acetone	14.8	0.69	-2.23 × 10^{-9}	9.203 × 10^{-10}	0.017	0.05	24.5	1.00	-2.00 × 10^{-9}	6.558 × 10^{-10}	0.013	-0.38	0.035	< 0.001	0.59
Benzaldehyde	14.5	0.66	-4.51 × 10^{-8}	4.662 × 10^{-7}	0.923	< 0.01	24.5	1.03	7.30 × 10^{-9}	3.314 × 10^{-7}	0.982	-0.39	0.036	< 0.001	0.58
Butanoic acid	13.9	0.69	5.38 × 10^{-9}	1.200 × 10^{-9}	< 0.001	0.037	23.4	1.05	3.10 × 10^{-9}	8.951 × 10^{-10}	< 0.001	-0.36	0.035	< 0.001	0.61
Dimethyl sulfone	15.2	0.61	-1.12 × 10^{-7}	4.850 × 10^{-8}	0.022	0.082	24.7	1.00	-7.30 × 10^{-8}	3.510 × 10^{-8}	0.041	-0.38	0.035	< 0.001	0.61
Ethyl acetate	13.9	0.68	2.89 × 10^{-7}	1.676 × 10^{-7}	0.088	0.06	24.0	1.03	1.68 × 10^{-7}	1.194 × 10^{-7}	0.163	-0.38	0.035	< 0.001	0.57
Ethyl butanoate	14.3	0.68	1.60 × 10^{-7}	5.650 × 10^{-8}	0.005	0.073	24.0	1.05	7.71 × 10^{-8}	4.160 × 10^{-8}	0.067	-0.38	0.036	< 0.001	0.58
Hexanal	14.9	0.66	-1.96 × 10^{-8}	1.001 × 10^{-8}	0.052	0.036	24.7	1.00	-1.80 × 10^{-8}	7.000 × 10^{-9}	0.011	-0.39	0.035	< 0.001	0.59
Hexanoic acid	14.0	0.69	4.34 × 10^{-9}	9.774 × 10^{-10}	< 0.001	0.155	23.4	1.04	2.60 × 10^{-9}	7.325 × 10^{-10}	< 0.001	-0.36	0.035	< 0.001	0.61
Octanoic acid	13.9	0.71	5.44 × 10^{-9}	1.200 × 10^{-9}	< 0.001	0.170	23.4	1.03	3.40 × 10^{-9}	8.793 × 10^{-10}	< 0.001	-0.36	0.034	< 0.001	0.62

Supplementary Table S6.7. Continued

Volatile metabolite (peak area)	Inter-cept	SE	Slope	SE	Slope *P*	R^2	Inter-cept	SE	Slope volatile	SE	Slope *P* volatile	Slope FPCM	SE	Slope *P* FPCM	R^2
Pentanoic acid	14.0	0.73	5.29×10^{-7}	1.590×10^{-7}	0.001	0.105	23.8	1.05	3.16×10^{-7}	1.164×10^{-7}	0.008	-0.37	0.035	< 0.001	0.60

(1) Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

(2) Peak area values (arbitrary unit of quantity).

(3) Parameters were extracted from the equation: methane intensity = a + b × volatile metabolite + e, where a is the intercept of the regression line, b is the slope of the regression line associated with the volatile metabolite, and e is the error.

(4) Parameters were extracted from the equation: methane intensity = a + b × volatile metabolite + c × FPCM + e, where a is the intercept of the regression line, b is the slope of the regression line associated with the volatile metabolite, c is the slope of the regression line associated with FPCM, and e is the error.

Supplementary Table S6.8. Linear regression between methane production (g/d) and non-volatile metabolites (relative peak area[1])

Non-volatile metabolite (relative peak area)	Intercept	SE	Slope	SE	Slope *P*	R^2
Acetate	332	24.3	161.2	75.97	0.036	0.13
Acetoacetate	333	22.7	304.2	124.15	0.016	0.12
Acetone	366	16.5	-63.9	104.16	0.541	< 0.01
Acetylcarnitine	414	18.8	-381.4	104.96	< 0.001	0.17
Betaine	356	21.0	20.3	50.76	0.691	< 0.01
β-hydroxybutyrate	340	22.4	176.3	110.16	0.112	0.05
Butyrate	353	17.4	20.0	13.78	0.149	0.02
Carnitine	364	28.0	-14.5	116.78	0.901	< 0.01
Choline	367	19.3	-6.8	15.04	0.652	< 0.01
Citrate	371	25.8	-3.7	7.53	0.628	< 0.01
Creatine	376	25.9	-39.7	58.23	0.497	< 0.01
Creatinine	358	28.0	47.3	285.35	0.869	< 0.01
Ethanol	353	23.3	111.0	212.12	0.602	< 0.01
Formate	352	19.6	388.9	402.13	0.336	0.01
Galactose-1-phosphate	368	17.8	-494.4	656.71	0.453	< 0.01
Glycerophosphocholine	352	19.5	10.9	14.59	0.458	< 0.01
Hippurate	334	21.9	410.9	179.56	0.024	0.07
Lactate	349	20.4	65.6	59.83	0.275	0.02
Lactose	375	38.6	-0.8	2.21	0.702	< 0.01
Malonate	342	22.6	207.7	157.19	0.189	0.03
Methylmalonate	341	22.8	157.8	101.46	0.123	0.07
N-acetylsugar A	335	23.6	93.9	55.10	0.091	0.05
N-acetylsugar B	382	22.5	-32.4	26.57	0.226	0.02
N-acetylsugar C	326	20.1	86.5	28.74	0.003	0.10
N-acetylsugar D	339	14.5	419.4	145.58	0.005	0.13
N-acetylsugar E	332	21.3	268.7	103.47	0.011	0.10
Orotate	378	20.3	-269.1	209.38	0.201	0.02
Oxaloacetate	331	23.1	447.4	170.71	0.010	0.14
Oxoglutarate	342	22.5	296.8	233.54	0.206	0.02
Phosphocreatine	339	19.6	440.1	232.71	0.061	0.04
Phosphorylcholine	347	15.8	91.7	39.66	0.023	0.05
Proline	344	22.2	114.4	119.75	0.230	0.02
Pyruvate	332	22.5	214.6	99.31	0.033	0.06
Succinate	334	22.3	388.7	193.22	0.047	0.05
Sugar A	355	24.0	287.5	827.68	0.729	< 0.01
Sugar B	345	16.6	610.9	325.67	0.063	0.04
Sugar C	352	14.9	466.3	214.55	0.032	0.05
UDP[2]-hexose A	377	20.1	-3550.8	3042.40	0.246	0.02
UDP-hexose B	374	19.9	-2000.2	1491.90	0.183	0.05
UDP-hexose C	345	21.6	224.4	169.76	0.189	0.04
UDP-hexose D	393	20.1	-1293.9	536.31	0.017	0.14

[1] Peak area relative to calibration standard 3-trimethylsilyl-2,2,3,3-tetraduteropropionate (TSP).

[2] Uridine diphosphate.

Supplementary Table S6.9. Linear regression between methane yield (g/kg DMI[1]) and non-volatile metabolites (relative peak area[2])

Non-volatile metabolite (relative peak area)	Intercept	SE	Slope	SE	Slope *P*	R^2
Acetate	23.2	0.80	-4.10	3.563	0.253	0.03
Acetoacetate	23.0	0.75	-5.95	6.171	0.337	0.02
Acetone	22.9	0.59	-6.96	5.319	0.194	0.02
Acetylcarnitine	23.5	0.90	-7.20	5.535	0.196	0.03
Betaine	22.3	0.89	0.55	2.618	0.833	< 0.01
β-hydroxybutyrate	23.2	0.83	-5.61	5.494	0.309	0.02
Butyrate	22.2	0.63	0.66	0.717	0.357	< 0.01
Carnitine	23.4	1.31	-4.74	6.002	0.431	< 0.01
Choline	22.3	0.80	0.28	0.777	0.720	< 0.01
Citrate	24.5	1.16	-0.74	0.383	0.057	0.04
Creatine	21.1	1.17	3.85	3.005	0.203	0.02
Creatinine	22.8	1.29	-3.59	14.574	0.806	< 0.01
Ethanol	23.5	0.87	-13.62	9.684	0.162	0.05
Formate	23.1	0.71	-25.24	20.103	0.212	0.02
Galactose-1-phosphate	22.8	0.67	-25.30	33.078	0.446	< 0.01
Glycerophosphocholine	22.3	0.82	0.22	0.754	0.766	< 0.01
Hippurate	22.8	0.80	-4.64	9.231	0.616	< 0.01
Lactate	23.0	0.74	-2.99	2.996	0.320	0.02
Lactose	21.9	1.74	0.03	0.105	0.744	< 0.01
Malonate	23.9	0.87	-14.92	7.777	0.058	0.05
Methylmalonate	22.5	0.83	-0.59	4.893	0.904	< 0.01
N-acetylsugar A	23.1	0.93	-2.26	2.787	0.419	0.01
N-acetylsugar B	22.7	1.01	-0.42	1.379	0.760	< 0.01
N-acetylsugar C	22.7	0.81	-0.64	1.527	0.678	< 0.01
N-acetylsugar D	22.3	0.65	2.98	7.559	0.694	< 0.01
N-acetylsugar E	23.2	0.74	-6.88	5.291	0.196	< 0.01
Orotate	22.2	0.84	4.57	10.923	0.676	< 0.01
Oxaloacetate	23.0	0.76	-7.90	8.514	0.356	0.02
Oxoglutarate	22.2	0.96	4.26	12.129	0.726	< 0.01
Phosphocreatine	22.8	0.81	-6.50	12.189	0.595	< 0.01
Phosphorylcholine	22.7	0.62	-1.72	2.088	0.413	< 0.01
Proline	22.9	0.92	-3.54	6.167	0.567	< 0.01
Pyruvate	23.1	0.87	-4.25	5.146	0.411	< 0.01
Succinate	22.9	0.88	-6.42	10.065	0.525	< 0.01
Sugar A	23.0	1.10	-24.84	42.686	0.562	< 0.01
Sugar B	22.6	0.70	-4.23	16.960	0.804	< 0.01
Sugar C	22.5	0.57	-1.67	11.284	0.883	< 0.01
UDP[3]-hexose A	21.8	0.88	148.02	156.250	0.345	0.01
UDP-hexose B	22.2	0.67	39.76	72.504	0.585	< 0.01
UDP-hexose C	23.7	0.72	-17.15	8.050	0.035	0.07
UDP-hexose D	22.9	0.79	-17.81	26.177	0.498	0.01

[1] Dry matter intake (kg/d).

[2] Peak area relative to calibration standard 3-trimethylsilyl-2,2,3,3-tetraduteropropionate (TSP).

[3] Uridine diphosphate.

Supplementary Table S6.10. Linear regression between methane intensity (g/kg FPCM[1]) and non-volatile metabolites (relative peak area[2]) and between methane intensity (g/kg FPCM) and non-volatile metabolites, including FPCM as a covariate

	Linear regression between methane intensity (g/kg FPCM) and non-volatile metabolites[3]						Linear regression between methane intensity (g/kg FPCM) and non-volatile metabolites, including FPCM as a covariate[4]								
Non-volatile metabolite (relative peak area)	Inter-cept	SE	Slope	SE	Slope *P*	R^2	Inter-cept	SE	Slope non-volatile	SE	Slope *P* non-vola-tile	Slope FPCM	SE	Slope *P* FPCM	R^2
Acetate	12.6	1.11	10.61	4.117	0.011	0.17	22.9	1.29	7.82	2.973	0.010	-0.385	0.035	< 0.001	0.65
Acetoacetate	13.0	0.98	16.07	6.838	0.021	< 0.01	23.2	1.21	12.74	4.885	0.010	-0.388	0.035	< 0.001	0.64
Acetone	15.0	0.73	-7.14	5.780	0.219	0.02	24.7	1.03	-4.77	4.112	0.248	-0.390	0.036	< 0.001	0.58
Acetylcarnitine	15.1	1.04	-3.91	6.094	0.523	< 0.01	27.1	1.19	-14.74	4.170	< 0.001	-0.415	0.035	< 0.001	0.62
Betaine	12.4	0.94	7.65	2.759	0.007	0.07	23.5	1.28	2.58	2.055	0.211	-0.382	0.037	< 0.001	0.60
β-hydroxybuty-rate	13.3	1.03	9.87	6.075	0.107	0.05	23.6	1.22	6.65	4.345	0.129	-0.390	0.036	< 0.001	0.60
Butyrate	12.9	0.76	3.52	0.708	< 0.001	0.20	22.7	1.12	2.10	0.537	< 0.001	-0.359	0.035	<0.001	0.63
Carnitine	13.7	1.45	3.89	6.517	0.551	< 0.01	24.6	1.43	-0.45	4.648	0.924	-0.393	0.036	< 0.001	0.58
Choline	13.4	0.87	1.46	0.833	0.082	0.03	24.5	1.22	0.04	0.611	0.944	-0.392	0.037	< 0.001	0.58
Citrate	16.9	1.27	-0.88	0.414	0.035	0.04	25.2	1.22	-0.33	0.302	0.276	-0.386	0.036	< 0.001	0.59
Creatine	12.4	1.29	5.91	3.232	0.070	0.03	24.9	1.50	-0.80	2.400	0.739	-0.396	0.037	< 0.001	0.58
Creatinine	10.7	1.43	46.57	15.311	0.003	0.11	23.0	1.58	14.70	11.613	0.208	-0.381	0.037	< 0.001	0.59
Ethanol	12.3	1.22	28.06	11.478	0.016	0.16	23.6	1.35	9.98	8.374	0.236	-0.386	0.036	< 0.001	0.60
Formate	13.5	0.82	41.45	21.869	0.061	0.05	23.8	1.15	21.90	15.828	0.169	-0.388	0.036	< 0.001	0.60
Galactose-1-phosphate	13.9	0.82	40.99	36.181	0.260	0.02	24.5	1.14	2.81	26.072	0.914	-0.392	0.036	< 0.001	0.58
Glycerophos-phocholine	14.1	0.94	0.48	0.816	0.557	< 0.01	24.2	1.14	0.33	0.579	0.568	-0.392	0.036	< 0.001	0.58
Hippurate	12.7	0.89	27.50	9.785	0.006	0.10	23.0	1.19	19.64	7.044	0.006	-0.384	0.035	< 0.001	0.64
Lactate	12.4	0.98	11.16	3.184	< 0.001	0.18	23.0	1.25	5.64	2.380	0.020	-0.375	0.036	< 0.001	0.62
Lactose	12.2	1.86	0.15	0.112	0.197	0.05	24.0	1.74	0.03	0.084	0.750	-0.391	0.036	< 0.001	0.58
Malonate	13.4	1.10	11.57	8.703	0.186	0.03	23.7	1.23	8.46	6.191	0.174	-0.391	0.036	< 0.001	0.59
Methylmalonate	11.5	1.21	23.12	5.333	< 0.001	0.37	22.2	1.36	12.73	4.029	0.002	-0.367	0.036	< 0.001	0.66

Supplementary Table S6.10. Continued

Non-volatile metabolite (relative peak area)	Inter-cept	SE	Slope	SE	Slope *P*	R^2	Inter-cept	SE	Slope non-volatile	SE	Slope *P* non-volatile	Slope FPCM	SE	Slope *P* FPCM	R^2
N-acetylsugar A	12.1	1.13	8.58	2.992	0.005	0.13	22.7	1.31	5.21	2.174	0.018	-0.381	0.035	< 0.001	0.62
N-acetylsugar B	12.5	1.10	3.25	1.467	0.029	0.05	24.7	1.41	-0.20	1.110	0.858	-0.395	0.037	< 0.001	0.58
N-acetylsugar C	14.5	0.93	0.13	1.661	0.936	< 0.01	23.8	1.08	2.73	1.173	0.021	-0.409	0.036	< 0.001	0.60
N-acetylsugar D	14.3	0.76	3.37	8.278	0.685	< 0.01	23.8	0.95	16.08	5.657	0.005	-0.406	0.035	< 0.001	0.60
N-acetylsugar E	14.1	0.92	3.83	5.865	0.515	< 0.01	23.7	1.12	9.15	4.131	0.029	-0.402	0.035	< 0.001	0.61
Orotate	14.3	0.95	3.58	11.830	0.763	< 0.01	24.9	1.18	-5.33	8.414	0.527	-0.395	0.036	< 0.001	0.58
Oxaloacetate	13.1	0.99	20.44	9.457	0.033	0.09	23.2	1.21	17.92	6.722	0.009	-0.390	0.035	< 0.001	0.64
Oxoglutarate	11.8	1.05	41.10	12.613	0.002	0.10	22.8	1.32	19.38	9.399	0.042	-0.377	0.036	< 0.001	0.60
Phospho-creatine	14.2	0.93	6.75	13.206	0.610	< 0.01	23.9	1.10	14.93	9.296	0.111	-0.398	0.036	< 0.001	0.59
Phosphoryl-choline	15.0	0.71	-3.22	2.251	0.155	0.02	24.5	1.01	2.37	1.674	0.160	-0.409	0.037	< 0.001	0.59
Proline	13.9	1.06	4.74	6.704	0.481	< 0.01	24.1	1.20	3.65	4.757	0.445	-0.393	0.036	< 0.001	0.59
Pyruvate	13.1	1.02	9.92	5.562	0.077	0.04	23.3	1.20	8.13	3.939	0.041	-0.390	0.035	< 0.001	0.61
Succinate	12.4	1.00	29.42	10.633	0.007	0.08	22.9	1.23	19.13	7.647	0.014	-0.383	0.035	< 0.001	0.62
Sugar A	12.6	1.18	84.33	45.795	0.068	0.03	24.5	1.32	36.44	33.030	0.272	-0.387	0.036	< 0.001	0.58
Sugar B	14.8	0.81	-10.24	18.384	0.579	< 0.01	24.2	1.01	20.60	13.230	0.122	-0.404	0.036	< 0.001	0.59
Sugar C	14.7	0.68	-7.52	12.206	0.539	< 0.01	24.6	1.00	15.92	8.818	0.074	-0.408	0.036	< 0.001	0.59
UDP-hexose A	13.7	0.99	188.60	169.480	0.268	0.02	24.8	1.23	-46.54	122.680	0.705	-0.395	0.036	< 0.001	0.58
UDP-hexose B	15.1	0.88	-90.23	81.645	0.271	0.03	25.0	1.12	-85.58	58.268	0.145	-0.393	0.036	< 0.001	0.60
UDP-hexose C	13.6	0.96	12.26	9.281	0.189	0.03	23.7	1.18	10.74	6.633	0.108	-0.392	0.036	< 0.001	0.61
UDP-hexose D	15.1	0.89	-23.88	28.827	0.409	0.02	25.6	1.13	-40.46	20.347	0.049	-0.398	0.035	< 0.001	0.62

(1) Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

(2) Peak area relative to calibration standard 3-trimethylsilyl-2,2,3,3-tetraduteropropionate (TSP).

(3) Parameters were extracted from the equation: methane intensity = a + b × non-volatile metabolite + e, where a is the intercept of the regression line, b is the slope of the regression line associated with the non-volatile metabolite, and e is the error.

(4) Parameters were extracted from the equation: methane intensity = a + b × non-volatile metabolite + c × FPCM + e, where a is the intercept of the regression line, b is the slope of the regression line associated with the non-volatile metabolite, c is the slope of the regression line associated with FPCM, and e is the error.

Supplementary Table S6.11. The variables included in the 10-fold cross validation prediction equations for methane production (g/d), yield (g/kg DMI[1]), and intensity (g/kg FPCM[2]) based on milk fatty acids, volatile or non-volatile metabolites alone, or all metabolites combined

	Methane production (g/d)			Methane yield (g/kg DMI)			Methane intensity (g/kg FPCM)		
	Item	Number in CV[3]	PredEq[4]	Item	Number in CV	PredEq	Item	Number in CV	PredEq
MFA[5]	C18:1 *trans*-15 +	9	yes	C18:1 *cis*-12	10	yes	*iso* C15:0	10	yes
	C18:2 *cis*-9, *trans*-11	7	yes	C18:3n-3	9	yes	C18:3n-3	9	yes
	C18:2n-6	5	no	C16:1 *trans*-9	1	no	C14:1 *cis*-9	3	no
	C18:3n-3	3	yes				C20:0	3	no
	C18:1 *trans*-11	2	no				C20:1 *cis*-11	3	yes
	C17:1 *cis*-9	1	no				C22:5n-3	3	yes
	C20:4n-3	1	no				C4:0	2	yes
							C13:0	1	no
							iso C14:0	1	no
							C16:0	1	no
							C20:2n-6	1	no
							C20:5n-3	1	no
V[6]	Hexanal	7	yes	Ethyl butanoate	9	yes	1-Pentanol	9	yes
	Acetone	1	no				2-Heptanone	9	yes
							Acetone	1	no
							Dimethyl sulfone	1	no
							Octanoic acid	1	no
							Pentanoic acid	1	no
NV[7]	Acetylcarnitine	9	yes	No models			Butyrate	7	yes
	UDP[8]-hexose D	6	yes				Citrate	6	no
	Oxaloacetate	5	yes				Methylmalonate	2	no
	Hippurate	3	no				*N*-acetylsugar A	2	no
	N-acetylsugar C	3	no				Hippurate	1	no
	Acetone	1	no				Malonate	1	no
	Choline	1	no				Oxoglutarate	1	no
	N-acetylsugar D	1	no						
	UDP-hexose C	1	no						

Supplementary Table S6.11. Continued

	Item	Number in CV	PredEq	Item	Number in CV	PredEq	Item	Number in CV	PredEq
ALL[9]	C18:1 trans-15 +	8	yes	C18:1 cis-12	10	yes	*iso* C15:0	9	yes
	C18:2 cis-9, trans-11	7	yes	C18:3n-3	8	yes	C18:3n-3	8	yes
	C18:2n-6	5	no	Benzaldehyde	2	no	2-Heptanone	5	yes
	C18:3n-3	3	yes	Ethyl butanoate	1	no	C22:5n-3	4	yes
	C17:1 cis-9	1	no				C20:1 *cis*-11	3	yes
	C18:1 trans-11	1	no				C16:0	2	no
	C20:4n-3	1	no				Betaine	2	no
	C20:5n-3	1	no				C4:0	1	no
	Hexanal	1	no				C6:0	1	no
	UDP-hexose A	1	no				C13:0	1	no
	UDP-hexose D	1	no				*iso* C14:0	1	no
							C14:1 *cis*-9	1	no
							C18:2n-6	1	no
							C20:2n-6	1	no
							C20:4n-6	1	no
							C20:5n-3	1	no
							C22:0	1	no
							Acetone	1	no
							Acetylcarnitine	1	yes
							Creatinine	1	no
							UDP-hexose B	1	no
							C24:0	0	yes

[1] Dry matter intake (kg/d).

[2] Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

[3] The number of times the metabolites was included in the 10-fold cross validation.

[4] Whether this metabolites is also included in the overall prediction equation, indicated by yes or no.

[5] Only milk fatty acids as selection variables; in g/100 g FA.

[6] Only volatile metabolites as selection variables; peak area value (arbitrary unit of quantity). Only 8 models were obtained for methane production (g/d) and only 9 models were obtained for methane yield (g/kg DMI).

Supplementary Table S6.11. Continued

[7] Only non-volatile metabolites as selection variables; peak area relative to calibration standard 3-trimethylsilyl-2,2,3,3-tetradeuteropropionate (TSP). No models were obtained for methane yield (g/kg DMI).
[8] Uridine diphosphate.
[9] All metabolites combined as selection variables.

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Chapter 7

Linseed oil and *DGAT1* K232A polymorphism: effects on methane emission, energy and N metabolism, lactation performance, ruminal fermentation, and rumen microbial composition of Holstein-Friesian cows

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ABSTRACT

Complex interactions between rumen microbiota, cow genetics, and diet composition may exist. Therefore, the effect of linseed oil, *DGAT1* K232A polymorphism (***DGAT1***), and the interaction between linseed oil and *DGAT1* on CH_4 and H_2 emission, energy and N metabolism, lactation performance, ruminal fermentation, and rumen bacterial and archaeal composition was investigated. Twenty-four lactating Holstein-Friesian cows (i.e., 12 with *DGAT1* KK genotype and 12 with *DGAT1* AA genotype) were fed, in a cross-over design, 2 diets: a control diet (**CON**) and a linseed oil diet (**LSO**) with a difference of 22 g/kg of dry matter (**DM**) in fat content between the 2 diets. Both diets consisted of 40% corn silage, 30% grass silage, and 30% concentrates (DM basis). Apparent digestibility, lactation performance, N and energy balance, and CH_4 emission were measured in climate respiration chambers, and rumen fluid samples were collected using the oral stomach tube technique. No linseed oil by *DGAT1* interactions were observed for digestibility, milk production and composition, energy and N balance, CH_4 and H_2 emissions, and rumen volatile fatty acid (**VFA**) concentrations. The *DGAT1* KK genotype was associated with a lower proportion of poly-unsaturated fatty acids (**FA**) in milk fat, and with a higher milk fat and protein content, and proportion of saturated FA in milk fat compared with the *DGAT1* AA genotype, whereas the fat- and protein-corrected milk yield was unaffected by *DGAT1*. Also, *DGAT1* did not affect nutrient digestibility, CH_4 or H_2 emission, ruminal fermentation or ruminal archaeal and bacterial concentrations. Rumen bacterial and archaeal composition was also unaffected in terms of the whole community, whereas at the genus level the relative abundances of some bacterial genera were found to be affected by *DGAT1*. The *DGAT1* KK genotype was associated with a lower metabolizability (i.e., ratio of metabolizable to gross energy intake), and with a tendency for a lower milk N efficiency compared with the *DGAT1* AA genotype. The LSO diet tended to decrease CH_4 production (g/d) by 8%, and significantly decreased CH_4 yield (g/kg of DM intake) by 6% and CH_4 intensity (g/kg of fat- and protein-corrected milk) by 11%, but did not affect H_2 emission. The LSO diet also decreased ruminal acetate molar proportion, the acetate to propionate ratio, and the archaea to bacteria ratio, whereas ruminal propionate molar proportion and milk N efficiency increased. Ruminal bacterial and archaeal composition tended to be affected by diet in terms of the whole community, with several bacterial genera found to be significantly affected by diet. These results indicate that *DGAT1* does not affect enteric CH_4 emission and production pathways, but that it does affect traits other than lactation characteristics, including metabolizability, N efficiency, and the relative abundance of *Bifidobacterium*. Additionally, linseed oil reduces CH_4 emission independent of *DGAT1* and affects the rumen microbiota and its fermentative activity.

Keywords: dairy cow, enteric methane production, linseed oil, *DGAT1* K232A polymorphism

INTRODUCTION

Several dietary strategies have been proposed to mitigate enteric CH_4 production, including the use of feed additives and improving forage quality (Beauchemin et al., 2009; Martin et al., 2010). Numerous studies have shown the potential of dietary lipid supplementation to reduce CH_4 emission, many of which have been reviewed by Grainger and Beauchemin (2011) and Hristov et al. (2013). To date, linseed is considered to be one of the most effective dietary lipid sources to reduce enteric CH_4 production from dairy cows (Beauchemin et al., 2009; Martin et al., 2010). Relatively few studies have considered the wider consequences of dietary linseed oil on the functioning of the rumen microbial ecosystem. Veneman et al. (2015) reported no effect of linseed oil supplementation on CH_4 emission or the rumen microbiota as a whole. Martin et al. (2016) reported significant decreases in CH_4 emissions upon extruded linseed supplementation for both corn silage-based and hay-based diets, whereas the abundance of rumen methanogens was not affected by linseed supply in the corn silage-based or hay-based diets.

Little is known whether host genetics can also influence the responses to dietary linseed oil. The acyl CoA:diacylglycerol acyltransferase 1 gene, located on chromosome 14, mediates the final step in triglyceride synthesis (Schennink et al., 2008). Many studies have investigated associations between the K232A polymorphism of this gene (i.e., a lysine to alanine substitution on the 232nd amino acid; ***DGAT1***) and milk production traits of dairy cows. Although *DGAT1* has no effect on fat- and protein-corrected milk (**FPCM**) yield, the *DGAT1* K allele is associated with a higher fat content, protein content, and fat yield, but lower milk production and protein and lactose yield (e.g., Banos et al., 2008; Näslund et al., 2008; Bovenhuis et al., 2015). Additionally, *DGAT1* has a marked effect on milk fatty acid (**MFA**) composition. The *DGAT1* K allele is associated with a larger fraction of C16:0, and smaller fractions of C18 UFA in milk fat (e.g., Schennink et al., 2007; Duchemin et al., 2013). Several of the MFA which have been associated with CH_4 emission (Van Gastelen and Dijkstra, 2016) are also affected by *DGAT1*, in particular C18 UFA in both the *cis* and *trans* isomers.

The *DGAT1* gene is expressed in the small intestine, liver, adipose tissue, and the mammary gland (DeVita and Pinto, 2013; Muise et al., 2014). Thus, effects of *DGAT1* on traits other than milk production might be expected. Van Engelen, S. (Wageningen University & Research, Wageningen, The Netherlands; unpublished data) performed a genome-wide association study (**GWAS**) to determine regions of the bovine genome that are associated with predicted CH_4 yield (g/kg of DMI) using the CH_4 prediction equations based on MFA profile published by Dijkstra et al. (2011) and Van Engelen et al. (2015). The association with *DGAT1* was significant in the GWAS for predicted CH_4 yield, suggesting that the *DGAT1* K allele is associated with higher predicted CH_4 yield. The association between *DGAT1* and CH_4 yield has not been studied before and could be of statistical and biological significance. To the best of our knowledge, no study has investigated if the genetic variation of dairy cows, namely *DGAT1*, affects the rumen bacterial and archaeal composition, one of the potential biological explanations for the relation between *DGAT1* and CH_4 yield. In addition, there is little information on the association of *DGAT1* with nutrient digestion or energy and N balance of dairy cattle.

Therefore, the objectives of the present study were to investigate the effects of dietary linseed oil, *DGAT1*, and the interaction between dietary linseed oil and *DGAT1* on CH_4 and H_2 emission, energy and N metabolism, lactation performance, ruminal fermentation, and rumen bacterial and archaeal composition of dairy cows.

MATERIALS AND METHODS

Experimental design

The experiment was conducted from January to April 2015, in accordance with Dutch law and approved by the Animal Care and Use Committee of Wageningen University & Research (Wageningen, The Netherlands). The experiment followed a cross-over design with 2 dietary treatments and 24 lactating Holstein-Friesian cows (i.e., 12 cows with *DGAT1* KK genotype and 12 cows with *DGAT1* AA genotype; each group had 6 primiparous and 6 multiparous cows). The 12 cows with *DGAT1* KK genotype were sired by 10 bulls, and the 12 cows with *DGAT1* AA genotype were sired by 9 bulls. Additionally, 1 bull sired 2 cows with *DGAT1* KK genotype and 1 cow with *DGAT1* AA genotype. At the start of the experiment, the cows with the *DGAT1* KK genotype and *DGAT1* AA genotype were, on average, 215 ± 65 (mean ± SD) and 216 ± 68 DIM and produced 23.9 ± 5.66 kg/d and 26.9 ± 5.87 kg milk/d, respectively. The cows were blocked in pairs according their *DGAT1* genotype, parity, DIM, and milk production. Within each block, cows were randomly allocated to a dietary treatment sequence in a cross-over design with 2 periods: a control diet (**CON**) and linseed oil diet (**LSO**). Treatment periods lasted 17 d and were composed of a 12 d adaptation period followed by a 5 d measurement period. There was a 14 d wash-out period between the treatment periods of the same block of cows.

Diets, feeding, and housing

Both the CON and LSO diet consisted of 40% corn silage, 30% grass silage, and 30% concentrates, on a DM basis. The ingredient and chemical composition of both diets are presented in Table 7.1. Linseed oil (Linagro NV, Lichtervelde, Belgium) was added to the concentrate of the LSO diet, substituting a part of the CON concentrate ingredients, to achieve a difference of 22 g/kg DM in fat content between the 2 diets. To determine apparent total-tract feed digestibility, Cr_2O_3 (1.5 g/kg of concentrate DM) was included in the concentrates of both diets as an external marker. Concentrates were produced by Research Diet Services (RDS BV, Wijk bij Duurstede, the Netherlands) in 1 batch and hence were assumed to be of uniform composition throughout the experiment. Diets were formulated to meet the requirements for maintenance and milk production of the lactating dairy cows. The NE_L was calculated with the VEM (feed unit lactation) system according to Van Es (1978), and intestinal digestible protein and rumen degradable protein balance were calculated according to Van Duinkerken et al. (2011).

Table 7.1. Chemical composition (g/kg of DM, unless otherwise stated) of the TMR ingredients (grass silage, corn silage, concentrates; analyzed) and of the complete TMR (calculated) for the control diet (CON) and the linseed oil (LSO) diet

	Silages		Concentrates		TMR	
Item	Grass[1]	Corn[2]	CON[3]	LSO[4]	CON[5]	LSO[6]
DM (g/kg of product)	558	318	878	890	468	469
OM	911	959	880	889	921	924
CP	140	83	394	361	194	184
Crude fat	33	34	33	108	34	56
Gross energy (MJ/ kg of DM)	18.3	18.4	18.0	19.7	18.2	18.7
NDF	546	330	203	178	357	349
ADF	303	185	101	91	195	192
ADL	15	9	17	16	13	13
Starch	_[7]	373	18	14	154	153
Reducing sugars	88	_[7]	137	124	67	63
Fatty acids						
C16:0	2.7	2.7	2.4	7.1	2.6	4.0
C18:0	0.26	0.44	0.48	2.9	0.40	1.1
C18:1 *cis*-9	0.30	3.2	3.8	17.9	2.5	6.8
C18:2n-6	2.3	8.3	6.8	19.7	6.1	9.9
C18:3n-3	8.5	1.0	1.0	35.4	3.2	13.5
Total fatty acids	16.2	16.1	14.4	86.8	15.6	37.3

[1] NE_L = 6.2 MJ/kg DM; intestinal digestible protein (DVE) = 64 g/kg of DM; rumen degraded protein balance (OEB) = 11 g/kg of DM; ensiling characteristics: acetic acid = 9 g/kg of DM, lactic acid = 13 g/kg of DM, ammonia-N = 6% of total N, and pH = 5.9.

[2] NE_L = 7.0 MJ/kg of DM; DVE = 56 g/kg of DM; OEB = -8 g/kg of DM; ensiling characteristics: acetic acid = 11 g/kg of DM, lactic acid = 55 g/kg of DM, ammonia-N = 8% of total N, and pH = 3.7.

[3] Concentrate added to the CON diet contained (g/kg of DM): soybean meal = 400, soybean meal, formaldehyde treated = 200, rapeseed meal = 100, rapeseed meal, formaldehyde treated = 100, sugar beet pulp = 119, sugarcane molasses = 40, $CaCO_3$ = 15, NaCl = 8.0, $NaHCO_3$ = 2.0, trace mineral and vitamin mix = 8.0, MgO = 7.0, and Cr_2O_3 = 1.5.

[4] Concentrate added to the LSO diet contained (g/kg of DM): soybean meal = 369, soybean meal, formaldehyde treated = 184, rapeseed meal = 92, rapeseed meal, formaldehyde treated = 92, sugar beet pulp = 109, sugarcane molasses = 37, $CaCO_3$ = 15, NaCl = 8.0, $NaHCO_3$ = 2.0, trace mineral and vitamin mix = 8.0, MgO = 7.0, Cr_2O_3 = 1.5, and linseed oil (Linagro NV, Lichtervelde Belgium) = 76.

[5] TMR contained grass silage, 300 g/kg of DM; corn silage, 400 g/kg of DM; CON concentrate, 300 g/kg of DM.

[6] TMR contained grass silage, 300 g/kg of DM; corn silage, 400 g/kg of DM; LSO concentrate, 300 g/kg of DM.

[7] Not determined.

Cows were fed and milked at 0600 and 1600 h. Just before milking, the feed refusals were weighed and a new portion of the diet provided. The diets were fed as a TMR in 2 equal daily portions. The concentrate was provided in meal form and manually mixed into the roughage mixture at the time of feeding. Cows had free access to clean drinking water throughout the experiment. Cows were fed individually and feed refusals were collected to determine DMI throughout the experiment. Cows were fed ad libitum during the first 8 d of the adaptation period. From d 9 onwards, feed intake was restricted per block to 95% of the ad libitum DMI of the cow within a block consuming the lowest amount of feed during d 5 to d 8 as described previously by Van Zijderveld et al. (2011a). The cows were fed restricted amounts

of feed to avoid confounding effects of DMI on enteric CH_4 production, similar to Van Zijderveld et al. (2011a). At all times, a minimum DMI of at least 82% of the ad libitum intake of the cow with the greatest DMI within each block was ensured.

During the 12-d adaptation period, the cows were individually housed in tie-stalls in order to become accustomed to the diet and restriction in movement. On d 13 (1400 h), after the adaptation period, 4 cows (2 blocks) were individually transported to 1 of 4 identical climate respiration chambers (**CRC**), located approximately 200m from the tie-stalls, for a 5-d period to determine gaseous exchange, energy and N balance, and apparent total-tract nutrient digestibility. A detailed description of the CRC design and gas measurements is reported by Heetkamp et al. (2015) and Van Gastelen et al. (2015). Briefly, in each CRC (i.e., an area of 11.8 m^2 and a volume of 34.5 m^3) relative humidity was maintained at 65% and temperature at 16°C. The CRC were equipped with thin walls with windows, to allow audio-visual contact in order to minimize the effect of social isolation on cow behavior and performance. Cows were exposed to 16 h of light per day (from 0530 to 2130 h) and housed in the CRC until d 17 (0900 h).

In addition to Van Gastelen et al. (2015), a H_2 analyzer (type MGA 3000 multi gas analyzer, ADC Gas Analysis Ltd, Hoddesdon, UK) was installed in series with the O_2, CO_2, and CH_4 gas analyzers. The H_2 concentrations were measured using an electrochemical cell technique which has a relatively slow response time compared to the nondispersive infrared method of the CO_2 and CH_4 gas analyzers and the paramagnetic method of the O_2 gas analyzer. Therefore, sampled air from the CRC was flushed through the gas analysis system for 180s before the analyzer readout was logged. To have as many measurements as possible, inlet air was not sampled in every sequence, but once per hour. Therefore, inlet and exhaust air of each CRC was sampled with an average interval of 12.5 min (i.e., 4 times 12-min intervals for each CRC and 1 interval of 15 min for inlet air) instead of the 10-min interval reported by Van Gastelen et al. (2015). Production of CO_2, H_2, and CH_4 and consumption of O_2 was calculated from the difference between inlet and exhaust gas volumes.

The ventilation rate within the CRC was 58 m^3/h to ensure that the H_2 peak after feeding was within the detection limit of the H_2 analyzer (i.e., 0-100 ppm). Staff entered the CRC twice daily at 0600 and 1600 h for approximately 30 min for feeding and milking. Van Gastelen et al. (2015) did not use the gas concentration data during these feeding and milking times. The H_2 concentration peak occurred directly after feeding when staff was still inside the CRC. Therefore, we calculated the daily CH_4 and H_2 production on 2 datasets: (1) without the gas concentration data during feeding/milking (as was done by Van Gastelen et al. (2015), partially missing the H_2 concentration peak after feeding), and (2) with the gas concentration data during feeding/milking (to capture the H_2 concentration peak directly after feeding). Excluding the gas concentration measurements during feeding/milking would have underestimated daily H_2 production by 15.2 ± 6.89%. Daily productions of CH_4 were unaffected when excluding these gas measurements compared with including these gas measurements (data not shown). Thus, for the present study it was decided to not discard the gas measurements during feeding and milking.

Gas concentrations and ventilation rates were corrected for pressure, temperature and relative humidity to arrive at standard temperature pressure dew point volumes of inlet and exhaust air. Once a day, calibration gasses were sampled for gas analysis instead of the inlet air,

and the analyzed and actual values of these calibration gasses were used to correct the measured gas concentrations from the inlet air and exhaust air of all compartments. Before the present experiment started, CRC were checked by releasing known amounts of CO_2 in each compartment and comparing these values with the data from the gas analysis system to calculate the recovery. The recovered amounts of CO_2 were between 99 and 101%.

Sample collection and measurements

Samples of grass silage, corn silage, and both concentrates were obtained when fresh feed was prepared (i.e., twice weekly). These samples were subsequently pooled per treatment period, subsampled and stored at -20°C pending analyses. During the 5-d period in the CRC, feed residues were collected twice daily (0600 and 1600h), weighed, and stored at 4°C. At the end of the 5-d period in the CRC, daily orts were pooled per cow, mixed, subsampled, and stored at −20°C pending analyses.

Rumen fluid samples (~1 L) were collected 1 h before and 4 h after morning feeding on d 12 and 4 h after morning feeding on d 17 using the oral stomach tube (**OST**) technique, similar to Shen et al. (2012). Rumen fluid samples could not be collected 1 h before morning feeding on d 17, because the cows were still housed in the CRC and the gas measurements were priority. To collect rumen fluid with the OST method, a probe was inserted in the ventral cranial part of the rumen via the esophagus. The probe was 190 cm long and the head of the probe consisted of small holes allowing only rumen fluid (i.e., no fibrous content) to be collected. Rumen fluid was collected by using a 500 mL suction pump, which was attached to the probe. The first 500 mL of rumen fluid was discarded to limit saliva contamination of the rumen fluid samples. Rumen pH was measured immediately after sampling using a calibrated portable electronic pH meter (pH electrode HI99141, Hanna Instruments, IJsselstein, The Netherlands) and 2 rumen fluid subsamples of 600 μL each were acidified with an equal volume of ortho-phosphoric acid, and directly stored at -20°C to stop microbial fermentation pending VFA analysis. On d 17, 4 h after morning feeding, 100 mL of rumen fluid was sampled, directly stored on dry ice and transferred to a -80°C freezer pending microbiota analysis.

Milk yield was recorded for each milking, both during the adaptation period in the tie-stalls and the measurement period in the CRC. Milk from cows in the CRC was collected twice daily at 0600 and 1600h. A milk sample (10 mL) of each milking event was collected in a tube containing sodium azide (5 μL) for preservation, stored no longer than 4 d at 4°C, and analyzed for fat, protein, lactose, and urea content. Milk composition was corrected for differences in milk yield between milking events on the same day, and the average milk composition on a daily basis was used for data analysis. An additional milk sample (5 g/kg milk) was collected at each milking event, pooled per cow for the entire period in the CRC, and stored at -20°C pending milk energy and N analyses. For MFA composition, milk samples were collected according to Van Gastelen et al. (2015), pooled per cow per period and stored at -20°C until analyses.

Measurements of CH_4, H_2, and CO_2 production, and O_2 consumption were based on data recorded from d 14 (0800 h) through d 17 (0800 h), whereas energy and N balance, and apparent total-tract feed digestibility were based on manure (mixture of feces and urine) and feces collections from d 13 (1400 h) through to d 17 (0900 h). Cows were weighed when entering

and leaving of the CRC. The feces and urine produced during the 5-d period in the CRC were quantitatively collected, weighed, mixed, subsampled and stored at -20°C pending analyses. In addition, samples of condensed water (i.e., collected from the heat exchanger) and 25% sulfuric acid solution w/w (i.e., through which the outflowing air was led to trap aerial ammonia) of each CRC were collected, because of N volatilization in the form of ammonia due to the mixing of feces and urine. During the 5 d in the CRC rectal grab samples (~300 g) were collected twice daily at 0600 and 1600h and stored at −20°C. Prior to analysis, the grab samples were thawed, pooled per cow and period, mixed, and subsampled.

Chemical analyses

Samples of feed, feed residues, manure, and rectal grab samples were thawed at room temperature, oven-dried at 60°C, ground to pass a 1-mm screen using a Wiley mill (Peppink 100AN, Olst, The Netherlands), and analyzed by wet chemistry [i.e., ash, DM, N, starch, reducing sugars (i.e., all carbohydrates with reducing properties and soluble in 40% ethanol), NDF, ADF, and ADL] as described by Abrahamse et al. (2008). Bomb calorimetry (ISO 9831; International Organization for Standardization, 1998) was used to determine gross energy (**GE**). Crude protein was calculated as N × 6.25, where N was determined using the Kjeldahl method with $CuSO_4$ as catalyst (ISO 5983; International Organization for Standardization, 2005). The N concentrations in manure and of roughages were determined in fresh material according to Klop et al. (2016). The FAME of the feed samples were determined as described by Khan et al. (2009) using GC (Carlo Erba 8560 HRGC, Rodano, Italy) with a fused silica capillary column (100 m × 0.250 mm and 0.2 μm film thickness; Supelco; SP2560, St. Louis, MO) and helium as the carrier gas. Crude fat content was analyzed according the gravimetric method NEN-ISO 1735 (ISO 1735; International Organization for Standardization, 2004) with modifications as described by Klop et al. (2017).

Grass silage, corn silage, and concentrates were analyzed for DM, ash, N, crude fat, starch (except for grass silage), sugars (except for corn silage), NDF, ADF, ADL, GE, and FAME. Feed residues were analyzed for DM, ash, and crude fat. Manure samples were analyzed for DM, N, and GE. The rectal grab samples were analyzed for DM, ash, N, crude fat, starch, NDF, and GE. Chromium oxide was determined in the concentrates and rectal grab samples using an atomic absorption spectrophotometer (AA240FS; Varian, Palo Alto, CA, USA) after oxidation by wet destruction as described in detail by Pellikaan et al. (2013).

The concentration of individual VFA in the rumen fluid samples of d 12 (i.e., 1 h before and 4 h after morning feeding) was determined using GC (Fisons HRGC Mega 2, CE Instruments, Milan, Italy) with a split/splitless injector and helium as carrier gas as described by Van Gastelen et al. (2015). Milk samples from individual milking events were analyzed for proximate composition (fat, protein and lactose content) by mid-infrared spectroscopy (ISO 9622; International Organization for Standardization, 1999), and for MUN using the pH difference technique (ISO 14637; International Organization for Standardization, 2004) at Qlip (Zutphen, The Netherlands). The MFA composition of the pooled milk samples was determined using GC (Thermo Electron Corporation, Waltham, MA) by Qlip with a split/splitless injector

and H_2 as carrier gas as described by Van Gastelen et al. (2015). The GE and N content of the pooled milk samples were analyzed as described above.

Microbiota analysis

Rumen fluid samples taken 4 h after morning feeding on d 17 were analyzed for bacterial and archaeal concentrations and community composition, using quatitative PCR (**qPCR**) and Illumina MiSeq sequencing of PCR-amplified 16S ribosomal RNA (**rRNA**) gene fragments. Total DNA was extracted from the rumen fluid samples using a protocol involving a combination of bead beating, stool transport and recovery buffer (Roche Diagnostics Nederland B.V, Almere, The Netherlands) and the Maxwell 16 Instrument (Promega, Leiden, The Netherlands) as described in detail by Van Lingen et al. (2017). For absolute quantification of bacteria and archaea, SYBR green qPCR assays were performed with sample DNA extracts using an iCycler iQ real-time detection system (Bio-Rad Laboratories B.V., Veenendaal, The Netherlands). The qPCR procedure, primers, cycling conditions, and standards used are described by Van Lingen et al. (2017). For combined bacterial and archaeal composition profiling, barcoded amplicons from the V4 region of 16S rRNA genes were generated from sample DNA extracts using a 2-step PCR strategy (Caporaso et al., 2012; Tian et al., 2016; Van Lingen et al., 2017). As described by Ramiro-Garcia et al. (2016), the 16S rRNA gene sequencing data were analyzed using NG-Tax, an in-house bioinformatics pipeline. Operational taxonomic units (**OTU**) were defined using an open reference approach, and taxonomy was assigned to those OTU using a SILVA 16S rRNA gene reference database (Quast et al., 2013). Preliminary analysis of the samples confirmed the necessity of excluding 3 of the rumen fluid samples from further microbial data analysis (i.e., both sequence and qPCR based analysis) due to issues associated with salivary contamination of the samples during collection.

Statistical analysis

All parameters related to feed intake, milk production, and milk composition while cows were housed in the CRC were averaged per cow over a 4-d period. The parameters related to energy and N balance were expressed per kg of metabolic bodyweight ($BW^{0.75}$) per d. All univariate data were subjected to ANOVA in a cross-over with a 2 period × 2 treatment design using the MIXED procedure in SAS (edition 9.2, SAS Institute Inc., Cary, NC). Treatments (i.e., diet and *DGAT1*), their interaction, diet sequence, and period were considered fixed effects. The model included block as random factor, and cow within diet × *DGAT1* was considered as subject. For all analyses, the fixed effect of CRC was initially included in the model, but was removed because it was found to be not significant. The fixed effect of diet sequence was found to be significant twice (i.e., MUN and C22:0) and a tendency was found once (i.e., C22:4n-6), but diet sequence was always kept in the model. The covariance structure compound symmetry provided best fit with the lowest overall Akaike's information criterion values. Ruminal VFA data were subjected to repeated measures ANOVA in order to take repeated sampling within the same cow per treatment period into account. Similar to the above described model, this model included treatments, their interactions, sequence, and period as fixed effects, and block as random effects. Again, the fixed effect of diet sequence was found to be significant once (i.e.,

total VFA concentration) and a tendency was found twice (i.e., molar proportions of acetate and butyrate), but diet sequence was always kept in the model. For both models, pairwise comparisons of means were tested with the Tukey-Kramer method. The Kenward-Roger option was used to estimate the denominator degrees of freedom. All results are reported as least square means with significance of effects declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$. No multiple testing correction was applied.

Permutational Multivariate Analysis of Variance (**PERMANOVA**; Anderson, 2001) was used to assess the significance of changes in the rumen bacterial and archaeal composition in terms of the microbiota as a whole community (with the OTU summarized to the genus level) with respect to different factors [i.e., diet (LSO and CON), *DGAT1* (*DGAT1* KK and AA genotype), and diet × *DGAT1*]. The PERMANOVA was applied on the Bray-Curtis distance matrices, and Bonferroni correction for multiple testing was applied on a nominal significance of 0.05. The Matlab Fathom toolbox (Jones, 2015) was used for calculations. The aforementioned ANOVA analysis was also used to determine the effect of diet, *DGAT1*, and diet × *DGAT1* interaction on the relative abundance of individual bacterial and archaeal genera that were (1) consistent in all animals and (2) were > 0.05% in terms of relative abundance.

Table 7.2. The effect of *DGAT1* K232A polymorphism (AA or KK) on intake and apparent total-tract digestibility of nutrients of lactating dairy cows fed diets with (LSO) or without (CON) linseed oil

	Diet		*DGAT1* genotype			*P*-value		
Item	CON	LSO	AA	KK	SEM	Diet	*DGAT1*	Diet × *DGAT1*
Nutrient intake (kg/d, unless otherwise stated)								
DM	18.0	17.5	17.8	17.6	0.69	0.649	0.825	0.935
OM	16.5	16.2	16.5	16.3	0.63	0.686	0.825	0.934
CP	3.48	3.22	3.37	3.33	0.130	0.165	0.927	0.939
Crude fat	0.60	0.99	0.80	0.79	0.032	< 0.001	0.817	0.892
Gross energy (MJ/d)	327.5	328.2	329.8	325.8	12.73	0.971	0.825	0.934
NDF	6.41	6.12	6.30	6.22	0.243	0.404	0.826	0.936
ADF	3.50	3.36	3.45	3.41	0.133	0.450	0.826	0.936
ADL	0.24	0.23	0.24	0.23	0.009	0.379	0.823	0.936
Starch	2.77	2.68	2.75	2.71	0.106	0.557	0.825	0.936
Reducing sugars	1.21	1.11	1.17	1.15	0.045	0.134	0.824	0.939
Fatty acid intake (g/d)								
C16:0	46.7	70.6	59.0	58.3	2.34	< 0.001	0.816	0.899
C18:0	7.1	19.7	13.5	13.3	0.59	< 0.001	0.816	0.866
C18:1 *cis*-9	45.2	118.3	82.3	81.2	3.53	< 0.001	0.815	0.869
C18:2n-6	108.7	173.7	142.1	140.3	5.68	< 0.001	0.816	0.896
C18:3n-3	57.9	236.9	148.5	146.3	6.82	< 0.001	0.819	0.854
Total fatty acids	280.4	651.6	469.3	462.7	18.92	< 0.001	0.815	0.873
Apparent digestibility (% of intake)								
DM	78.8	79.2	79.4	78.6	0.54	0.626	0.306	0.786
OM	80.3	80.8	80.9	80.2	0.49	0.553	0.344	0.635
Crude protein	77.0	77.0	77.3	76.7	0.58	0.999	0.422	0.687
Crude fat	68.8	77.1	73.8	72.1	0.93	< 0.001	0.203	0.975
Gross energy	78.3	79.0	79.0	78.2	0.52	0.373	0.267	0.576
NDF	70.8	71.0	71.6	70.2	0.82	0.858	0.215	0.498
Starch	99.0	99.0	99.0	99.0	0.04	0.999	0.999	0.165

Table 7.3. The effect of *DGAT1* K232A polymorphism (AA or KK) on milk production and milk composition of lactating dairy cows fed diets with (LSO) or without (CON) linseed oil

	Diet		*DGAT1* genotype			*P*-value		
Item	CON	LSO	AA	KK	SEM	Diet	*DGAT1*	Diet × *DGAT1*
Milk production (kg/d)	24.1	26.3	26.7	23.7	1.15	0.202	0.078	0.686
FPCM[1] (kg/d)	26.8	27.3	27.2	26.9	1.08	0.726	0.816	0.628
Milk fat content (%)	4.93	4.44	4.22	5.16	0.102	0.002	< 0.001	0.803
Milk protein content (%)	3.66	3.48	3.44	3.70	0.063	0.045	0.005	0.529
Milk lactose content (%)	4.64	4.68	4.68	4.63	0.033	0.384	0.347	0.991
Fat yield (g/d)	1,156	1,126	1,102	1,180	45.0	0.643	0.230	0.583
Protein yield (g/d)	882	892	900	874	35.4	0.837	0.601	0.702
Lactose yield (g/d)	1,121	1,229	1,251	1,099	55.9	0.171	0.058	0.728
MUN (mg/dL)	13.3	7.8	10.9	10.2	0.39	< 0.001	0.195	0.652

[1] Fat- and protein-corrected milk = (0.337 + 0.116 × fat % + 0.06 × protein %) × milk yield (kg/d).

Table 7.4. The effect of *DGAT1* K232A polymorphism (AA or KK) on milk fatty acid composition of lactating dairy cows fed diets with (LSO) or without (CON) linseed oil

	Diet		*DGAT1* genotype			*P*-value		
FA (g/100 g of FA)	Control	Linseed	AA	KK	SEM	Diet	*DGAT1*	Diet × *DGAT1*
C4:0	3.40	3.58	3.52	3.46	0.067	0.068	0.505	0.282
C6:0	2.29	2.00	2.12	2.17	0.039	< 0.001	0.451	0.198
C8:0	1.32	1.03	1.17	1.18	0.029	< 0.001	0.732	0.280
C10:0	3.15	2.15	2.67	2.63	0.086	< 0.001	0.738	0.258
C12:0	3.69	2.39	3.06	3.01	0.106	< 0.001	0.718	0.440
iso C14:0	0.09	0.07	0.08	0.08	0.003	< 0.001	0.854	0.580
C14:0[1]	11.8	9.3	10.8	10.3	0.17	< 0.001	0.026	0.022
C14:1 *cis*-9	1.03	0.76	0.83	0.96	0.032	< 0.001	0.008	0.900
iso C15:0	0.26	0.20	0.23	0.23	0.006	< 0.001	0.964	0.893
anteiso C15:0	0.44	0.37	0.41	0.40	0.009	< 0.001	0.804	0.901
C15:0[2]	1.06	0.81	0.87	1.01	0.024	< 0.001	< 0.001	0.037
iso C16:0	0.22	0.18	0.20	0.19	0.007	< 0.001	0.543	0.655
C16:0	32.4	22.4	26.0	28.9	0.43	< 0.001	< 0.001	0.852
C16:1 *trans*-9	0.18	0.17	0.18	0.17	0.005	0.448	0.448	0.861
C16:1 *cis*-9	1.59	1.11	1.20	1.50	0.043	< 0.001	< 0.001	0.409
iso C17:0	0.37	0.34	0.37	0.35	0.006	0.004	0.027	0.635
anteiso C17:0	0.42	0.35	0.39	0.38	0.008	< 0.001	0.329	0.689
C17:0	0.61	0.50	0.56	0.55	0.007	< 0.001	0.873	0.268
C17:1 *cis*-9	0.24	0.19	0.21	0.22	0.010	< 0.001	0.471	0.840
C18:0	9.0	13.7	11.5	11.2	0.20	< 0.001	0.341	0.607
C18:1 *trans*-6	0.19	0.51	0.37	0.33	0.012	< 0.001	0.075	0.483
C18:1 *trans*-9	0.15	0.30	0.23	0.22	0.007	< 0.001	0.194	0.726
C18:1 *trans*-10	0.19	0.49	0.33	0.35	0.032	< 0.001	0.623	0.572
C18:1 *trans*-11	0.88	2.60	1.77	1.71	0.104	< 0.001	0.654	0.754
C18:1 *trans*-15 + C18:1 *cis*-11	0.74	1.25	1.02	0.97	0.021	< 0.001	0.147	0.794
C18:1 *cis*-9[3]	18.2	23.7	21.9	20.0	0.54	< 0.001	0.016	0.492
C18:1 *cis*-12[4]	0.21	0.57	0.41	0.37	0.012	< 0.001	0.012	0.098
C18:1 *cis*-13	0.10	0.20	0.15	0.15	0.005	< 0.001	0.484	0.567
C18:2 *cis*-9, *trans*-11	0.42	1.01	0.76	0.68	0.046	< 0.001	0.238	0.399

Table 7.4. Continued

FA (g/100 g of FA)	Control	Linseed	AA	KK	SEM	Diet	*DGAT1*	Diet × *DGAT1*
C18:2n-6	1.71	1.67	1.79	1.60	0.053	0.597	0.015	0.620
C18:3n-3	0.47	0.75	0.65	0.57	0.023	< 0.001	0.017	0.478
C18:3n-6	0.07	0.06	0.06	0.06	0.002	< 0.001	0.067	0.397
C20:0	0.13	0.14	0.14	0.14	0.002	0.002	0.634	0.158
C20:1 cis-11	0.04	0.10	0.07	0.07	0.008	< 0.001	0.969	0.419
C20:2n-6	0.04	0.02	0.03	0.03	0.002	< 0.001	0.625	0.807
C20:3n-6	0.09	0.05	0.06	0.07	0.003	< 0.001	0.103	0.535
C20:4n-3	0.01	0.04	0.02	0.02	0.003	< 0.001	0.499	0.821
C20:4n-6	0.11	0.07	0.09	0.09	0.003	< 0.001	0.203	0.394
C20:5n-3	0.05	0.05	0.05	0.05	0.001	0.179	0.651	0.651
C22:0	0.05	0.04	0.04	0.05	0.002	< 0.001	0.016	0.661
C22:5n-3	0.09	0.07	0.08	0.08	0.003	< 0.001	0.483	0.196
C24:0	0.03	0.01	0.01	0.03	0.003	< 0.001	0.019	0.576
OBCFA[5]	3.46	2.83	3.09	3.19	0.056	< 0.001	0.090	0.090
SFA[6]	70.8	59.6	64.1	66.2	0.68	< 0.001	0.034	0.353
MUFA[7]	23.0	30.7	27.6	26.0	0.59	< 0.001	0.062	0.451
PUFA[8]	3.02	3.76	3.56	3.22	0.107	< 0.001	0.030	0.844
n-6 to n-3 ratio[9]	3.20	2.06	2.63	2.63	0.039	< 0.001	0.903	0.467

[1] Diet × *DGAT1* interaction; CON AA = 12.35, LSO AA = 9.33, CON KK = 11.24, LSO KK = 9.34.

[2] Diet × *DGAT1* interaction; CON AA = 0.96, LSO AA = 0.78, CON KK = 1.17, LSO KK = 0.84.

[3] C18:1 *cis*-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12, as these 2 FA could not be separated in the analysis. The portion of C18:1 *trans*-12 is considered to be negligible, as this FA is always present in small amounts.

[4] Diet × *DGAT1* interaction; CON AA = 0.22, LSO AA = 0.60, CON KK = 0.20, LSO KK = 0.53.

[5] Sum of all odd- and branched-chain fatty acids (i.e., *iso* C14:0, *iso* C15:0, *anteiso* C15:0, C15:0, *iso* C16:0, *iso* C17:0, *anteiso* C17:0, and C17:0). Diet × *DGAT1* interaction; CON AA = 3.36, LSO AA = 2.83, CON KK = 3.56, LSO KK = 2.83.

[6] Sum of all saturated fatty acids.

[7] Sum of all mono-unsaturated fatty acids.

[8] Sum of all poly-unsaturated fatty acids.

[9] Ratio between the sum of all n-6 FA (i.e., C18:2n-6, C18:3n-6, C20:3n-6, and C20:4n-6) and the sum of all n-3 FA (i.e., C18:3n-3, C20:4n-3, C20:5n-3, and C22:5n-3) reported in this table.

RESULTS AND DISCUSSION

Intake and digestibility of nutrients

Nutrient intake, DMI, fatty acid (**FA**) intake, and apparent total-tract digestibility of nutrients were not affected by *DGAT1* polymorphism and diet × *DGAT1* interaction. Feeding the LSO diet resulted in an increased crude fat intake ($P < 0.001$), intake of individual FA ($P < 0.001$), and intake of total FA ($P < 0.001$) compared to the CON diet (Table 7.2). These results are in line with the difference in chemical composition between the LSO diet and CON diet; linseed oil increased the dietary fat content from 34 g/kg DM to 56 g/kg DM (Table 7.1). The LSO diet (2.3% linseed oil on DM basis) increased apparent total-tract digestibility of crude fat compared to the CON diet ($P < 0.001$), but the apparent total-tract digestibility of the other nutrients was unaffected (Table 7.2). The increased crude fat digestibility of the LSO diet may have been the result of UFA from linseed having a higher total-tract digestibility than SFA (Van Zijderveld et al., 2011b). Several studies also reported the effect of linseed oil on apparent total-tract digestibility of nutrients, but the results reported are variable. Similar to this study, Benchaar et al. (2012) did not find an effect of adding increasing amounts of linseed oil to the diet [i.e., grass silage and corn silage; 50:50 forage:concentrate (**F:C**); DM basis] on apparent total-tract digestibility of DM, OM, CP, NDF, starch and GE. Martin et al. (2008), however, observed a decreased DM, OM, and NDF digestibility when supplementing 5.7% linseed oil to a forage rich diet (i.e., corn silage and grass hay; 65:35 F:C; DM basis). Ueda et al. (2003) reported an increase in OM and NDF digestibility when 3% linseed oil was added to a forage rich diet (i.e., grass hay; 65:35 F:C; DM basis), whereas the digestibility of these nutrients decreased when 3% linseed oil was added to a concentrate rich diet (35:65 F:C; DM basis). Benchaar et al. (2015) observed a decreased DM, OM, NDF, and GE digestibility when 4% linseed oil was added to a corn silage-based diet (60:40 F:C; DM basis), whereas DM, OM, and GE digestibility increased and NDF digestibility was unaffected when linseed oil was supplemented to a red clover silage-based diet (60:40 F:C; DM basis). Taken together, these results suggest that the effect of linseed oil on nutrient digestibility may vary with the source of forage in the basal diet, the forage to concentrate ratio, as well as the amount of linseed oil added.

Lactation performance and milk fatty acid profile

No diet × *DGAT1* interaction effect on milk production and milk composition was observed. Compared with the *DGAT1* AA genotype, the *DGAT1* KK genotype was associated with a higher milk fat and protein content ($P < 0.001$ and $P = 0.005$, respectively; Table 7.3), and tended to have a lower milk yield and lactose yield. The FPCM yield did not differ between the *DGAT1* KK and *DGAT1* AA genotype, which is consistent with a similar DMI, nutrient intake, and gross energy intake (**GEI**) between the 2 *DGAT1* genotypes. The major effect of *DGAT1* on milk production traits has been often observed, with the K allele associated with a higher milk fat and protein content, but lower milk production than the A allele (e.g., Schennink et al., 2007; Bovenhuis et al., 2015) which is in line with the present study. Many studies also reported the K allele to be associated with a higher fat yield and lower protein yield, although the reduced protein yield is not consistently reported (e.g., Näslund et al., 2008). This is not confirmed by the results of the present study (Table 7.3). In the present study, compared with the *DGAT1* AA

genotype, fat yield of the *DGAT1* KK genotype was only numerically higher (1,180 versus 1,102 g/d) and protein yield numerically lower (874 versus 900 g/d).

The LSO diet resulted in a decreased milk fat content, milk protein content, and MUN ($P < 0.045$; Table 7.3). The decrease in milk protein content was also observed by Benchaar et al. (2012, 2015) and may be the result of a dilution effect rather than a direct negative effect of the increased dietary fat content (Schroeder et al., 2004), as the milk yield numerically increased and protein yield was unaffected by the linseed oil. The decreased MUN content may have been a consequence of the lower CP content of the LSO diet. In general, a decreased MUN content is associated with an improved milk N efficiency (Spek et al., 2013), and the significantly higher milk N efficiency with the LSO diet (27.5%) compared with the CON diet (24.6%; discussed in section *energy and nitrogen retention*) diet is in line with the decrease in MUN. The decrease in milk fat content in the present study is in agreement with Martin et al. (2008), Ferlay et al. (2013), and Benchaar et al. (2015), but in contrast to others (e.g., Benchaar et al., 2012; Livingstone et al., 2015). The response of milk fat content to linseed oil is the result of the balance between a decrease in de novo FA synthesis and an increase in exogenous FA uptake and secretion by the mammary gland (Schroeder et al., 2004). The UFA from linseed oil have been reported to inhibit ruminal fibrolytic activity and subsequently decrease production of acetate and butyrate, which are precursors of de novo synthesized short- and medium-chain MFA (Bauman and Griinari, 2003). Also, these dietary UFA and several *trans* FA, the latter are formed from ruminal biohydrogenation of the UFA, are potent inhibitors of de novo milk fat synthesis in the mammary gland (Bauman et al., 2011). In turn, dietary UFA increase the content of long-chain UFA in milk (Schroeder et al., 2004; Benchaar et al., 2012). In the present study, the inhibitory effects of linseed oil on de novo synthesized FA is greater than the increase in long-chain FA in milk (Table 7.4).

A diet × *DGAT1* interaction for the individual MFA C14:0 and C15:0 was observed (Table 7.4). Previously, Van Vuuren et al. (2013) also observed an interaction between *DGAT1* and linseed oil supplementation for certain MFA, although these were different from the ones reported in the present study, namely C18:0 and C20:1n-11. In addition, compared with the *DGAT1* AA genotype, the *DGAT1* KK genotype was associated with a higher C14:1 *cis*-9, C16:0, C16:1 *cis*-9, C22:0, C24:0, and total SFA content ($P < 0.034$), and a lower *iso* C17:0, C18:1 *cis*-9, C18:1 *cis*-12, C18:2n-6, C18:3n-3, and total PUFA content ($P < 0.030$) in the milk (Table 7.4). These results are largely in agreement with data in the literature. According to Schennink et al. (2007), Van Arendonk et al. (2009), Duchemin et al. (2013), and Bovenhuis et al. (2016) the *DGAT1* K allele is associated with a larger fraction of C16:0, and smaller fractions of C14:0 (also reported by Lu et al., 2015), and C18 UFA in milk. In addition, Duchemin et al. (2013) reported an increase in milk C14:1 *cis*-9, C16:1 *cis*-9, and total SFA, and a decrease in total milk UFA for the *DGAT1* K allele. Furthermore, according to Van Arendonk et al. (2009), Lu et al. (2015), and Bovenhuis et al. (2016), the *DGAT1* K allele is associated with increased contents of C15:0 and C17:0 in milk.

The LSO diet of the present study resulted in a lower content of short- and medium-chain fatty acids (**SMCFA**) in the milk, with the exceptions of C4:0 and C16:1 *trans*-9 (Table 7.4). These results are generally in line with Chilliard et al. (2009), Benchaar et al. (2012), and Saliba

et al. (2014). As previously stated, dietary UFA and several *trans* FA are potent inhibitors of de novo milk fat synthesis in the mammary gland, and the UFA from the linseed oil potentially inhibit ruminal fibrolytic activity, thereby decreasing the precursors for SMCFA synthesis (Bauman and Griinari, 2003). Milk SMCFA are synthesized de novo in the mammary gland primarily from acetate, and ruminal acetate proportion was decreased upon feeding linseed oil (discussed in section *ruminal fermentation*). Only MFA C4:0 does not require acetate for its production as it can be produced directly from β-hydroxybutyrate derived from the blood (Bernard et al., 2008).

The LSO diet resulted in lower contents of all odd- and branched-chain fatty acids (**OBCFA**) in milk compared the CON diet ($P < 0.004$; Table 7.4). This is in line with Chilliard et al. (2009) and, in regard to C15:0 and C17:0, also with Benchaar et al. (2012). The odd-chain FA C15:0 and C17:0 are mainly synthesized de novo by ruminal bacteria from propionate. However, despite the increase in propionate proportions (Table 7.7), this ruminal synthesis of C15:0 and C17:0 may decrease when cows are fed dietary fat. This is because rumen bacteria preferably use preformed FA available in the ruminal environment (Byers and Schelling, 1988).

The intake of C18:3n-3 increased with the LSO diet ($P < 0.001$; Table 7.2). This increase is associated with an increase of C18:3n-3, as well as the biohydrogenation intermediates (e.g., C18:1 *trans*-11) and end-products (i.e., C18:0) in milk upon feeding linseed oil ($P < 0.001$; Table 7.4), suggesting high levels of biohydrogenation, and is in line with Benchaar et al. (2012), Ferlay et al. (2013), and Livingstone et al. (2015). Despite the increased intake of C18:2n-6 with the LSO diet ($P < 0.001$; Table 7.2), C18:2n-6 does not increase in milk upon feeding linseed oil, which is in agreement with Ferlay et al. (2013) and Saliba et al. (2014). Milk C18:2 *cis*-9, *trans*-11 did increase with the LSO diet ($P < 0.001$; Table 7.4), suggesting high levels of biohydrogenation of C18:2n-6 in the rumen as well as increased endogenous production of C18:2 *cis*-9, *trans*-11 in the mammary gland using C18:1 *trans*-11 produced in the rumen as substrate (Griinari et al., 2000).

In the present study, both C18:1 *trans*-10 and C18:1 *trans*-11 increased with the LSO diet ($P < 0.001$; Table 7.4), which is consistent with Benchaar et al. (2012) and Saliba et al. (2014). The increase in C18:1 *trans*-11 content in milk may be the result of its production during the biohydrogenation of dietary C18:2n-6 and C18:3n-3. An increase in C18:1 *trans*-10 in milk is generally associated with milk fat depression, and occurs when low fiber diets or diets supplemented with PUFA rich plant oils are fed, resulting in a shift in rumen microbial composition and a changed biohydrogenation pathway (Griinari and Bauman, 1999). In the present study, milk fat content (%) decreased, but intake of NDF and starch, ruminal pH, and milk fat yield (g/d) were unaffected with the LSO diet (Tables 7.2, 7.3, and 7.7). The increase of C18:1 *trans*-11 was greater compared with C18:1 *trans*-10 as the C18:1 *trans*-11 to C18:1 *trans*-10 ratio increased with the LSO diet (i.e., 4.6 ± 0.19 for the CON diet and 6.0 ± 0.46 for the LSO diet; $P = 0.009$). This indicates that there was a change in the rumen biohydrogenation pathway, potentially the result of the increased rumen UFA load (Lock, 2010) causing changes in microbiota composition or activity, and thus changes in ruminal fermentation characteristics.

Energy and nitrogen retention

No diet × *DGAT1* interaction was observed for the energy balance characteristics of the cows (Table 7.5). The ME intake (**MEI**) to GEI ratio was lower for the *DGAT1* KK genotype (66.8%) compared with the *DGAT1* AA genotype (67.9%; $P = 0.023$). To the best of our knowledge, the effects of *DGAT1* on energy balance measured in CRC have not been quantified previously. The energy output in milk was unaffected by *DGAT1* in the present study, which is in agreement with Bovenhuis et al. (2015) who only observed a difference in milk energy output for the *DGAT1* AK genotype compared with *DGAT1* AA and KK genotypes in parity 1. Bovenhuis et al. (2015) suggested that, in view of the absence of differences in energy output in milk, no large differences in energy balance between cows with different *DGAT1* alleles would be expected. In the present study, total energy retention was unaffected by *DGAT1* (Table 7.5), supporting the hypothesis of Bovenhuis et al. (2015). This is in agreement with Banos et al. (2008), who reported only a small positive effect of the *DGAT1* K allele on cumulative effective energy balance estimated based on live weight and BCS.

The LSO diet resulted in a decreased energy loss in the form of CH_4 ($P = 0.022$; Table 7.5) and an increased MEI to GEI ratio ($P = 0.006$; Table 7.5). The decreasing effect of linseed oil on energy loss in the form of CH_4, together with the tendency of reduced GE digestibility, may explain the difference in MEI to GEI ratio, because the GEI was unaffected by feeding linseed oil (Table 7.5).

The mean N balance was not affected by diet, *DGAT1*, and diet × *DGAT1* interaction (Table 7.5). Milk N efficiency tended ($P = 0.076$) to be higher for the *DGAT1* AA genotype (26.6%) compared with the *DGAT1* KK genotype (25.5%). A few studies have reported the effect of dietary linseed oil on N balance, which have been reviewed by Hristov and Jouany (2005). These authors indicated that the effects of fat supplementation on the N balance of cattle are inconsistent in the literature. In the present study, the LSO diet reduced N excretion in manure ($P = 0.010$; Table 7.5), which is similar to Benchaar et al. (2015) and might be the result of the tendency observed for a lower N intake of cows fed the LSO diet. The LSO diet resulted in a greater efficiency of dietary N utilization for milk N production ($P < 0.001$). This is in contrast with Benchaar et al. (2015), who observed no effect of linseed oil supplementation on N efficiency.

Table 7.5. The effect of *DGAT1* K232A polymorphism (AA or KK) on energy and nitrogen balance of lactating dairy cows fed diets with (LSO) or without (CON) linseed oil

	Diet		*DGAT1* genotype			*P*-value		
Item	CON	LSO	AA	KK	SEM	Diet	*DGAT1*	Diet × *DGAT1*
Metabolic BW[1] ($kg^{0.75}$)	123	123	123	122	2.0	0.951	0.736	0.632
Energy balance (kJ/kg of $BW^{0.75}$ per day, unless otherwise stated)								
GEI[2]	2,662	2,665	2,667	2,660	78.0	0.984	0.950	0.964
CH_4 production	185	169	178	176	4.7	0.022	0.697	0.591
Energy in manure	704	684	680	708	23.4	0.549	0.406	0.681
DEI[3]	2,083	2,103	2,108	2,079	61.7	0.824	0.744	0.730
MEI[4]	1,773	1,811	1,809	1,776	52.7	0.614	0.668	0.981
MEI to GEI ratio (%)	66.7	68.0	67.9	66.8	0.32	0.006	0.023	0.346
Heat production[5]	982	968	974	976	19.6	0.611	0.939	0.795
Energy in milk	678	711	701	688	23.0	0.309	0.688	0.475
ER total[6]	114	132	134	112	29.5	0.657	0.615	0.493
ER protein[7]	96	88	95	89	6.9	0.404	0.518	0.418
ER fat[8]	17	44	38	23	24.2	0.437	0.667	0.544
Nitrogen balance (mg/kg of $BW^{0.75}$ per day)								
N intake	4,556	4,232	4,409	4,380	134.0	0.095	0.878	0.974
N manure	2,663	2,360	2,477	2,545	79.4	0.010	0.548	0.960
N milk	1,120	1,160	1,168	1,111	37.1	0.455	0.282	0.471
N condense + N acid	121	116	117	120	7.8	0.684	0.817	0.152
N balance	652	597	646	603	46.6	0.404	0.518	0.418
N efficiency[9]	24.6	27.5	26.6	25.5	0.44	< 0.001	0.076	0.262

[1] The mean BW per cow per balance period was used to calculate metabolic BW ($BW^{0.75}$).

[2] GEI = Gross energy intake.

[3] DEI (digestible energy intake) = GEI × apparent total-tract digestibility of GE (% of intake).

[4] MEI (metabolizable energy intake) = GEI - methane production - energy in manure (feces and urine).

[5] Heat production (kJ/d) = 16.175 × VO_2 (L/d) + 5.021 × VCO_2 (L/d), where VO_2 = volumes of O_2 consumed, and VCO_2 = volumes of CO_2 produced (Gerrits et al.,2015).

[6] Energy retention total = MEI - heat production - energy in milk.

[7] Energy retention protein = protein gain (N × 6.25) × 23.6 kJ/g (energetic value of protein).

[8] Energy retention fat = energy retention total - energy retention protein.

[9] N efficiency = N milk / N feed (%).

Table 7.6. The effect of *DGAT1* K232A polymorphism (AA or KK) on gaseous exchange of lactating dairy cows fed diets with (LSO) or without (CON) linseed oil

	Diet		*DGAT1* genotype			*P*-value		
Item	CON	LSO	AA	KK	SEM	Diet	*DGAT1*	Diet × *DGAT1*
O_2 consumption (g/d)	7,987	7,926	7,996	7,917	241.3	0.859	0.819	0.922
CO_2 production (g/d)	12,351	12,001	12,184	12,168	379.2	0.517	0.975	0.997
Respiratory quotient	1.12	1.10	1.10	1.11	0.005	< 0.001	0.178	0.580
H_2								
g/d	1.79	1.65	1.71	1.73	0.097	0.310	0.906	0.196
g/kg of DMI	0.103	0.096	0.098	0.101	0.0063	0.455	0.748	0.286
g/kg of FPCM[1]	0.069	0.061	0.064	0.066	0.0041	0.211	0.718	0.460
CH_4								
g/d	409	375	396	388	13.7	0.085	0.658	0.863
g/kg of DMI	22.9	21.5	22.3	22.1	0.32	0.006	0.688	0.572
g/kg of FPCM[1]	15.5	13.8	14.7	14.6	0.35	0.002	0.925	0.430
% of GEI[2]	6.96	6.38	6.70	6.64	0.096	< 0.001	0.680	0.552
CH_4 to CO_2 ratio[3]	0.033	0.031	0.032	0.032	0.0004	0.001	0.248	0.552
H_2 to CH_4 ratio[4]	0.0045	0.0045	0.0044	0.0046	0.00028	0.926	0.660	0.352

[1] Fat- and protein-corrected milk = (0.337 + 0.116 × fat % + 0.06 × protein %) × milk yield (kg/d).

[2] Gross energy intake.

[3] Ratio between CH_4 (g/d) and CO_2 (g/d).

[4] Ratio between H_2 (g/d) and CH_4 (g/d).

Table 7.7. The effect of *DGAT1* K232A polymorphism (AA or KK) on ruminal pH, total VFA concentration, and VFA molar proportions of lactating dairy cows fed diets with (LSO) or without (CON) linseed oil[1]

	Diet		*DGAT1* genotype			*P*-value			
Item	CON	LSO	AA	KK	SEM	Diet	*DGAT1*	Diet × *DGAT1*	Time[2]
pH	6.76	6.76	6.75	6.77	0.028	0.929	0.575	0.147	< 0.001
Total VFA (m*M*)	93	93	94	92	2.1	0.913	0.536	0.469	< 0.001
VFA (% of total VFA)									
Acetate	67.4	66.6	67.1	66.9	0.18	0.004	0.425	0.502	< 0.001
Propionate	16.5	17.2	16.7	17.0	0.18	0.010	0.226	0.654	< 0.001
Butyrate	12.6	12.7	12.7	12.6	0.12	0.763	0.647	0.150	< 0.001
Isobutyrate	0.88	0.89	0.89	0.88	0.026	0.771	0.444	0.788	< 0.001
Valerate	1.19	1.20	1.20	1.19	0.016	0.432	0.559	0.746	< 0.001
Isovalerate	1.43	1.47	1.45	1.46	0.056	0.633	0.946	0.570	0.367
Acetate to Propionate ratio	4.16	3.93	4.08	4.01	0.051	< 0.001	0.236	0.766	< 0.001

[1] Values shown represent the mean of values on d 12 (i.e., 1 h before and 4 h after morning feeding).

[2] Time = effect of time of sampling on d 12; 1 h before and 4 h after morning feeding.

Table 7.8. The effect of *DGAT1* K232A polymorphism (AA or KK) on total concentrations and genus level relative abundances of archaea and bacteria in the rumen of lactating dairy cows fed diets with (LSO) or without (CON) linseed oil[1]

	Diet		*DGAT1* genotype			*P*-value		
Microbiota	CON	LSO	AA	KK	SEM	Diet	*DGAT1*	Diet × *DGAT1*
qPCR analysis								
Ruminal concentration (log_{10} 16S copies/mL of rumen fluid)								
Archaea	8.73	8.69	8.70	8.73	0.030	0.271	0.503	0.947
Bacteria	10.22	10.25	10.21	10.26	0.029	0.545	0.187	0.938
Archaea to bacteria ratio	0.034	0.028	0.032	0.030	0.0017	0.029	0.474	0.936
Universal 16S rRNA gene sequencing								
Relative abundance archaea (%)								
Methanobrevibacter[2]	7.7	7.3	7.5	7.5	0.40	0.537	0.965	0.095
Methanosphaera	0.40	0.40	0.42	0.39	0.025	0.919	0.466	0.290
Relative abundance bacteria (%)								
Acetitomaculum	2.0	1.8	2.1	1.7	0.15	0.450	0.069	0.596
Bifidobacterium[3]	0.28	0.39	0.47	0.19	0.088	0.366	0.039	0.068
Blautia	0.13	0.22	0.16	0.19	0.047	0.206	0.778	0.257
BS11 gut group, g-NA[4]	0.99	0.78	1.05	0.71	0.164	0.419	0.164	0.898
Butyrivibrio[5]	5.1	4.8	5.1	4.7	0.25	0.449	0.381	0.089
Christensenellaceae, g-NA	4.1	4.1	4.4	3.8	0.39	0.846	0.259	0.744
Desulfovibrio	0.09	0.09	0.09	0.08	0.016	0.901	0.551	0.850
Erysipelotrichaceae, Incertae Sedis	0.12	0.28	0.21	0.19	0.089	0.209	0.833	0.928
Erysipelotrichaceae, g-NA	0.38	0.24	0.19	0.44	0.120	0.376	0.151	0.986
Fibrobacter	0.65	0.95	0.92	0.69	0.153	0.150	0.254	0.419
Lachnospiraceae, Incertae Sedis	2.8	2.9	2.7	3.0	0.19	0.638	0.194	0.781
Lachnospiraceae, g-NA	1.7	1.8	1.6	1.9	0.22	0.679	0.236	0.727
Leuconostoc	0.11	0.09	0.08	0.12	0.025	0.472	0.209	0.484
Mogibacterium	1.6	1.5	1.5	1.6	0.11	0.784	0.264	0.381
Moryella	0.09	0.11	0.11	0.09	0.028	0.611	0.657	0.969
Prevotella	28.4	29.3	28.8	28.9	1.04	0.503	0.975	0.811
Prevotellaceae, g-NA	3.7	4.3	4.2	3.8	0.38	0.292	0.496	0.434
Pseudobutyrivibrio	0.72	0.81	0.76	0.77	0.070	0.434	0.935	0.660

Table 7.8. Continued

Microbiota	CON	LSO	AA	KK	SEM	Diet	DGAT1	Diet × DGAT1
RF16, g-NA	1.32	0.58	1.06	0.82	0.129	< 0.001	0.269	0.420
Rikenellaceae RC9 gut group	2.9	1.8	2.4	2.3	0.16	< 0.001	0.554	0.823
Ruminobacter	0.39	0.37	0.35	0.40	0.086	0.877	0.707	0.400
Ruminococcaceae, Incertae Sedis	1.4	1.5	1.4	1.6	0.23	0.989	0.526	0.630
Ruminococcaceae, g-NA	4.9	4.8	4.8	4.9	0.25	0.827	0.637	0.743
Ruminococcus6	10.1	9.5	9.0	10.7	0.65	0.443	0.078	0.087
Saccharofermentans	1.4	1.8	1.5	1.7	0.10	0.016	0.240	0.655
Schwartzia	0.07	0.07	0.06	0.08	0.016	0.997	0.373	0.472
Selenomonas	0.54	0.55	0.50	0.59	0.075	0.943	0.435	0.845
Streptococcus	0.34	0.34	0.33	0.34	0.063	0.952	0.860	0.465
Succiniclasticum	3.5	3.3	3.3	3.5	0.14	0.438	0.240	0.882
Sutterella	0.20	0.13	0.20	0.13	0.032	0.236	0.157	0.814
Treponema	0.13	0.14	0.13	0.15	0.035	0.930	0.705	0.860
Victivallis	0.05	0.09	0.06	0.08	0.017	0.112	0.618	0.598
Xylanibacter	3.3	3.2	3.6	2.9	0.27	0.884	0.097	0.419

[1] Values shown represent d 17, 4 h after morning feeding, and that of individual bacterial and archaeal genera that were (a) consistent in all animals and (b) were > 0.05% in terms of relative abundance.

[2] Diet × DGAT1 interaction; CON AA = 8.1, LSO AA = 6.8, CON KK =7.2, LSO KK = 7.8.

[3] Diet × *DGAT1* interaction; CON AA = 0.30, LSO AA = 0.65, CON KK = 0.25, LSO KK = 0.14.

[4] Taxonomic association of the genus level phylogenetic grouping could not be assigned.

[5] Diet × *DGAT1* interaction; CON AA = 5.5, LSO AA = 4.6, CON KK = 4.5, LSO KK = 4.9.

[6] Diet × *DGAT1* interaction; CON AA = 10.1, LSO AA = 7.8, CON KK = 10.2, LSO KK = 11.0.

Gas exchange

No diet × *DGAT1* interaction was found for O_2 consumption and CO_2, CH_4, and H_2 production, and gaseous ratios (Table 7.6). None of the gas exchange characteristics were affected by *DGAT1*. The results indicate that *DGAT1* does not affect CH_4 emission of dairy cows nor the response in CH_4 emission of dairy cows to dietary linseed oil. As mentioned before, Van Engelen, S. (Wageningen University & Research, Wageningen, The Netherlands; unpublished data) performed a GWAS to determine regions of the bovine genome that are associated with predicted CH_4 yield (g/kg DMI) using the equations published by Dijkstra et al. (2011) and Van Engelen et al. (2015). The association with *DGAT1* was significant in the GWAS for predicted CH_4 yield, suggesting that the K allele is associated with higher predicted CH_4 yield. The results of the present study suggest that the proposed relation between *DGAT1* and CH_4 based on predicted CH_4 yield using MFA is not in line with actual observations on CH_4 emission. Presumably, the relationship observed by Van Engelen, S. (Wageningen University & Research, Wageningen, The Netherlands; unpublished data) is due to the association between *DGAT1* and the MFA that were used to predict CH_4 yield. The CH_4 prediction equations, used in the GWAS by Van Engelen, S. (Wageningen University & Research, Wageningen, The Netherlands; unpublished data), included several C18 UFA (both *cis* and *trans* isomers), which were affected by *DGAT1* in the present study (Table 7.4) as well as in other studies (e.g., Schennink et al., 2007; Duchemin et al., 2013).

The LSO diet did not affect H_2 production (g/d), yield (g/kg DMI), and intensity (g/kg FPCM), which is consistent with Veneman et al. (2015). Similarly, the unaffected O_2 consumption and CO_2 production upon the LSO diet in the present study is consistent with Livingstone et al. (2015). The LSO diet did not affect the H_2 to CH_4 ratio, but decreased the respiration quotient (**RQ**; $P < 0.001$), CH_4 emissions (i.e., g/kg DMI, g/kg FPCM, and % of GEI; $P < 0.006$), and CH_4 to CO_2 ratio ($P = 0.001$; Table 7.6). The RQ is lower or equal to 1.0 when only substrate oxidation occurs (Gerrits et al., 2015). However, de novo fatty acid synthesis and ruminal anaerobic fermentation of dietary carbohydrates can result in a RQ larger than 1.0 (Gerrits et al., 2015). The decreased RQ found for the LSO diet may result from the higher dietary fat intake and fat digestibility (Table 7.2), because fat is not fermented in the rumen (Beauchemin et al., 2008) and an increased absorption of FA reduces the need to synthesize FA from carbohydrates in the intermediary metabolism.

The decrease in CH_4 emissions with the LSO diet in the present study is in line with previous studies (e.g., Martin et al., 2008; Benchaar et al., 2015). According to Grainger and Beauchemin (2011), a 10 g/kg of DM increase in dietary fat should result in a decreased CH_4 yield by 1 g/kg of DMI in cattle. In the present study, the average increase in dietary fat content (22 g/kg DM) was associated with a significant decrease of 1.4 g/kg DMI in CH_4 yield. This is lower than that reported by Grainger and Beauchemin (2011) in their meta-analysis, but higher than some other studies (e.g., Veneman et al., 2015). Fat analyses of the feed residuals suggest that the cows in the present experiment were not selecting against the concentrate supplemented with linseed oil included in the TMR (results not shown; fat content in residual feed was not different from the fat content in TMR offered). Benchaar et al. (2015) suggested that the forage of the basal diets affected the extent of CH_4 mitigation of linseed oil supplementation. For the

red-clover-based diet, an increase in dietary fat content of 27 g/kg of DM decreased CH_4 yield with 1.7 g/kg of DMI, whereas for the corn-silage-based diet, an increase in dietary fat content of 36 g/kg of DM decreased CH_4 yield with 4.0 g/kg of DMI (Benchaar et al., 2015). The forages used in the present experiment (i.e., a mixture of grass silage and corn silage) may have influenced the extent of CH_4 mitigation of dietary linseed oil. In addition, the 2 diets differed in CP content (194 and 184 g/kg of DM for CON and LSO, respectively; Table 7.1), but the effect of dietary CP on CH_4 emission is reported variable in literature. Ellis et al. (2009) found a positive relationship between dietary CP content and CH_4 emission of beef cattle, whereas Reynolds et al. (2010) did not observe differences in CH_4 yield with different dietary CP contents. The 10 g/kg of DM difference in dietary CP content between the 2 diets in the present study may not be expected to significantly affect CH_4 emission, especially because the dietary CP content (i.e., higher than 180 g/kg of DM; Table 7.1) was above calculated requirements for the dairy cows involved. Overall, it seems likely that the basal diet may have played an important role in the CH_4 mitigation effect of dietary linseed oil in the present study.

Ruminal fermentation

Three rumen fluid samples per cow within diet were collected: 1 h before and 4 h after morning feeding on d 12, and 4 h after morning feeding on d 17. None of the ruminal fermentation parameters significantly differed between d 12 and 17 at 4 h after morning feeding, with the exception of ruminal pH ($P < 0.001$, 6.56 and 6.68, respectively). To have a balanced design as well as the ability to distinguish between time and day effects, it was decided to use only the samples of 1 h before and 4 h after morning feeding of d 12 for ruminal fermentation data analysis.

Time of sampling (i.e., 1 h before feeding or 4 h after feeding) affected pH, total VFA concentration, and VFA molar proportions ($P < 0.001$; Table 7.7), except for isovalerate. As expected, ruminal pH declined after feeding and total VFA concentration was higher 4 h after feeding compared to 1 h before feeding (data not shown). Total VFA concentration was unaffected by diet, *DGAT1*, and diet × *DGAT1* interaction. The lack of an effect of the LSO diet is in agreement with Ueda et al. (2003), Doreau et al. (2009), and Benchaar et al. (2012), and all these results are consistent with the lack of effect of diet, *DGAT1*, and diet × *DGAT1* interaction on ruminal pH in the present study.

In general, ruminal pH in the present study seems relatively high compared to the total VFA concentration (Table 7.7). According to the prediction equation derived by Dijkstra et al. (2012), pH should be around 6.2 with a VFA concentration of 100 m*M*. In the present study, however, pH was around 6.76 with a VFA concentration of approximately 93 m*M*, probably as a result of collecting rumen fluid with the OST sampling technique. Duffield et al. (2004) and Wang et al. (2016) reported a higher ruminal pH when sampling rumen fluid using OST compared with rumen cannulation. Using the OST technique in the present study resulted in collection of rumen fluid from the ventral cranial part of the rumen or the reticulum. The pH within these regions of the reticulo-rumen is generally higher compared with other sites (e.g., Duffield et al., 2004; Li et al., 2009) as a result of rumination and the consequent entry of saliva.

The proportions of the individual VFA were unaffected by *DGAT1* and diet × *DGAT1* interaction (Table 7.7). Feeding the LSO diet resulted in an increased proportion of propionate ($P = 0.010$), whereas the proportion of acetate and the acetate to propionate ratio decreased compared with the CON diet ($P = 0.004$ and $P < 0.001$, respectively). Several other studies have also reported a shift in VFA pattern towards proportionally more propionate and less acetate when a linseed oil containing diet was fed (e.g., Benchaar et al., 2012, 2015; Ivan et al., 2013; Martin et al., 2016), which is also consistent with the reduction of CH_4 emission observed for the LSO diet (Table 7.6). This shift in fermentation toward propionate at the expense of acetate supports the key role of the redox state of NAD in rumen fermentation and CH_4 production (Van Lingen et al., 2017). As discussed before, dietary linseed oil may reduce fiber degradation in the rumen, whereas degradation of other carbohydrates (e.g., starch) remains unaffected (Doreau and Chilliard, 1997). This results in proportionally more propionate and less acetate. The apparent total-tract digestibility of NDF in the present study was unaffected by the LSO diet, but a decrease in rumen fiber digestion can be partially compensated for by digestion in the large intestine (Martin et al., 2008).

Rumen microbiota

Bacterial and archaeal concentrations. The concentration of archaea and bacterial 16S rRNA genes in the rumen were unaffected by diet, *DGAT1*, and diet × *DGAT1* interaction (Table 7.8). An absence of effect of linseed oil on archaeal concentrations is in agreement with Veneman et al. (2015) and Martin et al. (2016). Similar to Martin et al. (2016), despite the unaffected archaeal concentration, CH_4 emission decreased with the LSO diet. The archaea to bacteria ratio decreased ($P = 0.029$, Table 7.8) with the LSO diet. This reduced ratio suggests that per unit substrate fermented by bacteria, a smaller archaeal concentration is present to form CH_4, helping to explain the observed reduction in CH_4 emission when feeding the LSO diet.

Numerous studies have repeatedly failed to find a correlation between CH_4 emission and archaeal concentration (e.g., Morgavi et al., 2010). In the present study, archaeal concentration (log_{10} 16S copies / mL rumen fluid) was not related to CH_4 production, but was related to CH_4 yield ($r = 0.34$, $P = 0.019$), and tended to be related with CH_4 intensity ($r = 0.28$, $P = 0.055$) without considering effects of linseed oil and *DGAT1*. Additionally, the archaea to bacteria ratio was not related to CH_4 production, but was related to CH_4 yield ($r = 0.43$, $P = 0.002$) and CH_4 intensity ($r = 0.48$, $P = 0.001$) without considering effects of linseed oil and *DGAT1*. For both CH_4 yield and CH_4 intensity, the archaea to bacteria ratio provided the strongest correlation, which is for CH_4 yield in agreement with Wallace et al. (2014).

Bacterial and archaeal composition. Bacteria (91.8 ± 2.1% of the obtained 16S rRNA gene sequences) were represented by 1,077 different OTU whereas the archaea (7.9 ± 2.0% of the 16S rRNA sequences) were represented by 16 different OTU. In agreement with previous studies, the total number of bacterial OTU was much higher than archaeal ones (e.g., Kittelman et al., 2013; Veneman et al., 2015). The 1,093 OTU could be summarized to 89 different genus-level phylogenetic groupings (87 for bacteria and 2 for archaea). The rumen bacterial and archaeal community as a whole (i.e., PERMANOVA results) tended to be affected by diet ($P = 0.081$), but was not affected by *DGAT1* ($P = 0.326$) and diet × *DGAT1* interaction

(P = 0.365). We also individually analyzed the relative abundances of bacterial and archaeal genera to examine if these were affected by diet, *DGAT1*, and diet × *DGAT1* interaction (Table 7.8). This was done because changes in taxa of low relative abundance can be masked when the total rumen microbiota is analyzed as a whole.

Methanobrevibacter was the most abundant archaeal genus, which is in line with other studies (e.g., Janssen and Kirs, 2008; Veneman et al., 2015). Both of the detected archaeal genera in this study were unaffected by diet, *DGAT1*, and diet × *DGAT1* interaction, with the exception of *Methanobrevibacter* for which a tendency for a diet × *DGAT1* interaction was observed. The lack of an effect of *DGAT1* and of the interaction between *DGAT1* and dietary linseed oil on archaeal genera is in line with lack of an effect of these factors on CH_4 emissions in the present study (Table 7.6). The lack of an effect of linseed oil on both archaeal genera is in agreement with Veneman et al. (2015) and consistent with the unaffected archaeal concentrations in the present study. These results are also in agreement with Monosi et al. (2008), who demonstrated that the long-chain FA of dietary linseed oil (such as linolenic acid) do not affect archaeal concentrations and relative abundances.

Prevotella was the most abundant bacterial genus in the present study, which was reported by others as well (e.g., Henderson et al., 2013; Veneman et al., 2015). The relative abundance of *Bifidobacterium* (P = 0.039) was lower for the *DGAT1* KK genotype compared with the *DGAT1* AA genotype (Table 7.8). *Bifidobacterium* is a sugar fermenting bacteria (Trovatelli and Matteuzzi, 1997) producing acetate. However, intake of reducing sugars (Table 7.2) as well as the molar proportions of acetate (Table 7.7) was not affected by *DGAT1*.

With the LSO diet, *Rikenellaceae* RC9 gut group and a non-assigned genus (**g-NA**) within the *RF16* decreased (P < 0.001 for both), whereas *Saccharofermentans* increased (P = 0.016; Table 7.8). There is no cultured representative of *RF16*, g-NA, and hence the lack of knowledge with respect to the physiology of this group makes it unclear why their relative abundance decreased with the LSO diet. The metabolic function and role of the *Rikenellaceae* RC9 gut group in the rumen microbiome remains to be defined, but Zened et al. (2013) already demonstrated that supplementation of sunflower oil decreased its relative abundance. *Saccharofermentans* is a sugar fermenting bacteria (Chen et al., 2010). The reducing sugar content and intake of the LSO diet was lower compared with the CON diet (Tables 7.1 and 7.2), which is not consistent with the increased relative abundance of sugar fermenting bacteria.

The PUFA present in linseed oil are believed to have a toxic effect on cellulolytic bacteria (Nagaraja et al., 1997; Martin et al., 2010). This negative effect of linseed supplementation on cellulolytic bacteria has not been confirmed in vivo in dairy cows by Veneman et al (2015). Additionally, in the present study, no effect was observed on cellulolytic genera (such as *Fibrobacter*, *Butyrivibrio*, and *Ruminococcus*, P > 0.150, Table 7.8). This indicates that dietary linseed oil at the level used in the present study does not have a toxic effect on cellulolytic bacteria, and thus does not affect their relative abundance, and that this is therefore not the mode of action of dietary linseed oil to decrease CH_4 production. The results of the present, however, do not reject the potential toxic effect of dietary linseed oil on the metabolic activity of cellulolytic bacteria.

Overall, the results indicate that several rumen bacterial genera were affected by dietary linseed oil, which could be linked to changes in dietary composition, differences in ruminal fermentation characteristics, and gaseous exchange. Further, several rumen bacterial genera were affected by *DGAT1*, but could not be linked with ruminal fermentation characteristics and gaseous exchange because the latter 2 were not affected by *DGAT1*. As a consequence, the biological implications of their change appear to be limited. Despite some bacterial genera being affected by diet, *DGAT1*, and diet × *DGAT1* interaction (i.e., the latter only tendencies), the bacterial and archaeal community as a whole was not significantly affected. This is perhaps because more than 75% of the bacterial genera analyzed were unaffected by diet, *DGAT1*, and diet × *DGAT1* interaction, and because the quantities of the affected bacterial genera were relatively small, therefore representing only a minor part of the rumen microbiota.

Implications

We acknowledge that the results of this study could have been different if the cows would have been fed ad libitum. We restricted feed intake to ensure similar feed intake between treatments, thus avoiding confounding effects of DMI on CH_4 production. Feed intake restriction ranged from 82 to 95% with an average of 92 ± 0.7%, with all cows being in positive energy balance and no difference in milk yield (25.5 ± 1.08 when fed ad libitum and 25.3 ± 1.16 when feed intake was restricted). We, therefore, consider the overall effect of restricted feeding to be minimal. However, when considering the individual treatments, this may be different. The LSO receiving cow was in 67% of the cases the one with the lowest ad libitum feed intake within a block, and thus the CON receiving cow was in 67% of the cases relatively more restricted in her feed intake. In other words, when fed ad libitum, the LSO diet could have resulted in a lower feed intake relatively to the CON diet. For *DGAT1*, we did not find an association between ad libitum feed intake and the *DGAT1* KK or AA genotype.

Additionally, we acknowledge that the absence of effects of *DGAT1* might be related to the number of animals used in the present study. Many genetic studies used hundreds to thousands of animals (e.g., Bovenhuis et al., 2016), whereas only 24 animals were used in the present study. It is known that *DGAT1* has major effects on milk yield and composition (such as milk fat content and milk protein content). The present study was able to confirm the difference in milk composition between the *DGAT1* KK and AA genotype, but for milk yield only a tendency was found. This shows that the number of animals in the present study might have been insufficient to find all known major effects of *DGAT1*. For marginal effects, such as CH_4 production which differed 8 g/d between the *DGAT1* AA and KK genotypes (2% relative difference), this study did not have sufficient power because of the relative small number of animals. Furthermore, no dairy cows with the heterozygous *DGAT1* AK genotype were included in the present study. We assumed the AK genotype would be in between the homozygous *DGAT1* AA and KK genotypes, because there is little evidence for dominance effects of *DGAT1* (Bovenhuis et al., 2015). Whether this is also the case for other parameters, as measured in the current study, remains to be investigated.

Overall, the results of the present study suggest that *DGAT1* does not affect enteric CH_4 emission and production pathways, but that it does affect traits other than lactation

characteristics, including metabolizability, N efficiency, and the relative abundance of *Bifidobacterium*. Additionally, linseed oil reduces CH_4 emission independent of *DGAT1* and affects the rumen microbiota and its fermentative activity.

CONCLUSIONS

Nutrient digestibility, CH_4 and H_2 emission, ruminal fermentation, rumen archaeal and bacterial concentrations, and the ruminal bacterial and archaeal community as a whole were not affected by *DGAT1*. The major effects of *DGAT1* on milk fat and protein content were independent of dietary linseed oil. Also, *DGAT1* affected other traits, including metabolizability, N efficiency, and the relative abundance of *Bifidobacterium*. Upon feeding linseed oil, H_2 emissions did not change, whereas CH_4 production (g/d) decreased with 8% (tendency only), CH_4 yield (g/kg of DMI) decreased with 6%, and CH_4 intensity (g/kg of FPCM) decreased with 11%, independent of *DGAT1*. In line with this, an increase in ruminal propionate proportion and a decrease in acetate proportion as well as acetate to propionate ratio was observed, and the archaea to bacteria ratio also decreased for the LSO diet. Linseed oil tended to affect the ruminal bacterial composition and affected the relative abundance of several bacterial genera.

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Chapter 8

Short communication: The potential of milk fatty acids to predict enteric methane production in dairy cows – the effect of linseed oil and *DGAT1* K232A polymorphism

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ABSTRACT

Several *in vivo* methane (**CH_4**) measurement techniques have been developed, but are not suitable for precise and accurate large scale measurements. Hence, proxies for CH_4 emissions in dairy cattle have been proposed, including the milk fatty acid (**MFA**) profile. The aim of the present study was to determine whether recently developed MFA-based prediction equations for CH_4 emission are applicable to dairy cows with different diacylglycerol o-acyltransferase 1 (***DGAT1***) K232A polymorphism and fed diets with and without linseed oil. Data from a cross-over design experiment were used, encompassing two dietary treatments (i.e., a control diet and a linseed oil diet, with a difference in dietary fat content of 22 g/kg dry matter) and 24 lactating Holstein-Friesian cows (i.e., 12 cows with *DGAT1* KK genotype and 12 cows with *DGAT1* AA genotype). Enteric CH_4 production was measured in climate respiration chambers and MFA analysed using gas chromatography. Observed CH_4 emissions were compared with CH_4 emissions predicted by previously developed MFA-based CH_4 prediction equations. The results indicate that different types of diets (i.e., with or without linseed oil), but not the *DGAT1* K232A polymorphism, affect the ability of previously derived prediction equations to predict CH_4 emission. The concordance correlation coefficient was however smaller than or equal to 0.30 for both dietary treatments separately, both *DGAT1* genotypes separately, and the complete dataset. It is therefore concluded that previously derived CH_4 prediction equations can neither accurately nor precisely predict CH_4 emissions of dairy cows housed under different conditions from those under which the prediction equations were developed.

Keywords: enteric methane production, milk fatty acid, linseed oil, *DGAT1* K232 polymorphism

SHORT COMMUNICATION

Enteric methane (**CH_4**) emission is one of the main targets of greenhouse gas mitigation strategies for the dairy cattle sector (Knapp et al., 2014). Accurate and repeatable measurements of CH_4 emission from individual dairy cows are required to evaluate emission factors used in national inventories of greenhouse gas emissions in agriculture, to assess efficacy of mitigation strategies, and to develop protocols for genetic selection for cows with reduced CH_4 emission (Hammond et al., 2016). The *in vivo* CH_4 measurement techniques available today are not suitable for precise and accurate large scale measurements (Hammond et al., 2016). Hence, proxies (i.e., indirect traits related to enteric CH_4 production) have been suggested, including the milk fatty acid (**MFA**) profile. Recently, it has been shown that the relationship between the MFA profile and CH_4 emission can be affected by dietary composition. The prediction equation developed on diets with a wide range of additives (Dijkstra et al., 2011) overpredicted CH_4 emission of cows fed sunflower, flax, and canola seeds (Mohammed et al., 2011), and the prediction equations developed on a wide range of diets including a wide variety of additives (Van Lingen et al., 2014) could not accurately predict CH_4 emission of dairy cows fed grass- or grass silage-based diets (Dijkstra et al., 2016). Castro-Montoya et al. (2017) analyzed data from 9 experiments and concluded that MFA did not reliably predict specific amounts of CH_4 emitted by dairy cows, whereas MFA hold a modest potential to differentiate individual dairy cows with high or low CH_4 emissions. The relationship between MFA profiles and CH_4

emission is not only affected by dietary composition, but also by lactation stage as demonstrated by Vanrobays et al. (2016). However, little is known whether host genetics can also influence this relationship.

The MFA profile can be affected by host genetics and dietary composition. The diacylglycerol o-acyltransferase 1 (***DGAT1***) K232A polymorphism affects not only major milk components, such as protein and fat content, but also their composition, including the MFA profile (e.g., Bovenhuis et al., 2016). Also feeding linseed oil, a dietary strategy to reduce enteric CH_4 production, affects the MFA profile (e.g., Kliem et al., 2017). Therefore, the aim of the present study was to determine whether recent MFA-based prediction equations for CH_4 emission are applicable to dairy cows with different *DGAT1* genotypes fed diets with and without linseed oil.

Individual cow data from a cross-over design experiment with two dietary treatments (i.e., a control diet and a linseed oil diet, with a difference in dietary fat content of 22 g/kg dry matter; **DM**) and 24 lactating Holstein-Friesian cows (i.e., 12 cows with *DGAT1* KK genotype and 12 cows with *DGAT1* AA genotype; each group had 6 primiparous and 6 multiparous cows) were used. The experiment has been described by Van Gastelen et al. (2017a) and was in accordance with Dutch law and approved by the Animal Care and Use Committee of Wageningen University & Research (Wageningen, The Netherlands). Dry matter intake (**DMI**), milk production and enteric CH_4 production of cows were measured climate respiration chambers described in detail by Van Gastelen et al. (2015). Daily CH_4 production was expressed in g/d, CH_4 yield was expressed in g/kg of DMI, and CH_4 intensity was expressed in g/kg of fat- and protein-corrected milk (**FPCM**), where FPCM (kg/day) = [0.337 + 0.116 × milk fat (g/100 g milk) + 0.06 × milk protein (g/100 g milk)] × milk production (kg/day). CH_4 production was 392 ± 76.4 g/d, CH_4 yield was 22.2 ± 1.73 g/kg DMI, and CH_4 intensity was 14.6 ± 1.93 g/kg FPCM. The MFA profile was elucidated using gas chromatography, as described by Van Gastelen et al. (2015), and expressed in g/100 g of total fatty acids.

The best MFA-based CH_4 prediction equations obtained by Dijkstra et al. (2011; CH_4 yield only), Van Lingen et al. (2014; CH_4 yield and CH_4 intensity only), Van Gastelen et al. (2017b), and Van Gastelen et al. (accepted) were used to predict CH_4 production, yield, and intensity of each individual cow. Other prediction equations were also considered for evaluation, but could not be used as these equations included specific MFA not measured in the current milk samples. The ability of these equations to predict CH_4 emission of dairy cows with different *DGAT1* genotypes, fed diets with and without linseed oil, was evaluated using the root mean square prediction error (**RMSPE**) and the concordance correlation coefficient (**CCC**), both described in detail by Ellis et al. (2010). The results of these analyses are shown in Table 8.1 for all data combined, in Table 8.2 for the control and linseed oil diet separately, and in Table 8.3 for the *DGAT1* KK genotype and *DGAT1* AA genotype separately.

Table 8.1. The root mean square prediction error (RMSPE) and concordance correlation coefficient (CCC) results of the MFA-based methane prediction models (complete dataset, n = 48)

	RMSPE (unit)[1]	CCC[2]	r[3]	C_b[4]	v[5]	μ[6]
Methane production (g/d)						
Van Gastelen et al. (2017b)	122	0.20	0.32	0.65	0.75	1.003
Van Gastelen et al. (accepted)	132	0.17	0.33	0.52	0.82	1.355
Methane yield (g/kg DMI[7])						
Dijkstra et al. (2011)	7.8	0.11	0.47	0.24	0.36	2.308
Van Lingen et al. (2014)	2.6	0.30	0.55	0.56	1.10	1.260
Van Gastelen et al. (2017b)	2.6	0.29	0.32	0.92	0.67	0.011
Van Gastelen et al. (accepted)	6.0	0.15	0.41	0.37	0.39	1.576
Methane intensity (g/kg FPCM[8])						
Van Lingen et al. (2014)	3.1	0.23	0.46	0.51	1.14	1.378
Van Gastelen et al. (2017b)	2.4	0.12	0.18	0.65	1.65	-0.892
Van Gastelen et al. (accepted)	3.5	0.29	0.38	0.78	0.53	0.389

[1] Root mean square prediction error expressed in g/d, g/kg DMI, and g/kg FPCM for methane production, yield, and intensity, respectively.

[2] Concordance correlation coefficient, where CCC = $r \times C_b$.

[3] Pearson correlation coefficient; a measure of precision.

[4] Bias correction factor; a measure of accuracy.

[5] Scale shift; change in standard deviation between predicted and observed methane emission.

[6] Location shift; if positive underprediction, if negative overprediction.

[7] Dry matter intake in kg/d.

[8] Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

With respect to the complete dataset (n = 48), the prediction equations did not predict CH_4 emissions satisfactorily. The RMSPE of CH_4 production was 122 to 132 g/d, the RMSPE of CH_4 yield was 2.6 to 7.8 g/kg DMI, and the RMSPE of CH_4 intensity was 2.4 to 3.5 g/kg FPCM. The CCC ranged from 0.17 to 0.20 for CH_4 production, from 0.11 to 0.30 for CH_4 yield, and from 0.12 to 0.29 for CH_4 intensity. These low CCC values were composed of consistently low values for precision ($r < 0.55$), whilst values for accuracy (C_b) ranged from low (0.24) to high (0.92). Most of the CH_4 prediction equations had a positive μ value (i.e., location shift), indicating a general underprediction of CH_4 emission, with the exception of the prediction equations of Van Gastelen et al. (2017b) for CH_4 yield (small bias only; consistent with high C_b value) and CH_4 intensity (overprediction of CH_4 emission).

As discussed by Ellis et al. (2010), the variable v (i.e., scale shift) measures the relative difference in standard deviation between predicted and observed values. The prediction equation of Van Lingen et al. (2014) for CH_4 yield and CH_4 intensity had a scale shift close to 1.0, whereas the scale shift of the prediction equation of Van Gastelen et al. (2017b) for CH_4 intensity was 1.65. The latter suggests that the variation in the predicted CH_4 emissions was smaller than the variation in the observed CH_4 emissions. Most of the CH_4 prediction equations, however, had a scale shift smaller than 1.0, suggesting that the variation in the predicted CH_4 emissions was larger than the variation in the observed CH_4 emissions.

Table 8.2. The root mean square prediction error (RMSPE) and concordance correlation coefficient (CCC) results of the MFA-based methane prediction models (control diet and linseed oil diet, both n = 24)

	RMSPE (unit)[1]	CCC[2]	r[3]	C_b[4]	v[5]	μ[6]
Control diet						
Methane production (g/d)						
Van Gastelen et al. (2017b)	65	0.20	0.40	0.50	3.47	0.506
Van Gastelen et al. (accepted)	74	0.26	0.54	0.49	2.53	1.072
Methane yield (g/kg DMI[7])						
Dijkstra et al. (2011)	3.4	0.05	0.17	0.33	1.18	2.018
Van Lingen et al. (2014)	2.1	0.16	0.35	0.46	1.98	1.363
Van Gastelen et al. (2017b)	2.0	0.11	0.24	0.45	2.24	-1.331
Van Gastelen et al. (accepted)	1.8	0.16	0.23	0.70	1.64	0.773
Methane intensity (g/kg FPCM[8])						
Van Lingen et al. (2014)	2.8	0.07	0.17	0.42	2.49	1.381
Van Gastelen et al. (2017b)	2.4	0.04	0.05	0.74	1.99	-0.453
Van Gastelen et al. (accepted)	2.8	0.04	0.05	0.82	1.28	-0.615
Linseed oil diet						
Methane production (g/d)						
Van Gastelen et al. (2017b)	159	0.05	0.17	0.29	0.98	2.189
Van Gastelen et al. (accepted)	172	0.01	0.07	0.20	1.29	2.808
Methane yield (g/kg DMI)						
Dijkstra et al. (2011)	10.5	0.02	0.43	0.06	0.84	5.696
Van Lingen et al. (2014)	3.0	0.16	0.49	0.33	1.53	1.954
Van Gastelen et al. (2017b)	3.0	-0.04	-0.05	0.76	0.80	0.760
Van Gastelen et al. (accepted)	8.2	0.01	0.14	0.11	0.77	4.100
Methane intensity (g/kg FPCM)						
Van Lingen et al. (2014)	3.4	0.02	0.13	0.15	1.86	3.307
Van Gastelen et al. (2017b)	2.5	0.11	0.25	0.45	1.03	-1.565
Van Gastelen et al. (accepted)	4.1	-0.01	-0.04	0.34	0.57	1.873

[1] Root mean square prediction error expressed in g/d, g/kg DMI, and g/kg FPCM for methane production, yield, and intensity, respectively.

[2] Concordance correlation coefficient, where CCC = $r \times C_b$.

[3] Pearson correlation coefficient; a measure of precision.

[4] Bias correction factor; a measure of accuracy.

[5] Scale shift; change in standard deviation between predicted and observed methane emission.

[6] Location shift; if positive underprediction, if negative overprediction.

[7] Dry matter intake in kg/d.

[8] Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

Table 8.3. The root mean square prediction error (RMSPE) and concordance correlation coefficient (CCC) results of the MFA-based methane prediction models (*DGAT1* AA genotype and *DGAT1* KK genotype, both n = 24)

	RMSPE (unit)[1]	CCC[2]	r[3]	C_b[4]	v[5]	μ[6]
DGAT1 KK genotype						
Methane production (g/d)						
Van Gastelen et al. (2017b)	114	0.20	0.30	0.68	0.70	0.897
Van Gastelen et al. (accepted)	118	0.18	0.32	0.56	0.83	1.244
Methane yield (g/kg DMI[7])						
Dijkstra et al. (2011)	7.6	0.10	0.38	0.25	0.37	2.229
Van Lingen et al. (2014)	2.4	0.33	0.54	0.62	1.09	1.101
Van Gastelen et al. (2017b)	2.4	0.29	0.30	0.94	0.73	-0.167
Van Gastelen et al. (accepted)	5.2	0.15	0.35	0.43	0.42	1.378
Methane intensity (g/kg FPCM[8])						
Van Lingen et al. (2014)	2.9	0.33	0.56	0.58	1.31	1.160
Van Gastelen et al. (2017b)	2.7	0.09	0.17	0.54	2.15	-1.047
Van Gastelen et al. (accepted)	3.5	0.38	0.45	0.84	0.57	0.252
DGAT1 AA genotype						
Methane production (g/d)						
Van Gastelen et al. (2017b)	129	0.21	0.34	0.62	0.78	1.088
Van Gastelen et al. (accepted)	145	0.18	0.36	0.49	0.83	1.443
Methane yield (g/kg DMI)						
Dijkstra et al. (2011)	8.1	0.13	0.55	0.23	0.36	2.343
Van Lingen et al. (2014)	2.8	0.29	0.58	0.50	1.13	1.400
Van Gastelen et al. (2017b)	2.7	0.31	0.35	0.89	0.63	0.173
Van Gastelen et al. (accepted)	6.6	0.16	0.49	0.32	0.36	1.749
Methane intensity (g/kg FPCM)						
Van Lingen et al. (2014)	3.3	0.15	0.36	0.43	0.97	1.634
Van Gastelen et al. (2017b)	2.1	0.17	0.22	0.76	1.29	-0.758
Van Gastelen et al. (accepted)	3.6	0.19	0.27	0.70	0.48	0.555

[1] Root mean square prediction error expressed in g/d, g/kg DMI, and g/kg FPCM for methane production, yield, and intensity, respectively.

[2] Concordance correlation coefficient, where CCC = $r \times C_b$.

[3] Pearson correlation coefficient; a measure of precision.

[4] Bias correction factor; a measure of accuracy.

[5] Scale shift; change in standard deviation between predicted and observed methane emission.

[6] Location shift; if positive underprediction, if negative overprediction.

[7] Dry matter intake in kg/d.

[8] Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

Interestingly, one would most likely interpret the RMSPE results differently than the CCC results for the control diet. As discussed in more detail below, both RMSPE and CCC decrease for the control diet relative to the complete dataset. This is contradictive, because a decrease in RMSPE implies an improvement of the CH_4 prediction (Bibby and Toutenburg, 1977), whereas a decrease in CCC implies a poorer CH_4 prediction (Lin, 1989). However, as demonstrated by Ellis et al. (2010) upon evaluating *in vivo* farm model for CH_4 prediction, when prediction equations are unable to describe adequate amounts of the observed variation, CCC analysis is likely the better evaluation tool. This also applies to the CH_4 prediction equations for

the control diet, with relatively high v values (ranging from 1.18 to 3.47; Table 8.2) indicating the inability of the CH_4 prediction equations to predict the range of observed CH_4 emissions. Therefore, we will focus mainly on the CCC results.

Upon dividing the dataset into a dataset representing the control diet (n = 24) and a dataset representing the linseed oil supplemented diet (n = 24), the equations predicting CH_4 emissions performed even less satisfactorily. This is evident by the lower, and sometimes negative, CCC values for both control and linseed oil dataset relative to the complete dataset. For the control diet, the scale shift (v) increased considerably (i.e., ranging from 1.18 to 3.47) relative to the complete dataset, indicating the inability of the CH_4 prediction equations to predict the range of observed CH_4 emissions. For the linseed oil supplemented diet, the scale shift varied between 0.57 and 1.87, which is of similar magnitude as for the complete dataset. The larger location shift (μ) values for the linseed oil supplemented diet relative to the complete dataset, indicates a biased (in general underpredicted) CH_4 prediction. Overall, these results suggest that the dietary composition affects the capability to predict CH_4 emissions of dairy cows of previously developed MFA-based CH_4 prediction equations.

Dividing the dataset into a dataset representing the *DGAT1* KK genotype (n = 24) and into a dataset representing the *DGAT1* AA genotype (n = 24), hardly affected the RMSPE and CCC results relative to the complete dataset. This indicates that the ability to predict CH_4 emissions of dairy cows of previously developed MFA-based CH_4 prediction equations was not affected by the *DGAT1* K232A polymorphism.

The results suggests that dietary composition (i.e., with or without supplementation of linseed oil) affects the prediction potential of previously derived MFA-based CH_4 prediction equations, whereas the effect of *DGAT1* K232A polymorphism seems small. This may be related to the effect of linseed oil and *DGAT1* K232A polymorphism on the MFA profile. For example, the prediction equation of Van Lingen et al. (2014) for CH_4 yield included *iso* C16:0 (positive), C18:1 *trans*-10+11 (negative) and C18:2n-6 (negative) as explanatory variables. Van Gastelen et al. (2017a) demonstrated that the first of these 2 MFA were substantially affected by linseed oil supplementation. Contrary, according to Van Gastelen et al. (2017a) *DGAT1* K232A polymorphism only affected C18:2n-6, whereas the other two MFA were not affected. The same patterns was observed for the prediction equation of Van Gastelen et al. (accepted), which included C18:1 *trans*-15 + C18:1 *cis*-11, C18:2 *cis*-9, *trans*-11, and C18:3n-3 (all negatively related) as explanatory variables for CH_4 production in g/d. Van Gastelen et al. (2017a) demonstrated that these three MFA substantially increased upon linseed oil supplementation. Contrary to the effect of dietary composition, only C18:3n-3 was significantly affected by the *DGAT1* K232A polymorphism, whereas the other two MFA were unaffected (Van Gastelen et al., 2017a). The effect of linseed oil supplementation on these specific MFA might explain why the CH_4 prediction potential of the MFA-based prediction equation of Van Lingen et al. (2014) and Van Gastelen et al. (accepted) is affected by dietary composition. The minor and no effect of *DGAT1* K232A polymorphism on these specific MFA might explain why the MFA-based prediction equation of Van Lingen et al. (2014) and Van Gastelen et al. (accepted) predicted CH_4 emission equally well for the complete dataset and the datasets for the *DGAT1* KK and *DGAT1* AA genotype separately.

Overall, the results of this short communication indicate that dietary composition (i.e., with or without linseed oil), but not the *DGAT1* K232A polymorphism, affect the ability of previously derived MFA-based CH_4 prediction equations to predict CH_4 emission of dairy cows. This dietary effect on the ability to predict CH_4 emissions seems to be the result of diet-induced changes in the relationship between MFA profiles and enteric CH_4 production. Hence, we conclude that CH_4 prediction equations may not be universal and might be valid only when applied to dairy cows housed under similar conditions as to those under which the prediction equations were developed.

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Chapter 9

Predicting enteric methane emission of dairy cows with milk Fourier-transform infrared spectra and gas chromatography-based milk fatty acid profiles

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ABSTRACT

The objective of the present study was to compare the prediction potential of milk Fourier-transform infrared spectroscopy (**FTIR**) for methane (CH_4) emissions of dairy cows with that of gas chromatography (**GC**)-based milk fatty acid (**MFA**). Data from 9 experiments with lactating Holstein-Friesian cows with a total of 30 dietary treatments and 218 observations were used. Methane emissions were measured for 3 consecutive days in climate respiration chambers and expressed as production (g/d), yield (g/kg dry matter intake; **DMI**), and intensity (g/kg fat- and protein-corrected milk; **FPCM**). Dry matter intake was 16.3 ± 2.18 kg/d, FPCM yield was 25.9 ± 5.06 kg/d, CH_4 production was 366 ± 53.9 g/d, CH_4 yield was 22.5 ± 2.10 g/kg DMI, and CH_4 intensity was 14.4 ± 2.58 g/kg FPCM. Milk was sampled during the same days and analyzed by GC and by FTIR. Multivariate GC-determined MFA-based and FTIR-based CH_4 prediction models were developed and, subsequently, the final CH_4 prediction models were evaluated with root mean square error of prediction (**RMSEP**) and concordance correlation coefficient (**CCC**) analysis. Further, we performed a random 10-fold cross validation to calculate the models performance parameters (e.g., the coefficient of determination of cross validation; **R^2CV**). The final GC-determined MFA-based CH_4 prediction models estimate CH_4 production, yield, and intensity with a RMSEP of 35.7 g/d, 1.6 g/kg DMI, and 1.6 g/kg FPCM, and with a CCC of 0.72, 0.59, and 0.77, respectively. The final FTIR-based CH_4 prediction models estimate CH_4 production, yield, and intensity with a RMSEP of 43.2 g/d, 1.9 g/kg DMI, and 1.7 g/kg FPCM, and with a CCC of 0.52, 0.40, and 0.72, respectively. The GC-determined MFA-based prediction models described a greater part of the observed variation in CH_4 emission than FTIR-based models. The cross validation results indicate that all CH_4 prediction models (both GC-determined MFA-based and FTIR-based) are robust, as the difference between R^2 and R^2CV ranged from 0.01 to 0.07. These results indicate that GC-determined MFA have a greater potential than FTIR spectra to estimate CH_4 production, yield, and intensity. Both techniques hold potential, but may not yet be ready to predict CH_4 emission of dairy cows in practice. Additional CH_4 measurements are therefore needed to improve the accuracy and robustness of both GC-determined MFA and FTIR spectra for CH_4 prediction.

Keywords: dairy cow, enteric methane production, milk fatty acid concentration, milk Fourier-transform infrared spectroscopy

INTRODUCTION

Enteric methane (**CH_4**) is produced in the gastrointestinal tract of livestock, mainly ruminants, and comprises ~40% of global CH_4 emissions (Gerber et al., 2013). Enteric CH_4 is one of the main targets of mitigation strategies in the dairy cattle sector (Knapp et al., 2014). Quantification of CH_4 emission is thus important. Several *in vivo* CH_4 measurement techniques have been developed, but are not suitable for precise and accurate large scale measurements (Hammond et al., 2016). Cost-effective, efficient, robust, and fast CH_4 measurement techniques applicable on a large scale to estimate CH_4 emission of individual dairy cows are required. Therefore, identifying proxies (i.e., indicators or indirect traits related to CH_4 emission), might serve as a good alternative (Negussie et al., 2017).

Milk fatty acid (**MFA**) profiles have been suggested as proxy to estimate CH_4 emission in dairy cattle, and many studies have evaluated this proposed relationship between MFA concentrations and CH_4 emission (e.g., Chilliard et al., 2009; Mohammed et al., 2011; Rico et al., 2016). However, the gas chromatography (**GC**) procedure required to obtain the MFA profiles is time consuming, labor intensive, and requires expensive instruments and trained personnel (Capuano et al., 2014), and is, therefore, unsuitable for large scale measurements. Fourier-transform infrared spectroscopy (**FTIR**), on the other hand, is a rapid, cost-effective, and high-throughput technique. Currently, major milk components such as fat, protein, lactose, and urea contents are routinely measured with FTIR by milk recording organizations. Diverse milk phenotypes can be estimated by FTIR, as illustrated by De Marchi et al. (2014), including MFA composition (e.g., Rutten et al., 2009; Soyeurt et al., 2011), milk protein composition (Bonfatti et al., 2011), technological properties of milk (DeMarchi et al., 2009), and cow health and energy status (Van Knegsel et al., 2010; McParland et al., 2011).

Dehareng et al. (2012) and Vanlierde et al. (2015) used FTIR to predict CH_4 emission of dairy cattle. However, the CH_4 predictions of Dehareng et al. (2012) at different stages of lactation were not biologically meaningful, and Vanlierde et al. (2015) demonstrated that a lactation stage dependent CH_4 prediction model was more robust and biologically more meaningful. The CH_4 prediction potential of FTIR spectra seems moderate (reviewed by Van Gastelen and Dijkstra, 2016), which is based on experiments only using the SF_6-tracer technique to measure CH_4 emission. To date, no research has assessed the CH_4 prediction potential of milk FTIR spectra for CH_4 data obtained in climate respiration chambers and for all 3 units of CH_4 emission, viz. CH_4 production (in g/d), CH_4 yield (in g/kg dry matter intake; **DMI**), and CH_4 intensity (in g/kg fat- and protein-corrected milk; **FPCM**). The objective of the present study was to compare the prediction potential for CH_4 production, yield, and intensity of milk FTIR spectra with that of the GC-determined MFA profile, using CH_4 data obtained in climate respiration chambers.

MATERIALS AND METHODS

Data collection

Data from 9 studies, designed as randomized block experiments, from Wageningen University & Research (Wageningen, The Netherlands) were used (Table 9.1). The experiments were conducted in accordance with Dutch law and approved by the Animal Care and Use Committee of Wageningen University & Research. The 9 studies represented 30 dietary treatments and 218 individual observations from lactating Holstein-Friesian cows. The dataset included multiple observations from a small number of dairy cows (218 individual observations from 189 unique dairy cows). We consider these particular observations as unique and not as repeated measurements, because of the large differences in conditions between the observations of the same dairy cows (i.e., different experiment, different dietary treatment, different parity, and different lactation stage). The experimental setup was similar for all experiments. After an adaptation period of 12 d, cows were housed individually in open circuit, indirect climate respiration chambers (described by Van Gastelen et al., 2015) for a 5 d period to determine CH_4

emission (expressed as production, yield, and intensity). Diets were fed twice daily and intake was restricted to 95% of the voluntarily DMI of the cow consuming the least within a block.

Cows were milked twice daily and water was freely available during the entire experiment. While housed in the climate respiration chambers, milk yield was recorded and representative milk samples (i.e., 5 g/kg of milk production from each cow) were collected at each milking according to Van Gastelen et al. (2015). These milk samples were pooled per period and cow and subsequently analyzed for MFA composition (g/100 g FA) using GC as described by Van Gastelen et al. (2015). The same pooled milk samples were also analyzed in the laboratory of Qlip B.V. (Zutphen, the Netherlands) to determine the content of fat, protein, and lactose according to regular test-day procedures using MilkoScan FT 6000 equipment with diamond cuvettes (Foss Analytical A/S, Hillerød, Denmark) using the manufacturer supplied basic calibration models in conformity with ISO 9622 (International Organization for Standardization, 2013). The applied reference methods were ISO 1211 (International Organization for Standardization, 2010) for fat, ISO 8968-1 (International Organization for Standardization, 2014) for total protein, and an HPLC method based on ISO 22662 (International Organization for Standardization, 2007) for lactose. The FTIR absorption spectra were collected, consisting of 1060 infrared frequencies (wavenumbers) representing infrared light absorption through the milk samples ranging from 925 to 5008 cm^{-1}.

Statistical analysis

Model development GC-determined MFA. Multivariate models were developed using a stepwise procedure (PROC GLMSELECT of SAS; SAS Institute Inc., Cary, NC, USA, version 9.2) with CH_4 emission (i.e., production, yield, and intensity) as the independent variable and stepwise selection of only GC-determined MFA (g/100 g total fatty acids). The significance level for a GC-determined MFA to enter or stay in the model was 0.01 and 0.05, respectively. The final models were selected based on the minimum Akaike's information criterion statistic. The selected models were evaluated in PROC REG in terms of multicollinearity (variation inflation factor > 10), but no multicollinearity was observed.

Model development FTIR. Prediction models for CH_4 production, yield, and intensity were developed only on pre-processed data of selected wavenumbers as linear regression models using Partial Least Squares (PLS) calculated with the SIMPLS algorithm of the PLS toolbox (Eigenvector Research Inc., Manson, WA, USA). In the PLS method, spectroscopic data were reduced to a set of orthogonal, uncorrelated components (viz. latent variables; **LV**). Selected wavenumbers (n = 218) were in the ranges 964 - 1581 cm^{-1}, 1715 – 1773 cm^{-1}, and 2814 - 2968 cm^{-1}. These wavenumbers were selected because these contain valuable information on milk composition and are thus most relevant for milk analysis (Capuano et al., 2014). Additionally, parts of the infrared spectrum that are disturbed by high water absorption were omitted, because these can interfere with the quantification of other major milk components (Capuano et al., 2014). The selected wavenumbers were pre-processed by applying the Savitzky-Golay (Savitzky and Golay,1964), first derivative with polynomial order 2 and window width 7, and subsequently mean centered.

Table 9.1. Data sources and characteristics of included studies

Study	Reference	n[1]	No. of treatments	Diet composition / treatments[2]
1	Warner et al. (2015)	25	4	15% concentrate, 85% grass herbage. Grass herbage was cut after 3 vs. 5 weeks of regrowth, after receiving low (20 kg of N/ha) vs. high (90 kg of N/ha) fertilization rate after initial cut.
2	Van Gastelen et al. (2015)	30	4	20% concentrate, 80% roughage. Roughage consisted of 100:0 vs. 67:33 vs. 33:67 vs. 0:100 grass silage:corn silage.
3	Warner et al. (2016)	42	6	20% concentrate, 80% grass silage. Grass silage received low (65 kg N/ha) vs. high (150 kg N/ha) fertilization rate preceding growth period 28 d vs. 41 d vs. 62 d of regrowth.
4	Klop et al. (2016)	6	1	30 % concentrate, 21 % grass silage, 49% corn silage. Control diet, with concentrate containing urea as nonprotein N source.
5	Warner et al. (2017)	55	8	20% concentrate, 10% corn silage, 70% grass silage. Grass silage was cut at four growth stages (leafy vs. boot vs. early heading vs. late heading) and fed at two intake levels (15.5 kg vs. 16.6 kg DMI[3]).
6	Hatew et al. (2016)	25	4	20% concentrate, 5% wheat straw, 75% corn silage. Whole-plant corn was harvested at very early (25% DM) vs. early (28% DM) vs. medium (32% DM) vs. and late (40% DM) stage of maturity.
7	Klop et al. (2017)	7	1	30% concentrate, 30% grass silage, 40% corn silage (control diet).
8	Van Lingen et al. (2017)	4	1	30% concentrate, 30% grass silage, 40% corn silage (control diet).
9	Van Gastelen et al. (2017)	24	1	30% concentrate, 30% grass silage, 40% corn silage (control diet). Cows with *DGAT1* KK vs. *DGAT1* AA genotype.

[1] The total number of observations, which equals the number of dairy cows, used for the present study.

[2] Proportion (%) on DM basis.

[3] Dry matter intake (kg/d).

Table 9.2. Descriptive statistics of animal performance, dietary characteristics, methane emission, and the milk fatty acid profile determined with gas chromatography (n = 218)

Variable	Mean	Median	SD	Minimum	Maximum
Animal performance					
Body weight	617	617	59.7	462	817
Parity	2.7	3.0	1.38	1.0	7.0
Days in milk	179	185	85.2	59	567
Milk yield (kg/d)	24.3	23.9	5.42	11.3	36.8
FPCM[1] (kg/d)	25.9	25.3	5.06	12.3	39.9
Milk fat content (g/100 g milk)	4.67	4.67	0.659	2.94	6.70
Milk crude protein content (g/100 g milk)	3.37	3.30	0.406	2.62	5.00
Milk anhydrous lactose content (g/100 g milk)	4.57	4.59	0.221	3.80	5.03
DMI[2] (kg/d)	16.3	16.1	2.18	10.8	24.5
Dietary characteristics (in g/kg DM, unless stated otherwise)					
Dry matter (g/kg)	502	502	101.5	306	797
Ash	77	79	13.5	53	103
Crude protein	176	172	40.1	82	251
NDF	380	372	49.9	242	501
ADF	221	218	25.7	183	291
ADL	14	14	4.2	6	26
Crude fat	31	33	6.7	21	46
Starch	118	79	85.5	5	326
Sugar	89	70	59.0	21	265
GE (MJ/kg DM)	18.6	18.6	0.41	17.6	19.3
NDF to starch ratio	8.2	4.8	15.76	1.0	86.2
Methane emission					
Production (g/d)	366	365	53.9	234	535
Yield (g/kg DMI)	22.5	22.6	2.10	17.2	28.0
Intensity (g/kg FPCM)	14.4	14.4	2.58	8.5	24.8
Milk fatty acids (g/100 g fatty acids) determined with gas chromatography					
C4:0	3.5	3.5	0.35	1.8	4.4
C6:0	2.1	2.2	0.21	1.5	2.6
C8:0	1.1	1.1	0.17	0.6	1.6
C10:0	2.5	2.4	0.53	1.1	4.1
C12:0	2.8	2.8	0.69	1.3	4.9
C14:0	10.4	10.5	1.39	6.7	14.1
iso C14:0	0.08	0.08	0.017	0.04	0.13
C14:1 *cis*-9	0.99	0.97	0.238	0.47	1.95
C15:0	0.97	0.97	0.168	0.53	1.56
iso C15:0	0.23	0.23	0.041	0.13	0.37
anteiso C15:0	0.40	0.40	0.068	0.24	0.62
C16:0	31.7	31.7	3.35	24.6	42.3
iso C16:0	0.18	0.18	0.035	0.12	0.34
C16:1 *trans*-9	0.21	0.21	0.037	0.13	0.35
C16:1 *cis*-9	1.9	1.8	0.38	1.0	3.0
C17:0	0.65	0.64	0.099	0.44	0.96
iso C17:0	0.40	0.39	0.060	0.25	0.63
anteiso C17:0	0.42	0.41	0.056	0.32	0.61
C17:1 *cis*-9	0.31	0.30	0.087	0.15	0.69

Table 9.2. Continued

Variable	Mean	Median	SD	Minimum	Maximum
C18:0	9.6	9.7	1.61	5.0	15.2
C18:1 *cis*-9[3]	21.0	20.7	3.83	12.3	30.5
C18:1 *cis*-12	0.18	0.15	0.075	0.07	0.47
C18:1 *cis*-13	0.13	0.13	0.037	0.05	0.27
C18:1 *trans*-6	0.20	0.19	0.051	0.06	0.42
C18:1 *trans*-9	0.15	0.14	0.026	0.08	0.25
C18:1 *trans*-10	0.19	0.16	0.091	0.00	0.65
C18:1 *trans*-11	0.89	0.88	0.221	0.17	2.18
C18:1 *trans*-15 + C18:1 *cis*-11	0.77	0.75	0.171	0.33	1.23
C18:2 *cis*-9, *trans*-11	0.42	0.40	0.116	0.20	1.29
C18:2n-6	1.5	1.5	0.24	0.9	2.4
C18:3n-3	0.47	0.48	0.154	0.14	0.98
C18:3n-6	0.07	0.07	0.014	0.04	0.13
C20:0	0.13	0.13	0.019	0.08	0.19
C20:1 *cis*-11	0.06	0.06	0.022	0.00	0.12
C20:2n-6	0.04	0.04	0.007	0.02	0.07
C20:3n-6	0.07	0.07	0.019	0.03	0.13
C20:4n-3	0.03	0.03	0.026	0.00	0.13
C20:4n-6	0.11	0.11	0.024	0.05	0.18
C20:5n-3	0.06	0.06	0.013	0.03	0.09
C22:0	0.06	0.06	0.014	0.00	0.11
C22:5n-3	0.08	0.08	0.019	0.04	0.14
C24:0	0.04	0.04	0.013	0.00	0.08

[1] Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

[2] Dry matter intake (kg/d).

[3] C18:1 *cis*-9 represents the sum of C18:1 *cis*-9 and C18:1 *trans*-12, as these 2 FA could not be separated in the analysis. The portion of C18:1 *trans*-12 is considered to be negligible, as this FA is always present in small amounts.

Model evaluation. All CH_4 prediction models, GC-determined MFA-based and FTIR-based, were evaluated using 2 methods. Firstly, the mean square error of prediction (**MSEP**), calculated as

$$\text{MSEP} = \sum_{i=1}^{n} (O_i - P_i)^2 / n,$$

where n is the total number of observations, O_i is the observed value and P_i is the predicted value. The square root of the MSEP (**RMSEP**) gives an estimate of the overall error of prediction and is expressed as percentage of the observed mean or expressed in g/d, g/kg DMI, and g/kg FPCM for CH_4 production, yield, and intensity, respectively. Secondly, concordance correlation coefficient analysis (**CCC**; Lin, 1989) was performed, where CCC is calculated as

$$\text{CCC} = r \times C_b,$$

where r is the correlation coefficient providing a measure of precision, and C_b is a bias correction factor providing a measure of accuracy. The C_b variable is calculated as

$$C_b = \frac{2}{[v + 1 / v + \mu^2]},$$

where

$$v = \frac{S_o}{S_p},$$

$$\mu = \frac{\bar{O} - \bar{P}}{(S_o \times S_p)^{0.5}},$$

where v provides a measure of scale shift, while μ provides a measure of location shift, S_o and S_p are the observed and predicted standard deviations, and $\bar{O}$ and $\bar{P}$ are the observed and predicted means. A CCC of 0.20 or lower indicates poor predictive ability, between 0.21 and 0.40 indicates fair predictive ability, between 0.41 and 0.60 indicates moderate predictive ability, between 0.61 and 0.80 indicates substantial predictive ability, and between 0.81 and 1.00 indicates accurate predictive ability (Altman, 1997). Furthermore, the predictive power of the calibration was evaluated through the ratio of performance to deviation (**RPD**) statistic, which is the ratio of the standard deviation of the original data to the standard error of cross validation (Dehareng et al., 2012). The RPD values are preferably as high as possible; RPD values between 5 and 10 are adequate for quality control, process control, and potentially suitable for application (Williams et al., 2014). Additionally, PROC CORR in SAS was used to determine the Pearson correlation between the MFA predicted CH_4 emissions and the FTIR predicted CH_4 emissions.

Cross validation MFA and FTIR. In order to calculate the models performance parameters [i.e., root mean square error of cross validation (**RMSECV**) and the coefficient of determination of cross validation (**R^2CV**)], we performed a random cross validation with 10 splits and 10 iterations as recommended by Rodriguez et al. (2010) for all MFA and FTIR-based CH_4 prediction models. For each iteration, a model was developed as described above using 9 splits of the dataset, and the selected model was subsequently evaluated as described above on the remaining part of the dataset (i.e., 1 split). With this approach, all observations were used for both calibration and validation, and each observation was used for validation exactly once. The cross validation performance values represent the average of the 10-fold cross validation.

This random 10-fold cross validation was also used for selection of the number of LV for the FTIR-based CH_4 prediction models. The selected number of LV for the final models was based on the suggestion by PLS toolbox and visual assessment of the graphs of the root means square error of calculation (**RMSEC**) and RMSECV against the number of LV. The number of LV before the RMSECV starts increasing or the RMSECV starts deviating considerably from the RMSEC was the number selected.

RESULTS

The descriptive statistics of animal performance, dietary characteristics, CH_4 emission, and GC-determined MFA concentrations are presented in Table 9.2. The GC-determined MFA-based CH_4 production, yield, and intensity prediction models are shown in Table 9.3. In the final models, considering the odd- and branched-chain fatty acids (**OBCFA**), CH_4 production was positively associated with C15:0 (P = 0.002), CH_4 yield was positively associated with *iso* C15:0

and C17:0 ($P < 0.003$), but negatively associated with *anteiso* C15:0 ($P < 0.001$), and CH_4 intensity was positively associated with both *iso* C15:0 and *iso* C17:0 ($P < 0.001$). The relation between CH_4 emissions and the C18:1, C18:2, C18:3 isomers was generally negative ($P < 0.010$), with the exception of the positive association between CH_4 production and C18:2n-6 ($P = 0.005$). Additionally, CH_4 production was negatively associated with C24:0 ($P = 0.007$) and positively associated with C20:4n-3 ($P = 0.002$), and CH_4 intensity was positively associated with C22:5n-3 ($P < 0.001$). The FTIR-based CH_4 prediction models are based on the regression between the wavenumbers and CH_4 production, yield, or intensity, as illustrated in Figure 9.1. Certain wavenumbers were not related with CH_4 emissions (i.e., regression vector close to 0), whereas other wavenumbers were clearly positively or negatively related with CH_4 emissions. Both the strength and the direction (positive or negative) of the correlations as well as the correlated wavenumbers differed between the different units of CH_4 emission (i.e., production, yield, and intensity; Figure 9.1).

The evaluation results (i.e., R^2, RMSEP, and CCC analysis) of the GC-determined MFA-based and FTIR-based CH_4 prediction models are shown in Table 9.4. The observed versus predicted CH_4 production, yield, and intensity plots of the GC-determined MFA-based and FTIR-based CH_4 prediction models are shown in Figures 9.2A and 9.3A, respectively. The residual (i.e., observed - predicted) versus predicted CH_4 production, yield, and intensity plots of the GC-determined MFA-based and FTIR-based CH_4 prediction models are shown in Figures 9.2B and 9.3B, respectively. The R^2, RMSEP (%), and CCC of the GC-determined MFA-based CH_4 prediction models ranged from 0.40 to 0.62, from 7.1% to 10.9%, and from 0.59 to 0.77, respectively (Table 9.4). The R^2, RMSEP (%), and CCC of the FTIR-based CH_4 prediction models ranged from 0.25 to 0.56, from 8.2% to 11.8%, and from 0.40 to 0.72, respectively. Based on the CCC, for both GC-determined MFA and FTIR, the prediction model for CH_4 yield had the lowest prediction potential (moderate predicting ability for both MFA and FTIR based models) and the prediction model for CH_4 intensity had the highest prediction potential (substantial predicting ability for both MFA and FTIR based models, respectively). The MFA and FTIR based prediction models for CH_4 production had substantial and moderate predicting ability, respectively. The variation in predicted CH_4 emission was smaller than that in the observed CH_4 emission, in particular for CH_4 yield, as indicated by the variable v (scale shift; the relative difference in standard deviation between predicted and observed values). The scale shift was greater for FTIR-based prediction models (v ranged from 1.33 to 2.00) than for GC-determined MFA-based prediction models (v ranged from 1.26 to 1.55).

The RPD statistic, that relates the standard error of prediction to the standard deviation of the original reference data, was smaller than 1.58 for the GC-determined MFA-based CH_4 prediction models and smaller than 1.39 for the FTIR-based CH_4 prediction models (Table 9.4), suggesting unsatisfactory prediction ability. The Pearson correlations between GC-determined MFA predicted and FTIR predicted CH_4 production, CH_4 yield, and CH_4 intensity were 0.62 ($P < 0.001$), 0.51 ($P < 0.001$), and 0.69 ($P < 0.001$), respectively (Figure 9.4).

The results of the internal cross validation of all GC-determined MFA-based and FTIR-based CH_4 prediction models are also shown in Table 9.4. The average number of GC-determined MFA included in the GC-determined MFA internal cross validation models varied

between 4 and 5, and the average number of LV in the FTIR internal cross validation models varied between 4 and 6. The R^2CV and the RMSECV of the GC-determined MFA-based CH_4 prediction models ranged from 0.38 to 0.63 and from 8.1% to 11.6%, respectively. The R^2CV and the RMSECV of the FTIR-based CH_4 prediction models ranged from 0.19 to 0.49 and from 8.6% to 12.8%, respectively.

Table 9.3. The prediction model developed for methane production (g/d), yield (g/kg DMI[1]), and intensity (g/kg FPCM[2]) based on milk fatty acids determined with gas chromatography

Methane emission	Milk fatty acids	Estimate	SE	*P*-value
Methane production (g/d)	Intercept	507.9	28.66	< 0.001
	C15:0	62.9	17.22	0.002
	C17:1 *cis*-9	-240.6	32.29	0.007
	C18:1 *trans*-10	-202.8	47.75	0.010
	C18:1 *trans*-11	-59.3	12.70	< 0.001
	C18:2n-6	48.1	14.08	0.005
	C18:3n-3	-187.1	24.40	< 0.001
	C20:4n-3	326.4	104.30	0.002
	C24:0	-816.8	230.89	0.007
Methane yield (g/kg DMI)	Intercept	22.9	1.3	< 0.001
	iso C15:0	20.9	4.2	0.003
	anteiso C15:0	-9.6	2.3	< 0.001
	C17:0	7.6	1.26	< 0.001
	C18:1 *trans*-11	-2.4	0.52	< 0.001
	C18:1 *trans*-15 + C18:1 *cis*-11	-2.7	0.84	< 0.001
	C18:3n-3	-4.4	0.81	< 0.001
Methane intensity(g/kg FPCM)	Intercept	8.0	1.13	< 0.001
	iso C15:0	24.8	3.66	< 0.001
	iso C17:0	10.3	2.30	< 0.001
	C18:1 *trans*-15 + C18:1 *cis*-11	-6.6	0.95	< 0.001
	C22:5n-3	22.7	6.61	< 0.001

[1] Dry matter intake (kg/d)

[2] Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

Table 9.4. The coefficient of determination (R^2) and concordance correlation coefficient (CCC) analysis of the prediction equations and the 10-fold cross validation results

	Overall									10-fold cross validation		
Methane emission	Adjusted R^2	RMSEP[5]	RMSEP %[6]	CCC[7]	r[8]	C_b[9]	γ[10]	μ[11]	RPD[12]	Number of LV or MFA[13]	R^2CV	RMSECV %
Methane production (g/d)												
GC-determined MFA[1]	0.54	35.7	9.8	0.72	0.75	0.96	1.34	0	1.27	4	0.47	11.6
FTIR[2]	0.36	43.2	11.8	0.52	0.60	0.88	1.68	0	1.19	4	0.30	12.4
Methane yield (g/kg DMI[3])												
GC-determined MFA	0.40	1.6	7.1	0.59	0.64	0.91	1.55	0	1.15	5	0.38	8.1
FTIR	0.25	1.9	8.2	0.40	0.50	0.80	2.00	0	1.09	5	0.19	8.6
Methane intensity (g/kg FPCM[4])												
GC-determined MFA	0.62	1.6	10.9	0.77	0.79	0.97	1.26	0	1.58	5	0.63	11.4
FTIR	0.56	1.7	11.8	0.72	0.75	0.96	1.33	0	1.39	6	0.49	12.8

[1] Milk fatty acids in g/100 g fatty acids determined with gas chromatography.

[2] Fourier-transform infrared spectra.

[3] Dry matter intake (kg/d)

[4] Fat- and protein-corrected milk (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

[5] Root mean squared error of prediction expressed in g/d, g/kg DMI, and g/kg FPCM for methane production, yield, and intensity, respectively.

[6] Root mean squared error of prediction expressed as a percentage of the observed mean.

[7] Concordance correlation coefficient, where CCC = $r \times C_b$.

[8] Pearson correlation coefficient; a measure of precision.

[9] Bias correction factor; a measure of accuracy.

[10] Scale shift; change in standard deviation between predicted and observed methane emission.

[11] Location shift; if positive under prediction, if negative over prediction.

[12] Ratio of performance to deviation.

[13] Number of latent variables included in the Fourier-transform infrared based models or the number of milk fatty acids included in the milk fatty acid based models.

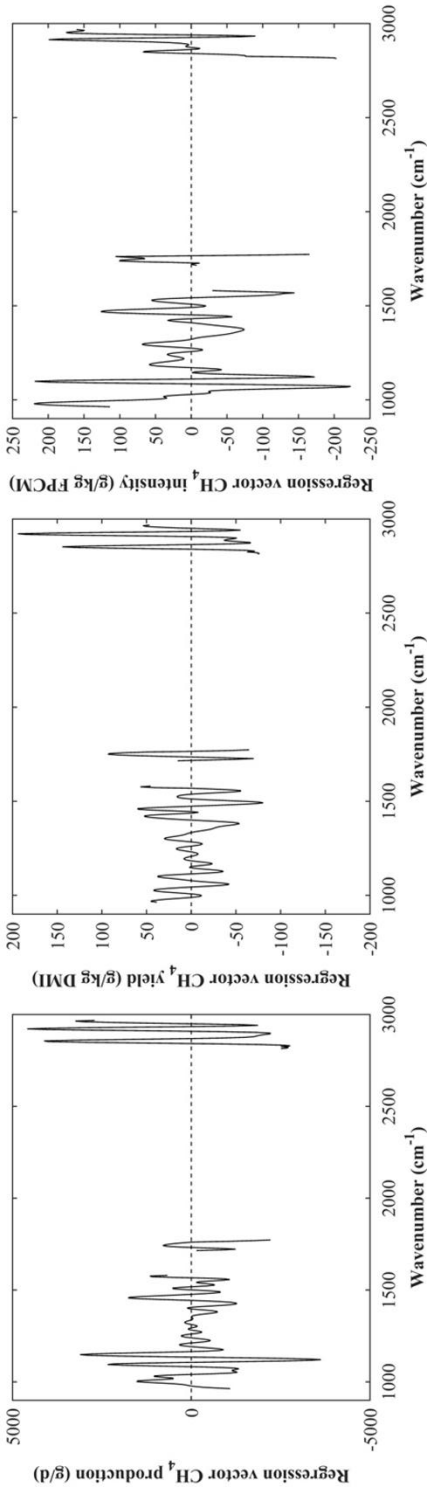

Figure 9.1. The regression vectors of the PLS models for methane production (g/d), yield (g/kg dry matter intake), and intensity (g/kg fat- and protein-corrected milk) plotted against wavenumbers (cm^{-1})

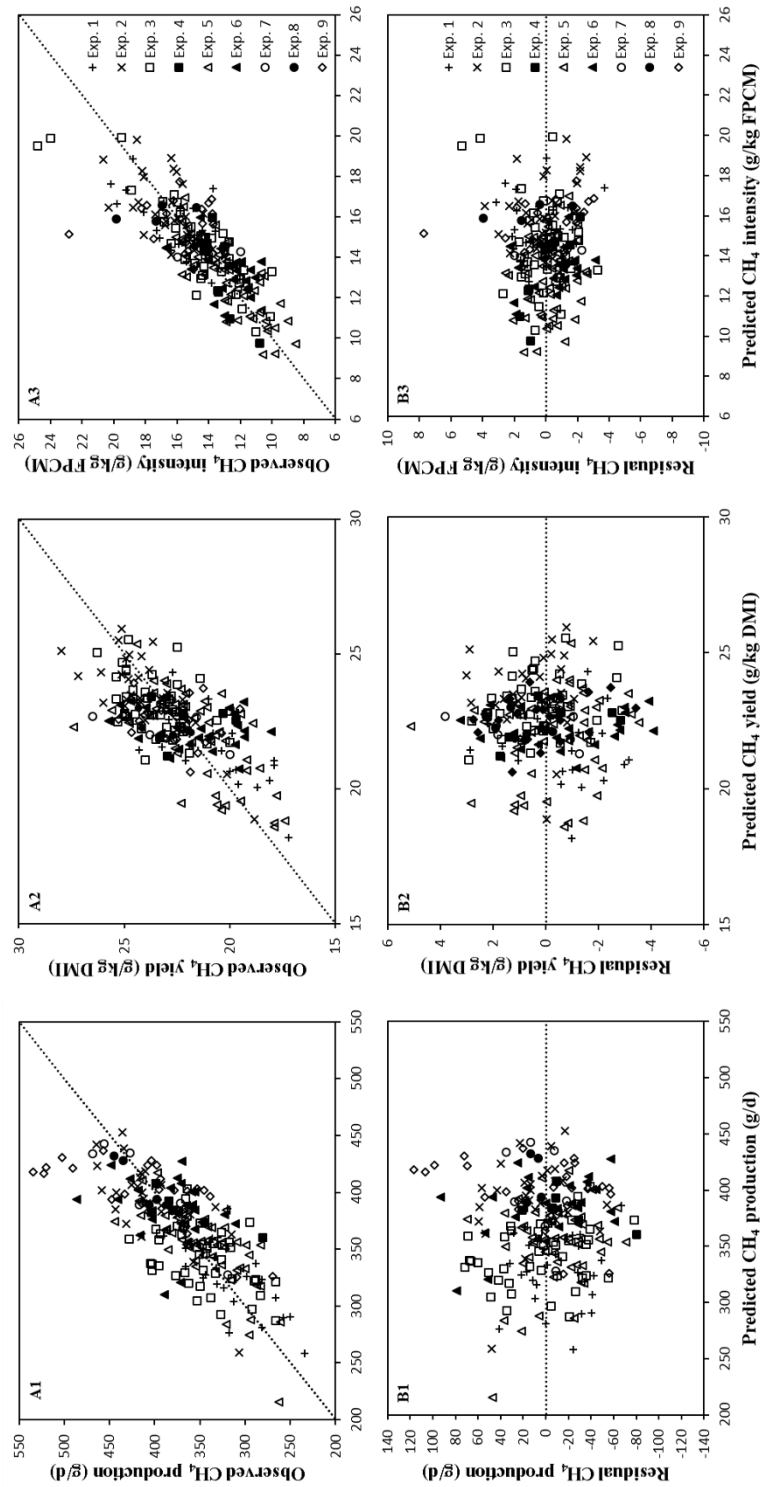

Figure 9.2. (A) Observed and predicted, and (B) residual (i.e., observed – predicted) (1) methane production (g/d), (2) methane yield (g/kg dry matter intake), and (3) methane intensity (g/kg fat- and protein-corrected milk) from the regression analyses based on milk fatty acid profiles (g/100 g fatty acids) determined with gas chromatography. The slope of residuals regressed on predicted values did not differ significantly from zero. The different symbols identify the 9 individual experiments described in Table 9.1.

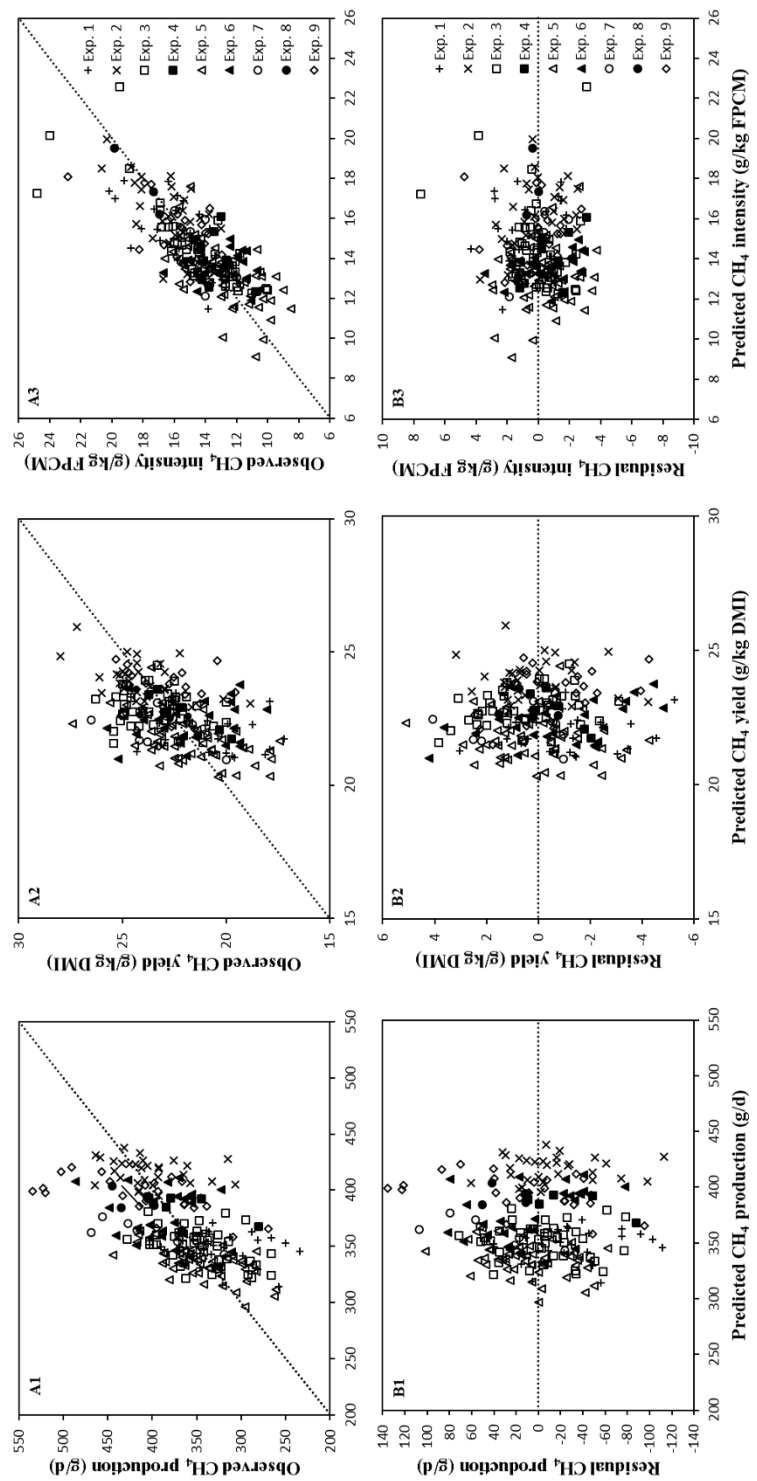

Figure 9.3. (A) Observed and predicted, and (B) residual (i.e., observed – predicted) (1) methane production (g/d), (2) methane yield (g/kg dry matter intake), and (3) methane intensity (g/kg fat- and protein-corrected milk) from the PLS regression analyses based on Fourier-transform infrared wavenumbers (cm^{-1}). The slope of residuals regressed on predicted values did not differ significantly from zero. The different symbols identify the 9 individual experiments described in Table 9.1.

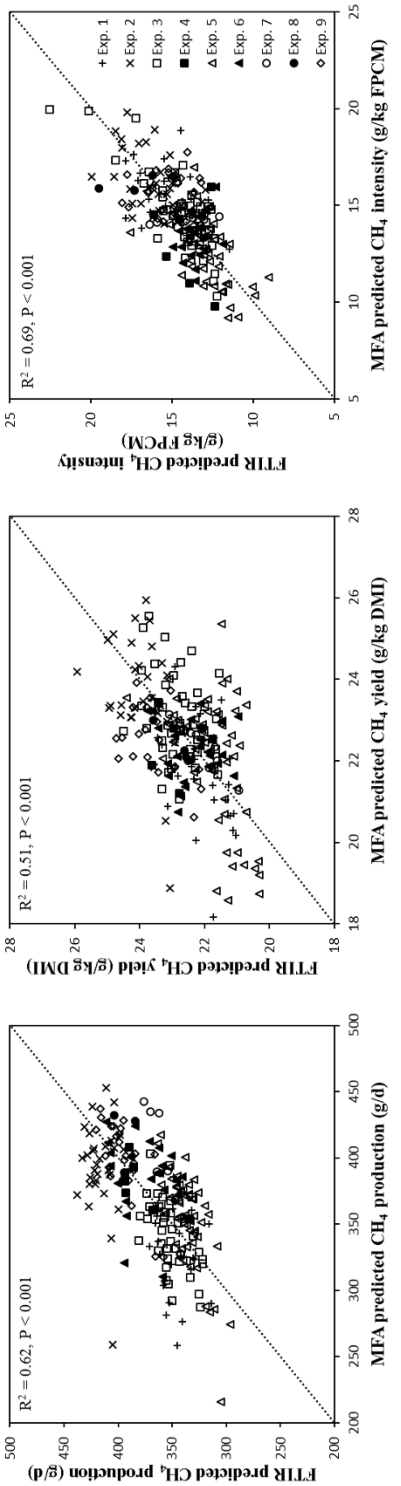

Figure 9.4. The relationship between methane production (g/d), yield (g/kg dry matter intake), and intensity (g/kg fat- and protein-corrected milk) predicted with milk fatty acid profiles determined with gas chromatography and predicted with milk Fourier-transform infrared spectra. The different symbols identify the 9 individual experiments described in Table 9.1.

DISCUSSION

This is the first study evaluating and comparing the CH_4 prediction potential of GC-determined MFA and milk FTIR spectra for CH_4 data obtained in climate respiration chambers. Data were obtained from dairy cattle experiments where type of forage, forage quality, and forage to concentrate ratio were varied, without use of CH_4 mitigating additives. Our results indicate that both GC-determined MFA-based and FTIR-based CH_4 prediction models are robust, and that both techniques can potentially be used to evaluate dietary CH_4 mitigation strategies and to breed for dairy cows with lower CH_4 emissions. The GC-determined MFA-based prediction models had a higher prediction potential than the FTIR-based models and described a larger amount of the observed variation in CH_4 emission.

GC-determined MFA-based methane prediction models

All CH_4 prediction models were based on OBCFA and long chain fatty acids (> 16 carbons). No short- and medium-straight, even-chain fatty acids (≤ 16 carbons) were included in any of the GC-determined MFA-based CH_4 prediction models, despite the fact that these are synthesized *de novo* in the mammary gland from acetate and β-hydroxybutyrate produced in the rumen, which are both reported to be positively associated with CH_4 emission (Ellis et al., 2008). As reviewed by Van Gastelen and Dijkstra et al. (2016), these short- and medium-straight, even-chain fatty acids were usually not included in the GC-determined MFA-based CH_4 prediction equations (n = 6) previously developed, except for C4:0 and C16:0 that were included in 1 equation each. The association of CH_4 emissions with both *iso* and *anteiso* OBCFA is in agreement with *iso* OBCFA being more abundant in fibrolytic bacteria and *anteiso* OBCFA being more abundant in amylolytic bacteria (Vlaeminck et al., 2006). Both C15:0 and C17:0 were found to be positively associated with CH_4 emissions, which is in disagreement with Vlaeminck et al. (2006) and Rico et al. (2016), but in agreement with Chilliard et al. (2009), Dijkstra et al. (2011) and Van Lingen et al. (2014). The negative relations between C18:1, C18:2, and C18:3 isomers in milk and CH_4 emission are in agreement with several other studies (e.g., Van Lingen et al., 2014 and Rico et al., 2016). The associations between CH_4 emissions and long-chain fatty acids have been reported before (i.e., Chilliard et al., 2009; Rico et al., 2016; Van Gastelen et al., 2017b), suggesting that these GC-determined MFA are important in terms of CH_4 prediction.

In general, the prediction potential of the GC-determined MFA-based CH_4 prediction models appears to be moderate to substantial, with the CCC ranging from 0.40 to 0.77. The observed R^2 values ranged from 0.40 to 0.62 and are lower than the ones reported by Dijkstra et al. (2011) for CH_4 yield, and by Chilliard et al. (2009), Mohammed et al. (2011), and Rico et al. (2016) for CH_4 production, but of similar magnitude as Van Lingen et al. (2014) and Van Gastelen et al. (2017b). The recent research, including the present study, on the relationship between GC-determined MFA and CH_4 emission gives inconsistent results. Where some studies found a clear and strong relation between GC-determined MFA and CH_4 emission (e.g., Chilliard et al., 2009, Dijkstra et al., 2011), other studies concluded that GC-determined MFA alone might not be suitable to develop universal CH_4 prediction models (e.g., Mohammed et al., 2011), and more recently, Castro-Montoya et al. (2017) concluded that GC-determined MFA are not reliable predictors for specific amounts of CH_4 emitted by a cow based on the coefficient of

determination of validation ranging from 0.18 to 0.41. Even the studies that do find a clear relation between GC-determined MFA and CH_4 emissions, do not describe similar prediction models using the same GC-determined MFA. The discrepancies between these studies have been reviewed by Van Gastelen and Dijkstra (2016). There are many factors that can influence GC-determined MFA concentrations and therefore the relation between GC-determined MFA and CH_4 emissions (Gengler et al., 2016), such as dietary composition (e.g., Mohammed et al., 2011 and Dijkstra et al., 2016) and lactation stage (Vanrobays et al., 2016). Moreover, it should be noted that previous analyses were often based on data of cattle fed lipid supplements or feed additives, whereas in the present study dietary contrasts included variation in forage to concentrate ratio, type of forage, and forage quality only.

The difference between R^2 and R^2CV for the GC-determined MFA-based CH_4 prediction models was small (0.07 for CH_4 production, 0.02 for CH_4 yield, and 0.01 for CH_4 intensity; Table 9.4). These small differences indicate that all GC-determined MFA-based CH_4 prediction models are robust in terms of CH_4 prediction. The GC-determined MFA-based CH_4 prediction models were also assessed for robustness in terms of composition of the prediction models. All 4 GC-determined MFA that were part of the overall prediction model for CH_4 intensity (Table 9.3) were also selected in the prediction models developed in the 10-fold cross validation (results not shown). Three of the 4 GC-determined MFA were included in all 10 models (i.e., *iso* C15:0, *iso* C17:0, and C18:1 *trans*-15 + C18:1 *cis*-11), which shows the robustness of the GC-determined MFA-based prediction model for CH_4 intensity in terms of composition. In comparison, all 6 GC-determined MFA of the MFA-based prediction model for CH_4 yield were selected in the 10-fold cross validation. Although only 1 GC-determined MFA of the GC-determined MFA-based model (i.e., C18:3n-3) was included in all 10 models of the cross validation, the other 5 GC-determined MFA were included in 6 to 8 of the 10 models. However, of the 8 GC-determined MFA in MFA-based prediction model for CH_4 production, only 5 were also selected in the 10-fold cross validation of which 1 GC-determined MFA (i.e., C18:3n-3) was included in all 10 models. Moreover, 3 of the GC-determined MFA in the GC-determined MFA-based CH_4 production prediction model were not selected in any of the 10 models of the cross validation (i.e., C18:1 *trans*-10, C18:2n-6, and C20:4n-3). This illustrates that the GC-determined MFA-based prediction model for CH_4 production in particular is less robust in comparison to the GC-determined MFA-based prediction model for CH_4 intensity and CH_4 yield.

FTIR-based methane prediction models

In general, the prediction potential of the FTIR-based CH_4 prediction models appears to be moderate to substantial, with the CCC ranging from 0.40 to 0.72 and the R^2 ranging from 0.25 to 0.56. From the regression vector (Figure 9.1) it appears that bands around 975 cm^{-1}, 1,075 – 1,150 cm^{-1}, 1,450 cm^{-1}, 1,500 – 1,575 cm^{-1}, 1,750 cm^{-1}, and 2,850 – 3,000 cm^{-1} are important for the prediction of CH_4 emissions. The latter region, and the bands around 1,175 cm^{-1} and 1,750 cm^{-1} are commonly used to quantify milk fat content (Safar et al., 1994; Dupuy et al., 1996; Yang and Irudayaraj, 2000). Protein is expected to have absorption peaks around wavenumbers 1,500 to 1,700 cm-1 (Osborn and Fearn, 1986; McQueen et al., 1995; Dufour et al., 1998), with the bands around 1,500 – 1,575 cm^{-1} coinciding with the amide II band (Etzion

et al., 2004). Additionally, the infrared region between 1,000 – 1,100 cm^{-1} provides information on sugar molecules (Hashimoto and Kameoka, 2008). This suggests that the bands of the FTIR spectra which are important to determine the milk composition, such as fat and protein content, are also important for the prediction of CH_4 emission. However, as illustrated by Negussie et al. (2017), milk fat and milk protein content have low CH_4 prediction potential. This is also observed in the present study, in which milk protein and milk fat content were relatively weakly associated with CH_4 emissions measured in the climate respiration chambers, except for CH_4 intensity which is calculated using milk fat and protein content. Methane yield was correlated with fat content ($r = 0.17$, $P = 0.010$) and tended to be related to protein content ($r = 0.12$, $P = 0.066$), whereas no significant correlations were observed for CH_4 production. However, as expected from the similarity in FTIR spectra bands, FTIR predicted CH_4 emissions were more strongly related to milk protein content ($r = 0.11$, $P = 0.096$ for CH_4 production; $r = 0.32$, $P < 0.001$ for CH_4 yield; $r = 0.64$, $P < 0.001$ for CH_4 intensity) and to milk fat content ($r = -0.11$, $P = 0.094$ for CH_4 production; $r = 0.37$, $P < 0.001$ for CH_4 yield; $r = 0.13$, $P = 0.053$ for CH_4 intensity).

The differences between R^2 and R^2CV for the milk FTIR-based CH_4 prediction models were 0.06 for CH_4 production, 0.06 for CH_4 yield, and 0.07 for CH_4 intensity (Table 9.4). For CH_4 yield and intensity, these differences between R^2 and R^2CV of FTIR-based models are somewhat larger than for GC-determined MFA-based models, indicating that GC-determined MFA-based models are slightly more robust. The number of studies on FTIR-based CH_4 prediction models is limited. Dehareng et al. (2012) reported FTIR-based prediction models for both CH_4 production and CH_4 intensity (g/kg milk) using the SF_6-tracer technique, involving 11 lactating dairy cows and 3 dietary treatments. The prediction potentials of the FTIR-based prediction models reported by Dehareng et al. (2012) were higher than the ones reported in the present study, with the R^2 ranging from 0.77 to 0.93 and the R^2CV ranging from 0.68 to 0.79. Additionally, Vanlierde et al. (2015) developed both lactation stage independent (i.e., including only FTIR spectra) and lactation stage dependent (i.e., including FTIR spectra and days in milk) CH_4 prediction models using the SF_6-tracer technique involving 142 lactating dairy cows fed a wide range of diets. Vanlierde et al. (2015) reported, for the lactation stage independent CH_4 prediction model (i.e., comparable to present study), a strong correlation ($R^2 = 0.77$) between observed and predicted CH_4 production, which is also higher than that in the present study. However, it is important to note that the previous studies developed the FTIR-based CH_4 prediction models using repeated measurements on the same cow. The study of Dehareng et al. (2012) involved 11 dairy cows, whereas the prediction models were developed using 77 observations, and the study of Vanlierde et al. (2015) involved 142 dairy cows, while the prediction models were developed using 446 observations. In contrast, the present study involved 218 dairy cows and the CH_4 prediction models were developed using 1 observation per cow only. The repeated measurements of Dehareng et al. (2012) and Vanlierde et al. (2015) could have positively influenced the performance parameters of the CH_4 prediction models, as repeated observations are more closely related than independent observations. This is also evident from the evaluation of the lactation stage independent model by Vanlierde et al. (2015) on an independent dataset, which showed a substantially decreased correlation (i.e., $r = 0.09$).

Additionally, the large range of CH_4 emissions measured using the SF_6-tracer technique might have contributed to the high prediction potentials found in both studies. In Dehareng et al. (2012) CH_4 production ranged from 218 to 653 g/d and CH_4 intensity ranged from 10.2 to 47.1 g/kg milk, and in Vanlierde et al. (2015) CH_4 production ranged from approximately 180 to 950 g/d, which are not within the range of CH_4 measurements reported in literature (Appuhamy et al., 2016).

Comparison of GC-determined MFA-based and FTIR-based methane prediction models

For all CH_4 emission units, but particularly for CH_4 production and CH_4 yield, GC-determined MFA-based prediction models had a higher prediction potential than the FTIR-based models. This is evident by the lower RMSEP values and higher R^2 and CCC values. The higher CCC values are caused by the higher accuracy (C_b) and, in particular, higher precision (r) of the GC-determined MFA-based CH_4 prediction models (Table 9.4). The relatively larger differences between the GC-determined MFA-based and FTIR-based prediction models for CH_4 production and CH_4 yield might be explained by GC-determined MFA being more closely linked to the ruminal CH_4 production pathways than FTIR spectra. It is known that GC-determined MFA are related to CH_4 production because of the common biochemical pathway between CH_4 and fatty acids in the rumen (Chilliard et al., 2009; Ellis et al., 2008). As discussed above, the FTIR spectra represent the absorbed light by vibrations at several wavelengths of many milk components, including GC-determined MFA, urea, citrate, free fatty acids, and fat, protein, and lactose content. The latter 3 solid major milk components have a low CH_4 prediction potential (Negussie et al., 2017) and do not seem to be directly linked with ruminal CH_4 pathways. The relatively small difference between the GC-determined MFA-based and FTIR based prediction models for CH_4 intensity might be explained by the fact that CH_4 intensity takes milk yield into account, which is directly associated with enteric CH_4 production by cows and reflected by both the FTIR spectral data and the GC-determined MFA profile, due to dilution effects (Dehareng et al., 2012). This is also illustrated by the somewhat stronger correlation between GC-determined MFA predicted CH_4 intensity and FTIR predicted CH_4 intensity ($r = 0.69$), compared with the correlation between both methods for CH_4 production ($r = 0.62$) and CH_4 yield ($r = 0.51$).

All CH_4 prediction models, both GC-determined MFA-based and FTIR-based, had a scale shift which was different from 1 (v). This indicates that there is a change in standard deviation between predicted and observed CH_4 values for all CH_4 prediction models, which is also visualized in Figures 9.2 and 9.3 for GC-determined MFA-based and FTIR-based models, respectively. The variation in predicted CH_4 values was clearly smaller than that in observed CH_4 values for all CH_4 prediction models. However, the scale shift was greater for all the FTIR-based CH_4 prediction models (v ranges from 1.33 to 2.00) than for the GC-determined MFA-based CH_4 prediction models (v ranges from 1.26 to 1.55), which indicates that GC-determined MFA-based CH_4 prediction models have the ability to describe more of the observed variation in CH_4 emissions compared with FTIR-based prediction models.

Table 9.5. Differences in methane emissions between 2 extreme dietary treatments within each study, measured in climate respiration chambers (CRC) and estimated with the MFA[1]-based and FTIR[2]-based prediction models

Study	Reference	Difference between treatments	Methane emission	Difference measured in CRC	Difference estimated	
					MFA	FTIR
1	Warner et al. (2015)	Grass herbage 5 weeks of regrowth receiving high fertilization compared with grass herbage 3 weeks of regrowth receiving low fertilization	Production (g/d)	+31	+36	+8
			Yield (g/kg DMI)	+2.0	+1.9	+0.4
			Intensity (g/kg FPCM)	+1.3	+1.3	+1.0
2	Van Gastelen et al. (2015)	Roughage consisting of 100% corn silage compared with roughage consisting of 100% grass silage	Production (g/d)	-12	-13	-15
			Yield (g/kg DMI)	-2.6	-2.9	-0.9
			Intensity (g/kg FPCM)	-1.3	-3.3	-0.8
3	Warner et al. (2016)	Grass silage 62 d of regrowth and high fertilization rate compared with grass silage 28 d of regrowth and low fertilization rate	Production (g/d)	-39	-4	+8
			Yield (g/kg DMI)	+1.6	+1.5	0.0
			Intensity (g/kg FPCM)	+4.6	+2.6	+1.6
5	Warner et al. (2017)	Late heading stage grass silage at low DMI compared with leafy stage grass silage at high DMI	Production (g/d)	+24	+32	-1
			Yield (g/kg DMI)	+5.0	+2.8	+1.5
			Intensity (g/kg FPCM)	+4.0	+3.1	+2.6
6	Hatew et al. (2016)	Late harvested whole-plant corn silage compared with early harvested whole-plant corn silage	Production (g/d)	-29	+13	-0.4
			Yield (g/kg DMI)	-1.6	0.0	+0.3
			Intensity (g/kg FPCM)	-0.9	-1.1	+0.4

[1] Milk fatty acids in g/100 g fatty acids determined with gas chromatography.

[2] Fourier-transform infrared spectra.

The RPD values from the present study are lower than the RPD values reported by Dehareng et al. (2012). The low RPD values from the present study (i.e., < 1.58 for the GC-determined MFA based CH_4 prediction models and < 1.39 for the FTIR-based CH_4 prediction models), suggest that the prediction ability of these models can be regarded as poor (Williams et al., 2014). According to Williams and Sobering (1993) a RPD value of 2.5 and above would suggest that the model is satisfactory for screening. A narrow range in the variability of the observations is known to negatively affect predictability of methods of interest (Manley, 2014). Indeed, the coefficient of variation (SD relative to mean) is highest for CH_4 intensity (17.9%) and the models for CH_4 intensity had relatively the best RPD. The lowest coefficient of variation is for CH_4 yield (9.3%) and the models for CH_4 yield had the smallest RPD values. Moreover, although the respiration chamber method is generally considered to be the golden standard for CH_4 measurements (Hammond et al., 2016), its reproducibility as compared with many chemical analyses for which the RPD statistic was originally developed, is much lower, hence reducing prediction accuracy of the prediction methods. The RPD values would suggest that the CH_4 prediction models presented in the current study, both GC-determined MFA-based and FTIR-based, would not be able to classify dairy cows from populations with low variation in CH_4 emission into low and high CH_4 producers. More variation in the dairy population under evaluation, such as greater variation in animal genetics, in dietary composition, and in production management, could potentially improve the ability of the models to predict CH_4 emission (Dehareng et al., 2012).

It is important to note though, that the present study did not take lactation stage into account. Although lactation stage is a poor CH_4 proxy when considered alone (Negussie et al., 2017), Vanlierde et al. (2015) demonstrated that lactation stage in combination with FTIR improved the CH_4 prediction model. Vanlierde et al. (2015) developed both lactation stage-independent and lactation stage-dependent CH_4 prediction models. The average CH_4 production (g/d) predicted by both models was similar (416 ± 63 g/d). However, in contrast to the lactation stage-independent prediction model, the lactation stage-dependent prediction model showed biologically meaningful behavior throughout lactation: an increase in CH_4 production (g/d) after calving up to approximately 100 DIM, followed by a gradual decline towards the end of lactation (Vanlierde et al., 2015). This effect of lactation stage could also be important for the MFA-based CH_4 prediction models, because Vanrobays et al. (2016) clearly demonstrated that the correlations between GC-determined MFA and CH_4 production in dairy cows vary according to lactation stage. We therefore acknowledge that the CH_4 prediction models of the present study may be improved in terms of predictive power and robustness, when combining GC-determined MFA or FTIR with lactation stage. We were, however, not able to confirm this, because differences in lactation stage were confounded by differences in dietary composition in the dataset used in the present study.

Application of methane prediction models in practice

In the present study, we show that GC-determined MFA have a higher prediction potential for CH_4 emissions than FTIR spectra. However, the gas chromatography procedure required to obtain the GC-determined MFA profile is unsuitable for routine milk recording,

whereas the prediction of CH_4 emission using FTIR has the potential for practical high throughput application.

Although the RPD results suggest that the GC-determined MFA-based and FTIR-based CH_4 prediction models currently have limited applicability, the CCC results demonstrated that the models had at least moderate predictive ability. Potential practical applications for these models include: (1) as a farm management tool, (2) to evaluate CH_4 mitigation strategies, and (3) as a tool to breed for dairy cows with lower CH_4 emissions (Cottle et al., 2011). When a dietary strategy is applied in practice, the proxy for CH_4 emission should be able to evaluate whether CH_4 emission is affected by the new dietary strategy. Therefore, within each study that had at least 2 dietary treatments, we evaluated whether the GC-determined MFA-based and FTIR-based CH_4 prediction models were able to estimate the same difference in CH_4 emission as measured in the climate respiration chambers, by comparing CH_4 emission at 2 extreme diets (i.e., furthest apart from one another in terms of dietary composition). The results of this evaluation are shown in Table 9.5. In general, all CH_4 prediction models predicted a difference in CH_4 emission similar to the climate respiration chambers in terms of trend (i.e., increase or decrease). There were only a few exceptions, viz. two for the GC-determined MFA-based and six for the FTIR-based CH_4 prediction models. Furthermore, the differences in CH_4 emission between the two diets as estimated by the GC-determined MFA-based CH_4 prediction models were generally more in line with the observed differences as measured in the climate respiration chambers, than that of the FTIR-based CH_4 prediction models compared with the difference measured in climate respiration chambers. This suggests that the FTIR-based CH_4 prediction models might have less accuracy relative to the GC-determined MFA-based CH_4 prediction models, both based on a single FTIR or a single GC measurement to determine the MFA profile of a 4-day combined milk sample, to evaluate the effect of forage level and quality on CH_4 emission of dairy cattle.

Breeding for reduced CH_4 emission can be achieved with, for example, improved productivity, increased longevity, or shorter calving interval (Bell et al., 2011), but also by breeding for actual lower enteric CH_4 production (Wall et al., 2010). Several studies have shown that CH_4 emissions of dairy cows have a genetic component, with heritability ranging from 0.20 to 0.30 (e.g., De Haas et al., 2011 for predicted CH_4 emission based on feed intake; Lassen and Løvendahl, 2016 for CH_4 emission measured with a portable air-sampler), indicating that breeding for dairy cows with lower CH_4 emission may be possible. Recently, Vanlierde et al. (2016) reported that FTIR can distinguish cows with low or high daily CH_4 emissions. Direct breeding for lower enteric CH_4 production requires CH_4 production measurements of a large number of individual dairy cows to determine the genetic component of the CH_4 phenotype as well as to determine the genetic correlations of CH_4 emissions with other traits. This can be facilitated by the FTIR technique as it can be used routinely to estimate CH_4 on commercial dairy farms.

CONCLUSIONS

This study is the first to assess and compare the CH_4 emission prediction potential of both GC-determined MFA profiles and FTIR spectra based on CH_4 emission data obtained in

climate respiration chambers and for three different units of CH_4 emission, viz. CH_4 production, yield, and intensity. For both GC-determined MFA and FTIR, the prediction model for CH_4 yield had the lowest prediction potential and the prediction model for CH_4 intensity had the highest prediction potential. For all CH_4 emission units, but particularly for CH_4 production and yield, GC-determined MFA-based prediction models had a higher prediction potential than the FTIR-based models, and GC-determined MFA-based prediction models described a greater part of the observed variation in CH_4 emission than FTIR-based models. Results indicate that the current GC-determined MFA-based and FTIR-based CH_4 prediction models have potential, but have limited current applicability. Additional CH_4 measurements are needed to improve prediction models in terms of accuracy and robustness of both GC-determined MFA and FTIR spectra for CH_4 prediction.

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Chapter 10

General discussion

INTRODUCTION

The dairy supply chain is associated with environmental costs (Baskaran et al., 2009), with methane (**CH_4**) emission from microbial fermentation of feed in the rumen and, to a smaller extent, the large intestines, being both an important contributor to global greenhouse gas (**GHG**) emissions and a potential loss of energy. This makes enteric CH_4 emission one of the main targets of the GHG mitigation objectives of the dairy cattle sector (Hristov et al., 2013). Diet changes and feed additives can be effective strategies to mitigate CH_4 emission (e.g., Beauchemin et al., 2009; Martin et al., 2010; Hristov et al., 2013), although their effects depend on continuous use of the diet or additive as well as an inability of the rumen microbiome to adapt to these strategies. Breeding for reduced CH_4 emission is another CH_4 mitigation strategy with a more permanent and cumulative effect (Wall et al., 2010), as several studies have shown that CH_4 emissions of dairy cows have a genetic component, with heritability ranging from 0.20 to 0.30 (e.g., De Haas et al., 2011; Lassen and Løvendahl, 2016). Dietary mitigation strategies together with breeding for reduced CH_4 emissions could therefore be effective in reducing the environmental impact of the dairy supply chain.

Accurate and repeatable measurements of CH_4 emission from individual dairy cows are required to assess the efficacy of possible mitigation strategies as well as to develop protocols for genetic selection for cows with reduced CH_4 emission (Hammond et al., 2016). Several techniques have been developed to measure CH_4 emissions from dairy cattle, with varying degrees of accuracy and repeatability [see Chapter 1 and Hammond et al. (2016)], but routine individual-animal measurements on a large scale are difficult to obtain (Pickering et al., 2015). Therefore, identifying proxies that are correlated with CH_4 emission but that are easy and relatively low cost to record on a large scale, is a much-needed alternative (Negussie et al., 2017). The research described in the present PhD thesis aimed to explore the possibility to develop a proxy, or combine a number of potential proxies, for CH_4 emission that can be measured in milk of dairy cows.

PROXIES FOR METHANE EMISSION IN MILK

As described in Chapter 1, a proxy for CH_4 emission of dairy cows is an indicator or indirect trait that is correlated with enteric CH_4 production. There are several criteria that a proxy needs to adhere to, in order to actually be valuable, as described in Chapter 1. From a practical point of view, a proxy should score satisfactory on the attributes simplicity, costs, invasiveness, and throughput. Additionally, from a technical point of view, it is important that a proxy is both accurate and precise when estimating CH_4 emission. It should however be noted that, despite the importance of precision, studies in general only focus the accuracy of the proxies for CH_4 emission without considering precision (e.g., Negussie et al., 2017). Additionally, the definition of a weak, moderate, and strong relation varies considerably in literature and some studies do not even define the R^2 values to differentiate between weak, moderate, and strong relation (e.g., Negussie et al., 2017). Therefore, to avoid confusion, in the current chapter, a relationship is considered to be weak when the R^2 is smaller than 0.30, moderate when the R^2 is between 0.30 and 0.70, and strong when the R^2 is larger than 0.70.

In this thesis, four potential proxies in milk for CH_4 emissions of dairy cows were investigated: (1) milk fatty acids (**MFA**), (2) volatile metabolites, (3) non-volatile metabolites, and (4) Fourier-transform infrared (**FTIR**) spectra. The MFA were considered to be the basis of the PhD work described in this thesis, because previous studies (e.g., Chilliard et al., 2009; Dijkstra et al., 2011) demonstrated that MFA hold potential to predict CH_4 emission of dairy cows. Besides MFA, milk also contains water, carbohydrates, proteins, vitamins, and minerals (Sundekilde et al., 2011). Stage of lactation, seasonal changes, genetic variability, health status of the cow, and nutrition have been shown to cause changes in major milk components (Walker et al., 2004; Heck et al., 2009), non-volatile metabolites (Klein et al., 2010), and volatile metabolites (Hettinga et al., 2008). It was therefore hypothesized that the addition of such other metabolites in a MFA-based CH_4 prediction equation would enhance its predictive power and thus would lead to a better proxy in milk for CH_4 emission of dairy cows. The CH_4 prediction potential of volatile and non-volatile metabolites in milk, both alone and in combination with MFA was therefore investigated. However, the principal method to determine the MFA profile (i.e., gas chromatography), the volatile metabolites (i.e., gas chromatography-mass spectroscopy), and non-volatile metabolites (i.e., nuclear magnetic resonance) are unsuitable for routine analysis. For MFA, this has led to the application of FTIR. Therefore, also the CH_4 prediction potential of FTIR was investigated.

Negussie et al. (2017) reviewed MFA in terms of their attributes with respect to their use as proxy for CH_4 emission of dairy cows. According to these authors, MFA have a medium score for simplicity because it involves gas-chromatography measurements, involves a medium level of costs, and represents a non-invasive proxy with a medium throughput. Additionally, Negussie et al. (2017) concluded that the level of accuracy (e.g., R^2) of MFA-based proxies for CH_4 emissions varied between medium and high. This is what was also found in Chapter 2, in which the recent research that related CH_4 emission with the MFA profile was reviewed. The predictive power of MFA-based CH_4 proxies ranged from 0.47 (Van Lingen et al., 2014) to 0.95 (Chilliard et al., 2009). In Chapter 5, in which MFA-based CH_4 proxies were developed for dairy cows fed roughage-based diets with varying levels of corn silage and grass silage, the adjusted R^2 of the MFA-based CH_4 proxies ranged from 0.47 for CH_4 intensity to 0.63 for CH_4 production. Additionally, the adjusted R^2 of the MFA-based CH_4 proxies for dairy cows fed a wide range of roughage-based diets, as described in Chapter 6, varied from 0.38 for CH_4 yield to 0.75 for CH_4 intensity. This pattern was also observed in Chapter 9 involving more observations and an even wider range of roughage-based diets. The adjusted R^2 in the latter chapter ranged from 0.40 for CH_4 yield to 0.62 for CH_4 intensity. The variation in accuracy with which the MFA can predict CH_4 emission, partially results from the different units in which CH_4 emission is expressed, as discussed in Chapter 2. Furthermore, there are many factors that can influence the relationship between MFA and CH_4 emissions (Gengler et al., 2016), such as dietary composition (e.g., Mohammed et al., 2011; Dijkstra et al., 2016) and lactation stage (Vanrobays et al., 2016), causing variable results.

Negussie et al. (2017) also reviewed FTIR in terms of its attributes and according to these authors, FTIR is simple to measure, involves a low level of costs, and represents a non-invasive proxy with a high throughput. Additionally, Negussie et al. (2017) concluded the level

of accuracy (e.g., R^2) of FTIR to directly predict CH_4 emissions (i.e., not via MFA) is high. The results presented in this thesis do, however, not support these findings. As stated in the review (Chapter 2), the major advantages of FTIR to predict CH_4 emission indeed include its simplicity and potential practical application on a large scale. However, disadvantages include the inability to predict important MFA for CH_4 prediction (as illustrated by Van Lingen et al., 2014), and the moderate predictive power for directly estimating CH_4 emission (based on Dehareng et al., 2012 and Vanlierde et al., 2015). Also in Chapter 9, describing FTIR-based CH_4 proxies, the adjusted R^2 ranged from 0.25 for CH_4 yield to 0.56 for CH_4 intensity. Thus, based on the work described in this thesis, FTIR is a good proxy from a practical point of view, but still lacks accuracy to be a good proxy from a technical point of view.

Negussie et al. (2017) did not review the attributes of the volatile metabolites and non-volatile metabolites in milk. The techniques required to determine the volatile metabolites (i.e., gas chromatography-mass spectroscopy) and non-volatile metabolites (i.e., nuclear magnetic resonance) in milk are not suitable for large-scale measurements and would score low in terms of simplicity and throughput. Additionally, the costs involved are medium to high, whereas both proxies can be considered non-invasive. It should be noted though, that rapid developments in metabolomics may offer tests and assay methodologies on milk samples that will provide a more practical tool for developing proxies for CH_4 emissions in dairy cattle in the future. However, at present, both volatile and non-volatile metabolites would not be interesting CH_4 proxies from a practical point of view. The results of Chapters 5 indicate that including volatile metabolites (CH_4 intensity only) and non-volatile metabolites increases the CH_4 emission prediction potential, whereas the results of Chapter 6 indicate that it is not worthwhile to further pursue research on the ability of both volatile and non-volatile metabolites in milk to estimate CH_4 emission of dairy cows, because of low adjusted R^2 values relative to the MFA profile.

Overall, the results presented in this thesis indicate that, of all the 4 potential CH_4 proxies in milk investigated (i.e., MFA, volatile metabolites, non-volatile metabolites, and FTIR), the MFA profile provides, thus far, the most accurate and precise proxy for CH_4 emission of dairy cows, irrespectively of the unit in which CH_4 emission is expressed. Also the FTIR spectra, although less accurate and precise than MFA, can serve as a proxy for CH_4 emission of dairy cows, especially because of its great practical application potential and, hence, repeated measurements. Thus both techniques, MFA and FTIR, hold potential to estimate CH_4 emissions of dairy cows.

Textbox 3. The statistical approach used

Multivariate models were developed using a stepwise procedure (PROC GLMSELECT of SAS) with CH_4 emission [i.e., production in g/d, yield in g/kg dry matter intake (**DMI**), and intensity in g/kg fat- and protein-corrected milk (**FPCM**)] as the independent variable and stepwise selection of lactation characteristics. The significance level for a variable to enter or stay in the model was 0.01 and 0.05, respectively. The final models were selected based on the minimum Akaike's information criterion statistic, and subsequently evaluated in PROC REG in terms of multicollinearity (variation inflation factor > 10), but no multicollinearity was observed. The final models were then evaluated with the concordance correlation coefficient (CCC; Lin, 1989) analysis. The new CH_4 prediction models developed in the general discussion are described in Table 10.1. The evaluation results (i.e., R^2 and CCC analysis) of these new developed CH_4 prediction models, as well as of the MFA- and FTIR-based prediction models from Chapter 9, are shown in Table 10.2.

The statistics described above, follow an empirical approach. When developing a CH_4 prediction model using a mechanistic approach, one would first determine which specific parameters would be of interest and subsequently start modelling the processes that occur to link the parameters of interest to CH_4 emission. However, in this research, the empirical approach was used, in which all parameters were related to CH_4 emission. This approach was chosen for two reasons: (1) for most MFA (e.g., short- and medium-straight even-chain MFA, odd- and branched-chain MFA, and long chain MFA) one can theoretically describe the processes that link these MFA to CH_4 emission, but it does not necessarily mean that these MFA are related with CH_4 emission, and (2) the main interest was to develop CH_4 prediction models with the highest prediction potential, describing as much of the variation in CH_4 emission as possible. This could only be achieved using an empirical approach.

As reported above, the significance level for a variable to enter or stay in the model was 0.01 and 0.05 and reflect an arbitrary decision. Initially, in Chapter 5, the significance levels of 0.05 and 0.10 were used, which represent the levels often used by others to indicate significant relationships and tendencies. However, it was decided to adjust these levels for two reasons: (1) developing models with more selection variables than observations increases the chance of overfitting in the CH_4 prediction model, and (2) lower significance levels would result in more robust CH_4 prediction models. To illustrate, combining MFA with lactation characteristics for CH_4 production resulted in a model with 7 variables (Table 10.1) and an R^2 of 0.72 (Table 10.2). However, when applying the significance levels of 0.05 and 0.10, it resulted in a model with 10 variables and an R^2 of 0.73. Additionally, when using no predetermined significance level for a variable to enter or stay in the model (i.e., default of SAS is 0.15 for a variable to enter and stay in the model), it resulted in a model with 15 variables and an R^2 of 0.76. Thus, less strict significance levels results in more variables to be included in the model with explaining only a limited amount of extra variation in CH_4 emission.

Table 10.1. Prediction models developed for methane production (g/d), methane yield (g/kg DMI[1]), and methane intensity (g/kg FPCM[2]) based on different sets of parameters

Methane production (g/d)				Methane yield (g/kg DMI)				Methane intensity (g/kg FPCM)			
Item	Estimate	SE	*P*-value	Item	Estimate	SE	*P*-value	Item	Estimate	SE	*P*-value
Lactation chatacteristics											
Intercept	-54	40.3	< 0.001	Intercept	20.3	0.50	< 0.001	Intercept	11.3	0.34	< 0.001
DIM	0.169	0.0446	< 0.001	DIM	0.005	0.0016	0.004	DIM	0.018	0.0017	< 0.001
Milk yield (kg/d)	8.69	0.682	< 0.001	Urea content (mg/dL)	0.076	0.0217	< 0.001				
Protein content (g/100 g milk)	53.0	9.56	< 0.001								
Milk composition											
No model obtained				Intercept	21.1	0.43	< 0.001	Intercept	31.1	3.53	< 0.001
				Urea content (mg/dL)	0.079	0.0220	< 0.001	Lactose content (g/100 g milk)	-3.63	0.770	< 0.001
MFA[3] selected according to Soyeurt et al. (2011)											
Intercept	591	21.6	< 0.001	Intercept	24.3	1.97	< 0.001	Intercept	27.1	1.12	< 0.001
∑*trans* C18:1	-44.0	9.27	< 0.001	C4:0	-1.13	0.366	0.002	C18:0	-0.387	0.1000	< 0.001
∑*cis* C18:1	-7.62	0.772	< 0.001	C16:0	0.16	0.039	< 0.001	C18:1 *cis*-9	14.5	1.68	< 0.001
				∑*trans* C18:1	-1.94	0.402	< 0.001	∑*cis* C18:1	-14.7	1.67	< 0.001
MFA selected according to Rutten et al. (2009)											
Intercept	524	17.3	< 0.001	Intercept	21.5	1.98	< 0.001	Intercept	27.1	1.67	< 0.001
C18:1 *cis*-9	-7.51	0.809	< 0.001	C4:0	-1.23	0.383	0.002	C4:0	-2.09	0.437	< 0.001
				C16:0	0.167	0.040	< 0.001	C18:1 *cis*-9	-0.252	0.0402	< 0.001
Dietary composition											
Intercept	464	18.0	< 0.001	Intercept	17.9	1.34	< 0.001	Intercept	52.2	7.62	< 0.001
Fat (g/kg DM)	-2.17	0.512	< 0.001	Crude protein (g/kg DM)	0.017	0.0037	0.005	Fat (g/kg DM)	-0.148	0.0256	< 0.001
Sugar (g/kg DM)	-0.352	0.0577	< 0.001	ADF (g/kg DM)	0.026	0.0049	0.001	Starch (g/kg DM)	-0.009	0.0018	< 0.001
				Fat (g/kg DM)	-0.133	0.0222	< 0.001	Gross energy (MJ/kg DM)	-1.73	0.424	< 0.001
Lactatation characteristics combined with MFA											
Intercept	169	42.5	< 0.001	Intercept	26.4	1.50	< 0.001	Intercept	-0.49	2.429	< 0.001
Milk yield (kg/d)	7.48	0.521	< 0.001	DIM	0.005	0.0014	0.001	DIM	0.010	0.0013	< 0.001

Table 10.1. Continued

Item	Estimate	SE	P-value	Item	Estimate	SE	P-value	Item	Estimate	SE	P-value
Protein content (g/100 g milk)	36.7	6.44	< 0.001	Urea content (mg/dL)	0.048	0.0174	0.006	*iso* C15:0	15.9	3.57	< 0.001
iso C15:0	315.4	60.87	< 0.001	LN(SCC)	-0.395	0.0928	0.002	C16:0	0.138	0.0471	0.005
C18:1 *trans*-11	-40.6	9.59	< 0.001	C17:0	5.58	1.614	< 0.001	*iso* C17:0	13.7	2.19	< 0.001
C18:1 *trans*-15 + C18:1 *cis*-11	-96.9	14.96	< 0.001	C18:1 *trans*-11	-1.99	0.539	0.001	C18:1 *trans*-15 + C18:1 *cis*-11	-4.50	1.006	< 0.001
C18:3n-6	-435.0	155.83	0.006	C18:1 *trans*-15 + C18:1 *cis*-11	-2.63	0.834	< 0.001	C20:0	15.3	5.61	0.006
C18:3n-3	-78.8	15.04	< 0.001	C18:1 *cis*-12	-8.43	2.455	0.002	C22:5n-3	15.9	6.10	0.010
				C18:3n-3	-4.78	0.908	< 0.001				
Dietary composition combined with MFA											
Intercept	508	28.7	< 0.001	Intercept	19.0	1.44	< 0.001	Intercept	5.4	1.25	< 0.001
C15:0	62.9	17.22	0.002	Crude protein (g/kg DM)	0.014	0.0033	< 0.001	Sugar (g/kg DM)	0.009	0.0018	< 0.001
C17:1 *cis*-9	-240.6	32.29	0.007	ADF (g/kg DM)	0.028	0.0044	< 0.001	NDF-to-starch ratio	-0.031	0.0075	0.004
C18:1 *trans*-10	-202.8	47.75	0.001	Fat (g/kg DM)	-0.099	0.0209	0.004	C14:1 *cis*-9	1.60	0.511	0.002
C18:1 *trans*-11	-59.3	12.7	< 0.001	*iso* C16:0	11.9	3.25	0.001	*iso* C15:0	23.7	3.71	< 0.001
C18:2n-6	48.1	14.08	0.005	C18:1 *trans*-11	-2.23	0.533	< 0.001	C18:1 *trans*-15 + C18:1 *cis*-11	-3.20	0.745	< 0.001
C18:3n-3	-187.1	24.4	< 0.001	C18:1 *trans*-15 + C18:1 *cis*-11	-3.10	0.721	< 0.001	C20:4n-6	16.8	4.78	< 0.001
C20:4n-3	326.4	104.3	0.002					C22:0	39.5	10.90	0.006
C24:0	-816.8	230.89	0.007								
Lactation characteristics combined with dietary composition and MFA											
Intercept	169	42.5	< 0.001	Intercept	17.4	1.60	< 0.001	Intercept	11.5	1.35	< 0.001
Milk yield (kg/d)	7.48	0.521	< 0.001	Crude protein (g/kg DM)	0.010	0.0028	0.005	ADL (g/kg DM)	0.077	0.0272	< 0.001
Protein content (g/100 g milk)	36.7	6.44	< 0.001	ADF (g/kg DM)	0.030	0.0059	< 0.001	Fat (g/kg DM)	-0.088	0.0180	< 0.001
iso C15:0	315.4	60.87	< 0.001	ADL (g/kg DM)	0.108	0.0344	0.002	DIM	0.010	0.0013	< 0.001

Table 10.1. Continued

Item	Estimate	SE	*P*-value	Item	Estimate	SE	*P*-value	Item	Estimate	SE	*P*-value
C18:1 *trans*-11	-40.6	9.59	< 0.001	Fat content (g/100 g milk)	0.580	0.1678	< 0.001	Urea content (mg/dL)	-0.048	0.0164	0.006
C18:1 *trans*-15 + C18:1 *cis*-11	-96.9	14.96	< 0.001	LN(SCC)	-0.362	0.0883	< 0.001	*iso* C15:0	23.6	3.16	< 0.001
C18:3n-6	-435.0	155.83	0.006	C18:1 *trans*-11	-1.91	0.516	< 0.001	C18:2n-6	-2.35	0.448	< 0.001
C18:3n-3	-78.8	15.04	< 0.001	C18:1 *trans*-15 + C18:1 *cis*-11	-4.59	0.683	< 0.001	C22:5n-3	21.5	6.45	0.002
				C24n-6	-26.5	7.02	0.002				

(1) Dry matter intake (kg/d).

(2) Fat- and protein-corrected milk = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d) (CVB, 2012).

(3) Milk fatty acids in g/100 g FA.

Table 10.2. The coefficient of determination (R^2) and concordance correlation coefficient (CCC) analysis of the prediction equations for methane emission

Item	Adjusted R^2	CCC[1]	r[2]	C_b[3]	v[4]	μ[5]
MFA[6] (Chapter 9)						
Methane production[7]	0.54	0.72	0.75	0.96	1.34	0.00
Methane yield[8]	0.40	0.59	0.64	0.91	1.55	0.00
Methane intensity[9]	0.62	0.77	0.79	0.97	1.26	0.00
FTIR[10] spectra (Chapter 9)						
Methane production	0.36	0.52	0.60	0.88	1.68	0.00
Methane yield	0.25	0.40	0.50	0.80	2.00	0.00
Methane intensity	0.56	0.72	0.75	0.96	1.33	0.00
Lactation characteristics						
Methane production	0.43	0.61	0.66	0.92	1.50	-0.01
Methane yield	0.09	0.18	0.30	0.60	3.01	0.03
Methane intensity	0.33	0.50	0.58	0.87	1.73	-0.01
Milk composition						
Methane production	n.a.[11]	n.a.	n.a.	n.a.	n.a.	n.a.
Methane yield	0.05	0.12	0.23	0.49	3.78	0.02
Methane intensity	0.09	0.17	0.30	0.56	3.23	-0.04
MFA selected according to Soyeurt et al. (2011)						
Methane production	0.35	0.52	0.59	0.88	1.68	0.00
Methane yield	0.20	0.35	0.46	0.76	2.16	0.00
Methane intensity	0.39	0.57	0.63	0.90	1.58	0.00
MFA selected according to Rutten et al. (2009)						
Methane production	0.28	0.44	0.53	0.83	1.87	0.00
Methane yield	0.12	0.23	0.36	0.64	2.79	0.00
Methane intensity	0.24	0.39	0.49	0.79	2.03	0.00
Dietary composition						
Methane production	0.18	0.30	0.42	0.72	2.37	0.00
Methane yield	0.24	0.39	0.50	0.80	2.02	0.00
Methane intensity	0.29	0.46	0.55	0.84	1.82	0.00
Lactation characteristics combined with MFA						
Methane production	0.72	0.84	0.85	0.99	1.17	-0.02
Methane yield	0.44	0.62	0.67	0.93	1.47	-0.01
Methane intensity	0.71	0.84	0.85	0.99	1.17	0.00
Dietary composition combined with MFA						
Methane production[12]	0.54	0.72	0.75	0.96	1.34	0.00
Methane yield	0.39	0.58	0.64	0.91	1.56	0.00
Methane intensity	0.66	0.81	0.82	0.98	1.22	0.00
Lactation characteristics combined with dietary composition and MFA						
Methane production[13]	0.72	0.84	0.85	0.99	1.17	-0.02
Methane yield	0.48	0.66	0.70	0.94	1.42	-0.01
Methane intensity	0.70	0.83	0.84	0.99	1.19	-0.01

[1] Concordance correlation coefficient, where CCC = $r \times C_b$.

[2] Pearson correlation coefficient; a measure of precision.

[3] Bias correction factor; a measure of accuracy.

[4] Scale shift; change in standard deviation between predicted and observed methane emission.

[5] Location shift; if positive under prediction, if negative over prediction.

[6] Milk fatty acids in g/100 g FA.

Table 10.2. Continued

(7) Production in g methane per day.

(8) Yield in g methane per kg dry matter intake.

(9) Intensity in g methane per kg fat- and protein-corrected milk (FPCM (kg/d) = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d); CVB, 2012).

(10) Fourier transform infrared spectra.

(11) Not applicable, because no model was obtained.

(12) Prediction model and evaluation results similar to model for methane production using only MFA (Chapter 9).

(13) Prediction model and evaluation results similar to model for methane production combing lactation characteristics and MFA.

LACTATION CHARACTERISTICS AS PROXY FOR METHANE EMISSION

Although information on lactation characteristics of individual cows is generally easily available because of the milk recording system, not much research is available investigating the CH_4 prediction potential of these lactation characteristics (i.e., milk yield and milk composition, including fat, protein, and lactose content). Moraes et al. (2014) identified milk fat content as a key explanatory variable for prediction of CH_4 emissions of dairy cattle. In contrast, Van Lingen et al. (2014) developed prediction equations for both CH_4 yield and CH_4 intensity of dairy cattle, and milk fat content and milk protein content were not selected in any of the prediction models. More recently, Negussie et al. (2017) reviewed the suitability of lactation parameters as CH_4 proxy of dairy cows and concluded that both milk yield and composition are simple to measure, involve low costs, are non-invasive, and have a high throughput. Milk yield was considered to have a medium to high accuracy in terms of CH_4 prediction, whereas that of major milk components was, however, considered to be low to medium (Negussie et al., 2017). To investigate the CH_4 prediction potential of only lactation characteristics and whether both MFA and FTIR have an added value in terms of CH_4 prediction potential relative to lactation characteristics, the same dataset as in Chapter 9 was used. The lactation characteristics include parity, days in milk (**DIM**), milk yield (kg/d), milk protein content (g/100 g milk), milk fat content (g/100 g milk), milk lactose content (g/100 g milk), milk urea content (mg/dL), and somatic cell count [natural logarithm; **LN(SCC)**]. All lactation characteristics were considered as continuous variables, with the exception of parity. Parity was considered as a class variable, with primiparous cows as one class, cows in second lactation as a second class, and cows in third or higher lactation as a third class. All of these lactation characteristics were used as independent variables for CH_4 production and CH_4 yield, but only parity, DIM, milk lactose content, milk urea content, and LN(SCC) for CH_4 intensity (other parameters were excluded because they are part of the FPCM calculation; CVB, 2012). The statistical method applied was similar to that of Chapter 9 and is shortly described in Textbox 3.

The CH_4 prediction models obtained when using only lactation characteristics as selection parameters had an adjusted R^2 ranging from 0.09 to 0.43 and a CCC ranging from 0.18 to 0.61 (Table 10.2). The model for CH_4 yield (g/kg DMI) performed the poorest and the model for CH_4 production (g/d) performed the best, as evident by the lowest and highest adjusted R^2 and CCC values, respectively. For both CH_4 yield and CH_4 intensity, the lactation characteristics-based prediction models performed clearly less satisfactory (i.e., lower adjusted R^2 and CCC

values) than the MFA- and FTIR-based prediction models. This indicates that both MFA and FTIR have a greater prediction potential for CH_4 yield and CH_4 intensity than lactation characteristics. This also holds for the MFA-based prediction model for CH_4 production, which performed better than the lactation characteristics. The only exception was the lactation characteristics-based model for CH_4 production which performed better than the FTIR-based model for CH_4 production, as evident by the higher adjusted R^2 and CCC values. Additionally, in comparison with the FTIR based model for CH_4 production, the r and C_b values of the lactation based model are higher, indicating a more precise and accurate prediction of CH_4 production. Moreover, the scale shift (v) is smaller, indicating that the lactation characteristics-based model can describe more of the observed variation in CH_4 production than the FTIR-based prediction model.

Three lactation characteristics were included in the model for CH_4 production, namely DIM, milk yield, and protein content, all with a positive slope (Table 10.2). Also in the prediction models for CH_4 yield and CH_4 intensity, DIM was included as explanatory variable. The positive association between CH_4 production and milk yield was expected. A higher milk yield is often associated with a higher DMI (Garnsworthy et al., 2012), and a higher DMI is often associated with a higher daily CH_4 production (e.g., Hristov et al., 2013; Bell et al., 2016; Charmley et al., 2016). Lactation stage (i.e., DIM) can also be related to CH_4 emissions from dairy cattle, based on a rough approximation of milk yield during lactation (Garnsworthy et al., 2012). However, milk yield increases after calving up to approximately 100 DIM, followed by a gradual decline toward the end of lactation, whereas the positive relationship found between DIM and CH_4 emission suggests that CH_4 emission is constantly increasing during lactation. Therefore, it seems that the relationship between DIM and CH_4 emission is a statistical rather than a biological relationship. Additionally, milk urea content was included in the prediction models for CH_4 yield with a positive slope. No biological explanation can be given for this association, and no other study reported a relation between CH_4 emission and milk urea content before.

The comparison made above is actually biased, because MFA and FTIR represent only milk composition and no other lactation parameters (parity, DIM and milk production). To examine whether MFA and FTIR have an added value relative to milk composition only, the same analysis was performed again, but only using milk composition [i.e., fat, protein, lactose, and urea content, and LN(SCC)]. Especially for FTIR this comparison is of interest, because it will show whether the complete FTIR spectra hold more information than only the major milk components estimated by FTIR. As illustrated in Table 10.1, no model could be obtained for CH_4 production. The significance level for a variable to enter the model was 0.01, whereas the significance level of the strongest correlation between a milk composition parameter [i.e., LN(SCC)] and CH_4 production was 0.072. The milk composition-based prediction models for CH_4 yield and CH_4 intensity performed worse than the MFA- and FTIR-based prediction models, as evident by the lower adjusted R^2 and CCC values. The results with respect to the CH_4 prediction potential of lactation characteristics, indicate (1) that MFA have a greater CH_4 prediction potential than milk composition, (2) that milk composition has a smaller CH_4 prediction potential than milk composition together with parity, DIM, and milk yield, and (3) that the complete FTIR spectrum holds more information and subsequently has a greater CH_4

prediction potential than only milk composition estimated by FTIR. The latter might be due to the fact that the FTIR spectrum combines the information of milk composition (i.e., major milk components), a selection of certain MFA, and other milk composition characteristics.

MILK FATTY ACIDS – INFRARED MEASUREMENTS AND UNITS

As indicated in Chapter 2, gas chromatography was until recently the principal method for MFA analysis. However, as gas chromatography is unsuitable for routine milk recording (Soyeurt et al., 2011), FTIR is also often applied to quantify MFA concentrations. Several studies investigated the potential use of FTIR to predict MFA composition of dairy cattle [e.g., Rutten et al. (2009) and Soyeurt et al. (2011)] and confirmed that FTIR can accurately predict several individual, particular higher abundant, MFA and groups of MFA, whereas a number of lower abundant MFA cannot be predicted by FTIR. In line with this, Fleming et al. (2017) reported recently that MFA appearing in negligible amounts did not predict well enough with FTIR to be useful. According to Rutten et al. (2009), individual MFA should have an average concentration of ≥ 2.45 g/100 g of FA in order to be predictable with reasonable accuracy by FTIR (i.e., C4:0, C10:0, C12:0, C14:0, C16:0, C18:0, and C18:1 *cis*-9). More recently, Soyeurt et al. (2011) reported that C4:0, C6:0, C8:0, C10:0, C12:0, C14:0, C16:0, C18:0, ∑*trans*-C18:1, C18:1 *cis*-9, ∑*cis*-C18:1, and some groups of MFA can be sufficiently accurately determined by FTIR. Similar to Van Lingen et al. (2014), the use of FTIR to estimate CH_4 emission of dairy cows by developing prediction models with a restricted selection of MFA based on the results of Rutten et al. (2009) and Soyeurt et al. (2011) was investigated, using the same statistical approach as described in Textbox 3.

The prediction model for CH_4 production (g/d) decreased in predictive power with a adjusted R^2 = 0.54 and CCC = 0.72 when all MFA were used (Chapter 9) to an adjusted R^2 = 0.35 and CCC = 0.52 when the MFA accurately determined by FTIR according to Soyeurt et al. (2011) were used, and to an adjusted R^2 = 0.28 and CCC = 0.44 when the MFA accurately determined by FTIR according to Rutten et al. (2009) were used. Similar patterns (i.e., decrease in adjusted R^2 and CCC values when using a more restricted number of MFA) were observed for CH_4 yield and CH_4 intensity (Table 10.2). These results are in agreement the results of Van Lingen et al. (2014), who also observed a decrease in predictive power when developing models with a restricted selection of MFA. The results thus indicate that, compared with gas chromatography, the performance of FTIR limits the potential for predicting CH_4 emission of dairy cows based on the MFA profile, because several MFA with lower concentration that appear in various CH_4 prediction models published previously (e.g., Chilliard et al., 2009; Dijkstra et al., 2011; Rico et al., 2016) are not available when the MFA profile is determined using FTIR.

An important note should also be made with respect the unit of the MFA. The work presented in this PhD thesis focussed on the MFA profile, also called MFA proportions, which refers to g/100 g of FA. One can imagine that when the proportion of a specific MFA increases, the proportion of another MFA (or multiple MFA) will decrease. This is an inherent characteristic of working with proportions. Therefore, in the previous chapters (i.e., 5, 6, and 9), the CH_4 prediction models were checked for multicollinearity. Multicollinearity refers to a phenomenon in which two or more of the variables in the CH_4 prediction models are correlated,

and can result in substantial changes in the predicted CH_4 emission in response to only small changes in the variables themselves. When working with proportions, there is an increased risk for multicollinearity. Multicollinearity was not observed in any of the developed MFA-based CH_4 prediction models in Chapters 5, 6, and 9. However, to investigate if the unit in which MFA are expressed affects the CH_4 prediction potential of MFA, MFA production (g MFA produced per day) and MFA portrait (g MFA per kg milk) were also considered. MFA production was calculated by dividing fat yield (g/d) with 100 and subsequently multiplying this with the MFA proportion, assuming that 100% of milk fat content consists of fatty acids. The MFA portrait was calculated by multiplying the milk fat content in g/kg milk with the MFA proportion and subsequently dividing this with 100. These calculations and subsequent development of CH_4 prediction models, provides an indication whether it might be better to work with a different unit than MFA profile. The adjusted R^2 of the MFA production-based prediction models were 0.18 for CH_4 production, 0.04 for CH_4 yield, and 0.16 for CH_4 intensity (CCC results not shown). These low adjusted R^2 values, relative to those of the MFA proportion-based CH_4 prediction models from Chapter 9 (see also Table 10.1), indicate that the production of MFA is less suitable for predicting CH_4 emission from dairy cows than MFA proportions. The adjusted R^2 of the MFA portrait-based prediction models were 0.53 for CH_4 production, 0.39 for CH_4 yield, and 0.61 for CH_4 intensity (CCC results not shown). These adjusted R^2 values are close the R^2 values reported for the MFA proportions (Table 10.2). These results indicate that both MFA proportions and portrait can better reflect the ratio between different processes occurring in the rumen, such as biohydrogenation and VFA production, than daily MFA production. Additionally, MFA production depends on milk yield, which has moderate potential as a proxy for CH_4 emission (Negussie et al., 2017). Thus, when developing MFA-based CH_4 prediction models, it is recommended to use MFA profile (i.e., proportions in g/100 g of FA) or MFA portrait (i.e., in g/kg milk), but always in combination with a multicollinearity test.

COMBINATION OF PROXIES

Although the results of this thesis show that both MFA and FTIR have the ability as a single proxy for CH_4 emission of dairy cows, there may be advantages in using two or more proxies in combination. According to Negussie et al. (2017), combining proxies might be more appropriate because (1) the proxies may describe independent sources of variation in CH_4 emissions, and (2) one proxy may allow correction for shortcomings in the way the other proxy describes CH_4 emissions. A clear example of the improvement when combining proxies is the study of Mohammed et al. (2011), who used measurements from the rumen (i.e., VFA, pH, and protozoa counts), feed intake (i.e., total DMI, forage DMI, and FA intake), and production parameters (i.e., milk yield and composition) in combination with MFA to develop CH_4 prediction models. The results of that study indicate that MFA predict CH_4 emission better (R^2 = 0.74) compared with only rumen variables, only feed intake, and only production parameters (R^2 < 0.58). However, combining MFA with feed intake and production parameters resulted in a model R^2 of 0.83, and combining MFA with feed intake, production, and rumen-related parameters resulted in a model R^2 of 0.90.

Similarly, although with different selection parameters, Vanlierde et al. (2015) took lactation stage into account when developing prediction equations, because of changing CH_4 emission prediction coefficients during lactation. Vanlierde et al. (2015) developed lactation stage-independent (i.e., including only FTIR spectra) and lactation stage-dependent (i.e., including FTIR and DIM to describe lactation stage) CH_4 prediction equations. The average CH_4 production (g/d) predicted by both models was similar (416 ± 63 g/d). However, in contrast to the lactation stage-independent prediction equation, the lactation stage-dependent prediction equation showed biologically meaningful CH_4 predictions throughout lactation, namely an increase in CH_4 production (g/d) after calving up to approximately 100 DIM, followed by a gradual decline toward the end of lactation (Vanlierde et al., 2015). These results indicate the importance of combining FTIR with lactation stage to improve the prediction of CH_4 emission in dairy cows. As shown in Table 10.1, DIM was an important explanatory variable in this thesis and perhaps also for Vanlierde et al. (2015). Hence, it would have been of great value if Vanlierde et al. (2015) also developed a CH_4 prediction model with only DIM to describe lactation stage as explanatory variable, because that would give an indication whether FTIR spectra have an added value relative to a simple measurable variable, such as DIM.

The importance of combining FTIR with lactation stage, as illustrated van Vanlierde et al. (2015), might also be important for further development of MFA-based CH_4 prediction models. Vanrobays et al. (2016) showed that correlations between CH_4 production (g/d) and MFA vary according to the lactation stage of the cow, a fact that is still often ignored when trying to predict CH_4 emission from dairy cows from the MFA profile. Based on the results of Mohammed et al. (2011) and Vanrobays et al. (2016), as well as the finding of Negussie et al. (2017), it was investigated whether the combination of MFA with lactation characteristics or dietary composition would improve the CH_4 prediction potential relative to only MFA. Because of the extreme complexity of combining FTIR spectra with other parameters, the principle of combining proxies was only investigated in combination with MFA to see whether this concept can improve CH_4 prediction.

The same statistical approach as before was applied, which is shortly described in Textbox 3. Three different sets of parameters were used: (1) MFA in g/100 g FA, (2) lactation characteristics including parity, DIM, milk yield (kg/d), fat, protein, and lactose content (all in g/100 g milk), urea-content (mg/dL), and LN(SCC), (3) dietary composition including DM (g/kg), ash, NDF, ADL, ADF, fat, starch (all in g/kg DM), gross energy (MJ/kg DM), and the NDF-to-starch ratio (dimensionless). Please note that DMI was not included as variable in combination with dietary composition, because the relation between DMI and CH_4 emissions has been described many times before (e.g., Hristov et al., 2013; Charmley et al., 2016; Rico et al., 2016). Methane prediction models were developed using the three datasets alone, combining the MFA dataset with either dataset 2 (lactation characteristics) or dataset 3 (dietary composition), and combining the MFA dataset with both datasets (lactation characteristics and dietary composition).

The CH_4 prediction models using only MFA or only lactation characteristics will not be discussed in detail, as these have already been discussed in Chapter 9 (i.e., MFA) and in an earlier section of the general discussion (i.e., lactation characteristics). When considering only

the dietary composition as selection parameters, dietary fat content is included in all three CH_4 prediction models (Table 10.1). The negative association between dietary fat content and CH_4 emission is as expected, because fat is known to reduce CH_4 emissions via multiple mechanisms as described in Chapter 7. A positive association is found between crude protein content and CH_4 yield. This is in agreement with Ellis et al. (2009), who observed a positive relationship between dietary crude protein content and CH_4 emission of beef cattle, but contrary to Dijkstra et al. (2011). The latter authors concluded that mitigation options aiming to reduce urinary nitrogen excretion, such as decreased nitrogen intake (i.e., based on CP intake), may result in elevated CH_4 emission levels, suggesting a negative association. Additionally, the positive association between ADF content and CH_4 yield is according to expectation that fermentation of fiber favors the ruminal production of acetate, which increases H_2 availability. Furthermore, the negative association between gross energy content and CH_4 intensity is most likely related to the positive association between gross energy and milk yield. Also the negative association between dietary starch content and CH_4 intensity was as expected, because the fermentation of starch favors the ruminal production of propionate at the expense of acetate and decreases rumen pH, which reduces H_2 availability and activity of rumen methanogens (Van Kessel and Russell, 1996; Hook et al., 2011).

Interestingly, despite the expected associations found between the dietary composition and CH_4 emissions, the CH_4 prediction potential of the dietary composition is rather limited. Both the adjusted R^2 values and the CCC values of the dietary composition-based CH_4 prediction models are lower than the ones from the MFA-based CH_4 prediction models (Table 10.2). Also, dietary composition seems to have a lower CH_4 prediction potential than lactation characteristics, with the exception of CH_4 yield. This was unexpected, because enteric CH_4 production is a natural by-product arising from microbial fermentation of feed within the rumen (Beauchemin et al., 2009). Possible reasons for not finding the expected CH_4 prediction potential might be the variation in the dietary composition, which was rather limited. All diets were roughage-based (> 700 g/kg DM) and the dietary treatments were limited to the roughage part of the diet (e.g., different qualities of grass herbage, grass silage, and corn silage). To illustrate, NDF content of the diets ranged from 242 to 501 g/kg DM and the starch content of the diets ranged from 5 to 326 g/kg DM (see also Table 9.2 of Chapter 9). Perhaps the CH_4 prediction potential of dietary composition would have been greater if the dataset would contain more dietary variation, for example different levels of concentrates, facilitating a larger range in dietary NDF and starch content. Furthermore, although the CH_4 prediction models may include dietary NDF and starch content, it does not take all characteristics of feed into account. The impact of feed on CH_4 emission, namely, is not only based on the dietary composition. According to Beauchemin et al. (2009), the quantity of CH_4 produced by an animal depends on many interacting factors that include: carbohydrate intake, chemical composition of the carbohydrate sources, retention time in the rumen, rate of ruminal fermentation, and rate of methanogenesis. Another reason for the low CH_4 prediction potential of dietary composition, might be related to other sources of variation. Cows receiving the exact same dietary treatment and thus dietary composition, showed considerable variation in CH_4 emissions. For example, the control diet of the experiment described in Chapter 7 has a NDF content of 357 g/kg DM, whereas the CH_4

emission of the dairy cows receiving this diet ranged from 270 to 535 g/d, from 19.5 to 25.3 g/kg DMI, and from 12.9 to 22.9 g/kg FPCM. This variation can be the results of many factors, including differences in DMI and lactation stage, hampering perhaps to find a clear relationship between dietary composition and CH_4 emission. The results demonstrate that dietary composition as such is not a satisfactory proxy for CH_4 emission. Perhaps the combination of dietary composition and the absolute intake of the different dietary components might increase the CH_4 prediction potential, because Ellis et al., (2010) already demonstrated that the more generalized CH_4 prediction models (i.e., based only on feed intake) performed worse than those that attempted to take important aspects of diet composition into account.

Similarly, when combining dietary composition with MFA, the prediction potential is almost similar or slightly higher than that of only MFA. For CH_4 production, there was no improvement observed at all, as no dietary composition parameters were selected resulting in a model identical to only MFA. For CH_4 yield, the adjusted R^2 value decreased from 0.40 to 0.39 upon combining MFA with dietary composition, similar as for the CCC value (i.e., decreased from 0.59 to 0.58). Only for CH_4 intensity a slight improvement in terms of prediction potential was observed, with the adjusted R^2 value increasing from 0.62 to 0.66 and the CCC value increasing from 0.77 to 0.81. Overall, these results indicate that, relative to MFA alone, CH_4 prediction potential does not increase when combining MFA with dietary composition

In contrast though, the CH_4 prediction potential increases considerably when combining lactation characteristics with MFA, relative to MFA alone. For CH_4 production, yield, and intensity, adjusted R^2 values increase from 0.54 to 0.72, from 0.40 to 0.44, and from 0.62 to 0.71, respectively. Additionally, the CCC values increase from 0.72 to 0.84, from 0.59 to 0.61, and from 0.77 to 0.84 for CH_4 production, yield, and intensity, respectively. This shows that the combination of lactation characteristics with MFA results in a more accurate and precise prediction of CH_4 emission of dairy cows. As expected, based on the previous results with respect to dietary composition, the CH_4 prediction potential does not improve when combining MFA with both lactation characteristics and dietary composition relative to the CH_4 prediction potential of the combination of lactation characteristics and MFA for CH_4 production and CH_4 intensity. For CH_4 yield, however, the adjusted R^2 and CCC increase, indicating a better prediction of CH_4 yield when combining dietary composition with both lactation characteristics and MFA. This makes sense, because, as indicated before, DMI is one of the most important determining factor for CH_4 production (Hristov et al., 2013), whereas both DIM and milk yield are the most important determining factors for CH_4 intensity (Garnsworthy et al., 2012). The factors (i.e., DMI, DIM, and milk yield) are not as important for CH_4 yield, perhaps explaining why the inclusion of dietary composition results in improved prediction potential only for CH_4 yield.

Overall, the results of the latter analysis indicate that, as proposed by Negussie et al. (2017), combining two proxies might have an advantage over a single proxy for CH_4 emissions of dairy cows. However, the correct combination of proxies is critical. As demonstrated, combining dietary composition with MFA does not always create synergy. A possible explanation for this is that MFA and dietary composition describe the same part of the variation in CH_4 emission. To illustrate, dietary composition is strongly related to the MFA profile (e.g.,

the relationship C18:1 *trans*-10 and crude protein content, NDF content, fat content, and starch content is significant; $P < 0.005$; data not shown) and it could, therefore, be that the variation in the dietary composition and the variation in the MFA profile describe the same variation in CH_4 emission. This is most likely the result of the common biochemical pathway between ruminal feed fermentation, CH_4 production, and MFA composition. Combining lactation characteristics and MFA composition did create synergy for all CH_4 units and thus resulted in a more accurate and precise prediction of CH_4 emission as well as a better description of the observed variation in CH_4 emissions relative to MFA alone and lactation characteristics alone. The results for CH_4 intensity, in which DIM was included in the prediction model, are in agreement with the findings of Vanrobays et al. (2016), who observed that the relationship between CH_4 emission and MFA is lactation stage dependent. It is therefore concluded that it is important to combine MFA composition with lactation characteristics to improve the prediction of CH_4 emission in dairy cows, although the magnitude of improvement depends on the unit of CH_4 emission. Similar holds for the combination of MFA composition, lactation characteristics, and dietary composition, which resulted in synergy for CH_4 yield only. This principle of synergy, although not investigated, would most likely also apply when combining the FTIR spectra with lactation characteristics. It should be noted though that some of the lactation parameters are actually determined by FTIR (e.g., protein and fat content) and, as described above, FTIR spectra contain more information of CH_4 emission than milk composition. Hence, the improvement of CH_4 emission prediction upon combination with lactation characteristics might be less for FTIR than for MFA, but overall also the combination of lactation characteristics and FTIR may result in a better proxy for CH_4 emission in dairy cows.

ROBUSTNESS OF PROXIES FOR METHANE EMISSION

In general, previously developed CH_4 prediction models have a great CH_4 prediction potential in the study in which they were developed. This, however, does not necessarily mean that one can extrapolate previously developed MFA-based CH_4 prediction equations to another situation. This was already demonstrated in Chapter 8, in which previously developed MFA-based prediction equations [e.g., Dijkstra et al. (2011) and Van Lingen et al. (2014)] did predict CH_4 emission of dairy cows with different *DGAT1* genotypes or fed diets with or without linseed oil neither accurately nor precisely. Furthermore, Mohammed et al. (2011) observed an over-prediction in CH_4 emission when comparing measured CH_4 emission with CH_4 emission predicted by the MFA-based equations of Chilliard et al. (2009) and Dijkstra et al. (2011). Dijkstra et al. (2016) compared observed CH_4 emission of dairy cattle fed grass- and grass silage-based diets with CH_4 emission predicted with the MFA-based equations developed by Van Lingen et al. (2014). The CH_4 prediction equations of Van Lingen et al. (2014) did not accurately predict CH_4 emission, indicating that the relationship between MFA and CH_4 emission in dairy cows fed grass- and grass silage-based diets differs from that of other types of diets.

To provide another, perhaps more extreme example, the observed CH_4 emissions from cows receiving nitrate, docosahexaenoic acid (**DHA**), or a combination of nitrate and DHA was compared with CH_4 emission predicted with the MFA-based prediction models from Chapter 9. As demonstrated by Klop et al. (2016), nitrate decreased CH_4 emission irrespective of the unit

in which it was expressed, whereas DHA did not affect CH_4 yield (g/kg DMI), but actually resulted in a higher CH_4 production (g/d; likely related to a significantly higher DMI compared with diets without DHA) and CH_4 intensity (g/kg FPCM; likely related to a trend of decreased FPCM production with DHA). Additionally, nitrate and, especially, DHA affected the MFA composition relative to the control diet, whereas the interaction between nitrate and DHA did not affect the MFA composition considerably.

Table 10.3 shows the observed and predicted mean CH_4 emissions and the corresponding CCC values. The MFA-based CH_4 prediction equations are not able to estimate CH_4 emission of dairy cows fed nitrate, DHA, or a combination of both. For nitrate supplemented cows, the CCC is highest for CH_4 production (0.26) and close to zero for CH_4 yield and intensity. The MFA-based prediction models are neither accurate (i.e., C_b) nor precise (i.e., r), and over-predict CH_4 emission as evident by the negative location shift (i.e., μ) values. For the DHA supplemented cows, the CCC is highest for CH_4 intensity (0.24) and close to zero for CH_4 production and yield. The MFA-based prediction models under-predicted CH_4 emission from cows receiving DHA, as evident by the relatively large positive μ values. The reason for predicted CH_4 production being negative for cows fed DHA can be explained by the effects of DHA on CH_4 emission and the MFA profile. As mentioned earlier, Klop et al. (2016) observed an increase in CH_4 production for DHA relative to the control diet. Additionally, the MFA C18:1 *trans*-10 and C18:1 *trans*-11 increased 19-fold and 4-fold, respectively, for DHA relative to the control diet. These two MFA were included in the MFA-based prediction model for CH_4 production with a negative slope. The pronounced increase in these MFA in combination with the negative slope resulted in negative values for CH_4 production. For the cows receiving a combination of nitrate and DHA, the results show a similar trend as for the DHA supplemented cows. The CCC is close to zero for CH_4 production and yield, and highest for CH_4 intensity (0.56), which is also the highest CCC value found in general for this dataset. The latter is accompanied by moderate to high values for precision and accuracy. For CH_4 intensity there is a small over-prediction, whereas for CH_4 production and yield there is a relatively large under-prediction. Overall, these results clearly indicate that the MFA-based CH_4 prediction models from Chapter 9, which were developed from data obtained of dairy cows fed a wide range of roughage-based diets without additives, are not able to predict CH_4 emissions from dairy cows fed nitrate, DHA, a combination of nitrate and DHA, and probably even feed additives in general.

The latter example, in combination with the findings of Chapter 8, Mohammed et al. (2011), and Dijkstra et al. (2016), shows clearly that the robustness of MFA-based CH_4 emissions is a problem. At present, several different MFA-based CH_4 prediction models have been developed for dairy cattle (e.g., Chilliard et al. 2009; Van Lingen et al., 2014; Rico et al., 2016). However, most of these models tend to be accurate only for the production system and the environmental conditions under which they were developed. Therefore, the greatest shortcoming today is the lack of robustness in the applicability of MFA-based CH_4 prediction models and, subsequently, attention should not only be directed to the accuracy of proxies but also to their robustness.

Table 10.3. The concordance correlation coefficient (CCC) results of the MFA[1]-based methane prediction models from Chapter 9 applied to dairy cows supplemented with nitrate, DHA[2], or a combination of nitrate and DHA

	Observed CH_4 emission	Predicted CH_4 emission	CCC[3]	r[4]	C_b[5]	v[6]	μ[7]
Nitrate							
Methane production[8]	263	318	0.26	0.75	0.34	0.88	-1.96
Methane yield[9]	16.9	21.7	-0.02	-0.30	0.08	3.06	-4.63
Methane intensity[10]	10.8	14.4	0.07	0.30	0.25	0.46	-2.33
DHA							
Methane production	369	-798	-0.01	-0.56	0.01	0.16	11.52
Methane yield	22.4	15.0	-0.02	-0.74	0.03	0.32	7.86
Methane intensity	15.4	13.6	0.24	0.48	0.49	1.88	1.30
Nitrate + DHA							
Methane production	298	-120	0.00	0.08	0.06	0.13	5.26
Methane yield	18.2	12.0	-0.02	-0.20	0.12	0.16	3.16
Methane intensity	12.6	12.9	0.56	0.60	0.93	1.25	-0.31

[1] Milk fatty acids in g/100 g FA.

[2] Docosahexaenoic acid.

[3] Concordance correlation coefficient, where CCC = $r \times C_b$.

[4] Pearson correlation coefficient; a measure of precision.

[5] Bias correction factor; a measure of accuracy.

[6] Scale shift; change in standard deviation between predicted and observed methane emission.

[7] Location shift; if positive under prediction, if negative over prediction.

[8] Production in g methane per day.

[9] Yield in g methane per kg dry matter intake.

[10] Intensity in g methane per kg fat- and protein-corrected milk (FPCM = [0.337 + 0.116 × fat (g/100 g milk) + 0.06 × protein (g/100 g milk)] × milk yield (kg/d); CVB, 2012).

THE BEST WAY FORWARD

Finding a proxy for enteric CH_4 production of dairy cattle is not as straightforward as expected from theory. There are indeed indicators and animal traits highly correlated with CH_4 emission (e.g., feed intake with CH_4 production; Negussie et al., 2017), as well as indicators and animal traits which are easy and relatively low cost to record on a large scale (e.g., milk yield; Negussie et al., 2017). But finding a proxy that performs well on both statistical and practical aspects, is a challenge. As already explained in the general introduction (Chapter 1), enteric CH_4 production is influenced by many factors, including dietary factors (such as the type and the amount of feed), animal factors (such as milk yield and genetic traits), management factors (such as feeding frequency), and environmental factors (such as seasons and temperature) (e.g., Hristov et al., 2013). These factors result in large variation in CH_4 emission of dairy cattle, making it a challenge to develop a universal and robust proxy for CH_4 emission.

Proxies for CH_4 emission can have great implications in the dairy chain, including dairy management (e.g., evaluating CH_4 mitigating potential of feeding strategies) and dairy breeding (e.g., identifying low and high CH_4 emitting dairy cows). The best way forward, in my opinion, would be to focus on CH_4 proxies that perform at least moderately well both in terms of the practical aspect and the statistical aspect. Of course, a proxy must be accurate and precise to

ensure unbiased estimates of CH_4 emission close to the truth. But, if one would only focus on the statistical aspect, not taking the complexity of certain techniques into account, one would most likely be better off measuring CH_4 emission rather than estimating CH_4 emission. For example, although interesting in terms of understanding CH_4 production, why would there be a focus on the rumen microbiome as CH_4 proxy if it cannot be measured easily, at low cost, and at large scale? Similarly the other way around, a CH_4 proxy that can easily be measured on a large scale, but cannot predict CH_4 emission with a certain accuracy and precision, would lead to incorrect mitigation recommendations and strategies.

The results show that MFA are the most accurate and precise CH_4 proxy investigated in this PhD work. However, its lack of robustness as demonstrated in Chapters 8 in this thesis, as well as by Mohammed et al. (2011) and Dijkstra et al. (2016), remains a concern. Additionally, MFA have restricted practical application, meaning that most MFA retained in the current CH_4 prediction models cannot be determined routinely because of the use of gas chromatography. The MFA that can be determined with the use of infrared spectroscopy are however no promising predictors for CH_4 emission. Furthermore, it should be noted that MFA have only a moderate CH_4 prediction potential with the R^2 ranging from 0.40 to 0.62, and Castro-Montoya et al. (2017) recently demonstrated that MFA are not yet reliable predictors of specific amounts of CH_4 emitted by a cow, while holding a modest potential to differentiate cases of high or low emissions. This together suggests that it might not be the best option to focus in the future on MFA alone as a proxy for CH_4 emission of dairy cows.

It is however questionable whether the FTIR spectra can serve as a more valuable CH_4 proxy. The CH_4 prediction potential of FTIR spectra in the research described in this thesis is low to moderate with the R^2 ranging from 0.25 to 0.56. This is considerably less accurate and precise than MFA. However, FTIR has a great potential for practical high throughput application, facilitating repeated measurements of the same cow. As illustrated by Negussie et al. (2017), certain proxies might be less accurate but random noise can be reduced when measuring repeatedly. This is visualized when comparing the CH_4 prediction potential of FTIR as described in Chapter 9 with the CH_4 prediction potential of FTIR by Dehareng et al. (2012) and Vanlierde et al. (2015). The latter two studies report higher R^2 values (> 0.77) than reported in Chapter 9 of this thesis, but developed their FTIR-based CH_4 prediction models on repeated measurements of the same cow. The study of Dehareng et al. (2012) involved 11 dairy cows, whereas the prediction models were developed using 77 observations, and the study of Vanlierde et al. (2015) involved 142 dairy cows, while the prediction models were developed using 446 observations. In contrast, the study described in Chapter 9 involved 218 dairy cows and the CH_4 prediction models were developed using 1 observation per cow only. It is important to note though, that not only the repeated measurements in the studies of Dehareng et al. (2012) and Vanlierde et al. (2015), but also a much larger variation in CH_4 emission in the dataset compared to that of Chapter 9, may have resulted in the higher R^2 values reported by those authors (see Chapter 9 for a more elaborate explanation). It remains therefore unclear how much more accurate and precise the CH_4 estimations of FTIR spectra can become upon repeated measurements of the same cows.

Furthermore, as demonstrated in Chapter 9, FTIR spectra evaluate the effect of forage level and quality on CH_4 emission of dairy cattle considerably less satisfactory than MFA. In 6 out of 18 situations (i.e., 6 studies each with 3 units of CH_4 emission), FTIR spectra were not able to predict the same trend (i.e., increase or decrease) in CH_4 emission between two diets as measured by the climate respiration chambers. This was only 2 out of 18 situations for MFA. This demonstrates that FTIR spectra do not have the power to detect differences in CH_4 emission between diets which are, in terms of forage level and quality, commonly fed in practice. Moreover, the robustness of FTIR spectra is currently unknown. Hence, it remains to be investigated whether FTIR spectra can predict CH_4 emissions of dairy cows fed additives (e.g., nitrate and DHA) or from dairy cows housed under different conditions from those under which the FTIR-based prediction equations were developed.

According to Negussie et al. (2017), milk FTIR in particular, along with covariates such as lactation stage, are a promising option for the prediction of CH_4 emission in dairy cows. The increase in CH_4 prediction potential upon combining lactation characteristics with MFA in the current Chapter, would most likely also apply when combining FTIR spectra with lactation characteristics, such as DIM. I therefore believe that FTIR spectra, along with lactation characteristics, is the best way forward when developing a proxy for CH_4 emission of dairy cows. The FTIR technique can be routinely measured in milk production registration for dairy herd improvement and farm management, and can thus potentially be incorporated with regular FTIR analysis or perhaps even at the farm level in the farm tank milk. However, more research is required to support these conclusions, including more observations, on farm measurements of CH_4 emission (with for example the GreenFeed system; Chapter 1), FTIR spectra, and lactation characteristics, and independent experiments and data to test the robustness of the FTIR spectra to predict CH_4 emission of dairy cows.

GENERAL CONCLUSIONS

Although both volatile and non-volatile metabolites can be related to ruminal feed fermentation and enteric CH_4 production, these milk metabolites hold no potential to predict CH_4 emission of dairy cows. This is both in terms of the statistical aspect, because of the low adjusted R^2 and CCC values, and the practical aspect, because gas chromatography-mass spectroscopy and nuclear magnetic resonance are complex techniques which require specialized personnel and which are not applicable at large scale. Additionally, combining these milk metabolites with MFA does not always improve the CH_4 prediction potential relative to MFA alone, because an improvement was observed when using a small dataset ($n = 29$) with a small range of forage-based diets but no improvement was observed when using a larger dataset ($n = 123$) with a larger range of forage-based diets. The MFA profile can predict enteric CH_4 production more accurately and precisely than volatile and non-volatile metabolites as well as FTIR spectra, but its lack of robustness remains a concern. The FTIR spectra have a greater potential for practical implementation than MFA, because FTIR is currently already routinely used in milk recording systems to predict fat, protein, lactose, and urea contents in milk, and hence can be potentially incorporated with regular FTIR analysis or perhaps even at the farm level in farm tank milk. However, the accuracy and precision to predict CH_4 emission using

FTIR spectra needs to increase, and the capacity of the FTIR spectra to evaluate the differences in CH_4 emission between dairy cows and different types of diets needs to improve, in order to actually be a valuable proxy for CH_4 emission of dairy cows.

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English summary,
Nederlandse samenvatting

ENGLISH SUMMARY

Enteric methane (**CH_4**) is produced as a result of microbial fermentation of feed components in the gastrointestinal tract of ruminant livestock. Methane has no nutritional value for the animal and is predominately released into the environment through eructation and breath. Therefore, CH_4 not only represents a greenhouse gas contributing to global warming, but also an energy loss, making enteric CH_4 production one of the main targets of greenhouse gas mitigation practices for the dairy industry. Obviously, reduction of CH_4 emission could be achieved by simply reducing livestock numbers. However, the global demand for dairy products has been growing rapidly and is expected to further grow in the future. Therefore, it is critical to minimize environmental impact to produce high-quality dairy products. The overall aim of this PhD research was, therefore, to develop a proxy for CH_4 emission that can be measured in milk of dairy cows.

There are currently a number of potentially effective dietary CH_4 mitigation practices available for the livestock sector. The results of Chapter 3 show that replacing fiber-rich grass silage with starch-rich corn silage in a common forage-based diet for dairy cattle offers an effective strategy to decrease enteric CH_4 production without negatively affecting dairy cow performance, although a critical level of starch in the diet seems to be needed. Little is known whether host genetics may influence the CH_4 emission response to changes in diet. Therefore, the interaction between host *DGAT1* K232A polymorphism with dietary linseed oil supplementation was evaluated in Chapter 7. The results of Chapter 7 indicate that *DGAT1* K232A polymorphism is associated with changes in milk composition, milk N efficiency, and diet metabolizability, but does not affect digestibility and enteric CH_4 emission, whereas linseed oil reduces CH_4 emission independent of the *DGAT1* K232A polymorphism.

Accurate and repeatable measurements of CH_4 emission from individual dairy cows are required to assess the efficacy of possible mitigation strategies. There are several techniques to estimate or measure enteric CH_4 production of dairy cows, including climate respiration chambers, but none of these techniques are suitable for large scale precise and accurate measurements. Therefore, the potential of various metabolites in milk, including milk fatty acids (**MFA**), as a proxy (i.e., indicators or animal traits that are correlated with enteric CH_4 production) for CH_4 emission of dairy cows gained interest. Until recently, gas chromatography was the principal method used to determine the MFA profile, but this technique is unsuitable for routine analysis. This has led to the application of Fourier-transform infrared spectroscopy (**FTIR**) for determination of the MFA profile. Chapter 2 provides an overview of the recent research that relates MFA with CH_4 emission, and discusses the opportunities and limitations of using FTIR to estimate, indirectly via MFA or directly, CH_4 emission of dairy cattle. The recent literature on the relationship between MFA and CH_4 emission gives inconsistent results. Where some studies found a clear and strong relation, other studies consider MFA to be unreliable predictors for CH_4 emitted by dairy cows. Even the studies that do find a clear relation between MFA and CH_4 emissions do not describe similar prediction models using the same MFA. These discrepancies can be the result of many factors, including dietary composition and lactation stage. Additionally, literature showed that the major advantages of using FTIR to predict CH_4 emission include its simplicity and potential practical application on a large scale. Disadvantages include

the inability to predict important MFA for the prediction of CH_4 emission, and the moderate power of FTIR to directly predict CH_4 emission. The latter was also demonstrated in Chapter 9, in which the CH_4 prediction potential of MFA was compared with that of FTIR using data from 9 experiments (n = 218 individual cow observations) covering a broad range of roughage-based diets. The results indicate that MFA have a greater potential than FTIR spectra to estimate CH_4 emissions, and that both techniques have potential to predict CH_4 emission of dairy cows, but also limited current applicability in practice. Much focus has been placed on the relationship between MFA and CH_4 emission, but milk also contains other metabolites;, such as volatile and non-volatile metabolites. Currently, milk volatile metabolites have been used for tracing animal feeding systems and milk non-volatile metabolites were shown to be related to the health status of cows. In Chapter 4, the relationship between CH_4 emission and both volatile and non-volatile metabolites was investigated, using data and milk samples obtained in the study described in Chapter 3. In general, the non-volatile metabolites were more closely related to CH_4 emissions than the volatile metabolites. More specifically, the results indicate that CH_4 intensity (g/kg fat- and protein-corrected milk; **FPCM**) may be related to lactose synthesis and energy metabolism in the mammary gland, as reflected by the milk non-volatile metabolites uridine diphosphate-hexose B and citrate. Methane yield (g/kg dry matter intake) on the other hand, may be related to glucogenic nutrient supply, as reflected by the milk non-volatile acetone. Based on the metabolic interpretations of these relationships, it was hypothesized that the addition of both volatile and non-volatile metabolites in a prediction model with only MFA would enhance its predictive power and, thus, leads to a better proxy in milk for enteric CH_4 production of dairy cows. This was investigated in Chapter 5, again using data and milk samples described in Chapter 3. The results indicate that MFA alone have moderate to good potential to estimate CH_4 emission. Furthermore, including volatile metabolites (CH_4 intensity only) and non-volatile metabolites increases the CH_4 emission prediction potential.

The work presented in Chapters 3, 4 and 5, was based upon a small range of diets (i.e., four roughage-based diets in which grass silage was replaced partly or fully by corn silage) of one experiment. Therefore, in Chapter 6, the relationship between CH_4 emission and the milk metabolome in dairy cattle was further quantified. Data (n = 123 individual cow observations) were used encompassing a large of roughage-based diets, with different qualities and proportions of grass, grass silage and corn silage. The results show that changes in individual milk metabolite concentrations can be related to the ruminal CH_4 production pathways. These relationships are most likely the result from changes in dietary composition that affect not only enteric CH_4 production, but also the profile of volatile and non-volatile metabolites in milk. Overall, the results indicate that both volatile and non-volatile metabolites in milk might provide useful information and increase our understanding of CH_4 emission of dairy cows. However, the development of CH_4 prediction models revealed that both volatile and non-volatile metabolites in milk hold little potential to predict CH_4 emissions despite the significant relationships found between individual non-volatile metabolites and CH_4 emissions. Additionally, combining MFA with milk volatile metabolites and non-volatile metabolites does not improve the CH_4 prediction potential relative to MFA alone. Hence, it is concluded that it is not worthwhile to determine the volatile and non-volatile metabolites in milk in order to estimate CH_4 emission of dairy cows.

Overall, in comparison with FTIR, volatile and non-volatile metabolites, the MFA are the most accurate and precise proxy in milk for CH_4 emission of dairy cows. However, most of MFA-based models to predict CH_4 emission tend to be accurate only for the production system and the environmental conditions under which they were developed. In Chapter 8 it was demonstrated that previously developed MFA-based prediction equations did not predict CH_4 emission satisfactory of dairy cows with different *DGAT1* genotypes or fed diets with or without linseed oil. Therefore, the greatest shortcoming today of MFA-based CH_4 prediction models is their lack of robustness. Additionally, MFA have restricted practical application, meaning that most MFA retained in the current CH_4 prediction models cannot be determined routinely because of the use of gas chromatography. The MFA that can be determined with the use of infrared spectroscopy are however no promising predictors for CH_4 emission. Furthermore, MFA have only a moderate CH_4 prediction potential. This together suggests that it might not be the best option to focus in the future on MFA alone as a proxy for CH_4 emission of dairy cows.

The FTIR technique has a low to moderate CH_4 prediction potential. However, FTIR has a great potential for practical high throughput application, facilitating repeated measurements of the same cow potentially reducing random noise. Results of this thesis also demonstrated that FTIR spectra do not have the potential to detect differences in CH_4 emission between diets which are, in terms of forage level and quality, commonly fed in practice. Moreover, the robustness of FTIR spectra is currently unknown. Hence, it remains to be investigated whether FTIR spectra can predict CH_4 emissions from dairy cows housed under different conditions from those under which the FTIR-based prediction equations were developed. It is therefore concluded that the accuracy and precision to predict CH_4 emission using FTIR needs to increase, and the capacity of FTIR to evaluate the differences in CH_4 emission between dairy cows and different types of diets needs to improve, in order to actually be a valuable proxy for CH_4 emission of dairy cows.

NEDERLANDSE SAMENVATTING

Enterisch methaan (**CH_4**) wordt in de pens van herkauwers gevormd als gevolg van microbiële fermentatie van het geconsumeerde voer. Dit CH_4 heeft geen voedingswaarde voor de melkkoe en wordt hoofdzakelijk uitgestoten via oprispingen en de adem. Hierdoor is enterische CH_4 emissie niet alleen de grootste bron van broeikasgassen in de melkveehouderij, maar vertegenwoordigt het ook een verlies van de opgenomen energie door de melkkoe. Vanwege deze grote gevolgen is onderzoek naar strategieën om enterische CH_4 emissie van melkkoeien te verlagen noodzakelijk. Verlaging van enterische CH_4 emissie zou eenvoudig bereikt kunnen worden door het aantal melkkoeien te verminderen. Echter, de vraag naar voedingsproducten van dierlijke oorsprong, waaronder melk, zal naar verwachting toenemen als gevolg van een toename van de wereldbevolking en inkomstenniveau van consumenten. Het is daarom van belang om zuivelproducten te produceren en gelijktijdig de impact op het klimaat te verminderen. Het overkoepelende doel van dit promotieonderzoek was daarom het ontwikkelen van een indicator voor CH_4 emissie die gemeten kan worden in de melk van melkkoeien.

Er zijn momenteel verschillende voederstrategieën beschikbaar om enterische CH_4 emissie van melkkoeien te verminderen. De resultaten in Hoofdstuk 3 geven aan dat het vervangen van vezelrijk kuilgras met zetmeelrijk snijmais in een ruwvoerrijk rantsoen een effectieve strategie is om enterische CH_4 emissie te verlagen zonder dat dit negatieve gevolgen heeft voor de productie van de koe. Echter, een minimum niveau van zetmeel in het rantsoen blijkt noodzakelijk. Er is weinig bekend of de genetische achtergrond van een koe het CH_4 verlagende effect van voederstrategieën kan beïnvloeden. Daarom is in Hoofdstuk 7 de interactie tussen het *DGAT1* K232A polymorfisme van de koe en de toevoeging van lijnzaadolie aan het rantsoen onderzocht. De resultaten van Hoofdstuk 7 geven aan dat het *DGAT1* K232A polymorfisme geassocieerd is met veranderingen in de melksamenstelling, stikstof efficiëntie en de metaboliseerbaarheid van het rantsoen, maar geen effect heeft op vertering en enterische CH_4 emissie, terwijl lijnzaadolie enterische CH_4 emissie effectief wel verlaagt ongeacht het *DGAT1* K232A polymorfisme van de koe.

Om mogelijke enterisch CH_4 verlagende strategieën te evalueren, zijn nauwkeurige methoden nodig om enterische CH_4 emissie van individuele melkkoeien te meten. Er zijn verschillende technieken beschikbaar waarmee deze emissie van melkkoeien geschat, dan wel, gemeten kan worden. Echter, geen van deze technieken is toepasbaar voor grootschalige en nauwkeurige CH_4 metingen in de praktijk. Vandaar dat onderzoekers zijn gaan kijken naar verschillende metabolieten in de melk die kunnen fungeren als indicatoren voor enterische CH_4 emissie van melkkoeien, zoals melkvetzuren. Een voorwaarde hiervoor is dat deze indicatoren gerelateerd zijn aan de enterische CH_4 emissie. Melkvetzuren werden tot voor kort standaard geanalyseerd door middel van gas chromatografie. Echter, deze techniek is niet geschikt voor routinematige analyses, wat resulteerde in de toepassing van Fourier Transform infrarood spectroscopie (**FTIR**). In Hoofdstuk 2 wordt een overzicht gegeven van de literatuur waarin melkvetzuren gerelateerd worden aan enterische CH_4 emissie van melkkoeien, waarnaast ook de voor- en nadelen van de FTIR techniek worden besproken. Het blijkt dat de resultaten in de literatuur over de relatie tussen melkvetzuren en enterische CH_4 emissie variabel zijn. Sommige studies rapporteren een sterke relatie, waar andere studies juist aangeven dat melkvetzuren geen

betrouwbare indicator zijn voor enterische CH_4 emissie. Zelfs de studies die wel een sterke relatie vinden tussen melkvetzuren en enterische CH_4 emissie, beschrijven enterische CH_4 voorspelformules die bestaan uit een andere set van melkvetzuren. Deze verschillen kunnen het gevolg zijn van een aantal factoren, waaronder rantsoensamenstelling en lactatiestadium van de koeien. Daarnaast komt in dit hoofdstuk naar voren dat de eenvoud van FTIR het grootste voordeel van deze methode is en daarbij de mogelijke toepasbaarheid in de praktijk. Daartegenover staat wel dat FTIR zelf een laag tot gemiddelde voorspelkracht heeft voor enterische CH_4 emissie en dat FTIR niet in staat is om melkvetzuren te voorspellen die belangrijk zijn voor de voorspelling van enterische CH_4 emissie. Dat eerste wordt ook duidelijk in Hoofdstuk 9, waarin de voorspelkracht van melkvetzuren vergeleken wordt met de voorspelkracht van FTIR. Hiervoor zijn data van 9 experimenten (n = 218 individuele koe observaties) gebruikt waarin een brede range van ruwvoer-rijke rantsoenen werd gevoerd. De resultaten geven aan dat melkvetzuren een grotere voorspelkracht voor enterische CH_4 emissie hebben dan FTIR en hoewel beide technieken (d.w.z. melkvetzuren en FTIR) potentieel enterische CH_4 emissie van melkkoeien kunnen voorspellen, geen van beide is momenteel geschikt om toegepast te worden in de praktijk.

Ondanks dat er veel focus is geweest op de relatie tussen melkvetzuren en enterische CH_4 emissie, is het belangrijk te realiseren dat melk nog meer metabolieten bevat zoals o.a. vluchtige en niet-vluchtige metabolieten. Momenteel worden vluchtige metabolieten in de melk bijvoorbeeld gebruikt om de voederstrategieën van de koe te achterhalen en niet-vluchtige metabolieten in de melk zijn bijvoorbeeld gerelateerd aan de gezondheid van de melkkoe. In Hoofdstuk 4 is onderzocht of vluchtige en niet-vluchtige metabolieten in de melk ook gerelateerd zijn aan enterische CH_4 emissie, waarbij gebruik is gemaakt van de data en melkmonsters van de studie beschreven in Hoofdstuk 3. In het algemeen blijken de niet-vluchtige metabolieten in de melk sterker gerelateerd te zijn aan enterische CH_4 emissie dan de vluchtige metabolieten in de melk. Daarnaast bleek dat CH_4 intensiteit (g CH_4 per kg vet- en eiwit-gecorrigeerde melkproductie) gerelateerd is aan lactose synthese en energiemetabolisme in het uier, wat naar voren kwam door de relatie tussen CH_4 intensiteit en de niet-vluchtige metabolieten uridinedifosfaat hexose B en citroenzuur. Methaan opbrengst (g CH_4 per kg voer opname) daarentegen is gerelateerd aan de toevoer van glycogene (d.w.z. energierijke) nutriënten, wat naar voren kwam door de relatie tussen CH_4 opbrengst en de niet-vluchtige metaboliet aceton.

Aan de hand van de hierboven beschreven relaties tussen enterische CH_4 emissie en zowel vluchtige als niet-vluchtige metabolieten in melk, werd verondersteld dat het meenemen van zowel vluchtige als niet-vluchtige metabolieten aan CH_4 voorspelformules met alleen melkvetzuren zou leiden tot een verbetering van de voorspelkracht met dus een betere indicator voor enterische CH_4 emissie van melkkoeien tot gevolg. Dit werd onderzocht in Hoofdstuk 5, waarbij wederom data en melkmonsters van de studie beschreven in Hoofdstuk 3 werden gebruikt. De resultaten geven aan dat melkvetzuren alleen een gemiddelde tot goede voorspelkracht voor enterische CH_4 emissie hebben. Deze voorspelkracht werd groter wanneer vluchtige en niet-vluchtige metabolieten toegevoegd werden, in het bijzonder bij de voorspelformule voor CH_4 intensiteit.

Het werk dat gepresenteerd is in Hoofdstukken 3, 4 en 5 is gebaseerd op een relatief kleine diversiteit aan rantsoenen, namelijk 4 ruwvoerrijke rantsoenen waarin kuilgras gedeeltelijk of volledig vervangen is door snijmais. Daarom is in Hoofdstuk 6 de relatie tussen enterische CH_4 emissie en zowel vluchtige als niet-vluchtige metabolieten in de melk verder uitgezocht, waarbij de data van 6 studies (n = 123 individuele koe observaties) zijn gebruikt die een brede range van ruwvoer-rijke rantsoenen omvatten met verschillende kwaliteiten en hoeveelheden gras, kuilgras en snijmais. De resultaten van dit hoofdstuk laten duidelijk zien dat zowel vluchtige als niet-vluchtige metabolieten in melk nuttige informatie bevatten die mogelijk onze kennis rondom enterische CH_4 emissie van melkkoeien kan vergroten. Echter, bij de ontwikkeling van voorspelformules voor enterische CH_4 emissie kwam naar voren dat zowel vluchtige als niet-vluchtige metabolieten in de melk, geen tot weinig voorspelkracht hebben. Ook wordt de voorspelkracht niet groter wanneer de vluchtige en niet-vluchtige metabolieten gecombineerd worden met melkvetzuren ten opzichte van melkvetzuren alleen. Aan de hand van deze bevindingen is de conclusie van Hoofdstuk 6 dat het niet de moeite waard is om vluchtige en niet-vluchtige metabolieten in melk te analyseren voor het voorspellen van enterische CH_4 emissie.

Alle resultaten die in dit proefschrift gepresenteerd zijn, laten zien dat ten opzichte van vluchtige metabolieten, niet vluchtige metabolieten en FTIR, melkvetzuren de meest nauwkeurige indicator in melk is voor enterische CH_4 emissie van melkkoeien. Echter, veel van de melkvetzuur-gebaseerde voorspelformules voor enterische CH_4 emissie zijn alleen in staat enterische CH_4 emissie nauwkeurig te voorspellen in het productiesysteem met dezelfde omgevingsfactoren waaronder ze ontwikkeld zijn. In Hoofdstuk 8 wordt het duidelijk dat reeds ontwikkelde melkvetzuur-gebaseerde voorspelformules voor enterische CH_4 emissie niet in staat zijn enterische CH_4 emissie goed te voorspellen voor melkkoeien met verschillende *DGAT1* genotypen of voor ruwvoerrijke rantsoenen met of zonder lijnzaadolie. Dit, in combinatie met andere bevindingen in de literatuur, geeft aan dat robuustheid op dit moment de grootste tekortkoming is van melkvetzuur-gebaseerde voorspelformules voor enterische CH_4 emissie. Daarnaast hebben melkvetzuren een beperkte praktische toepasbaarheid, aangezien veel van de melkvetzuren in de melkvetzuur-gebaseerde voorspelformules niet routinematig bepaald kunnen worden vanwege het gebruik van gas chromatografie. De melkvetzuren die eventueel wel met infrarood bepaald kunnen worden, zijn echter niet van belang voor de voorspelling van enterische CH_4 emissie. Verder is de voorspelkracht van de melkvetzuren voor enterische CH_4 emissie van melkkoeien slechts gemiddeld. Dit gezamenlijk geeft aan dat het wellicht niet het beste is om toekomstig onderzoek te richten op alleen melkvetzuren als indicator voor enterische CH_4 emissie van melkkoeien.

De voorspelkracht van FTIR voor enterische CH_4 emissie is laag tot gemiddeld. Echter, FTIR zou relatief eenvoudig toegepast kunnen worden in de praktijk waardoor herhaalde waarnemingen van dezelfde melkkoe mogelijk gemaakt worden met potentieel minder ruis in de voorspelde enterische CH_4 emissie. Er is ook gebleken dat FTIR niet in staat is om verschillen in enterische CH_4 emissie tussen rantsoenen die verschillen in ruwvoerkwaliteit en ruwvoer hoeveelheid, in kaart te brengen. Daarnaast is de robuustheid van FTIR momenteel onbekend, wat vraagt naar onderzoek waarin bekeken wordt of FTIR in staat is enterische CH_4 emissie van

melkkoeien te voorspellen die onder andere omstandigheden gehuisvest worden dan de omstandigheden waaronder de FTIR-gebaseerde CH_4 voorspelformules ontwikkeld zijn. Uiteindelijk wordt geconcludeerd dat FTIR veel potentie heeft voor het voorspellen van enterische CH_4 emissie van melkkoeien, in het bijzonder omdat het een snelle goedkope methode is. Echter, de CH_4 voorspelkracht van FTIR moet beter zijn dan nu en de capaciteit om verschillen in CH_4 emissie tussen dieren en rantsoenen goed te voorspellen moet beter zijn dan nu. Pas dan kan FTIR een waardevolle proxy zijn voor enterische CH_4 emissie van melkkoeien.

List of abbreviations

^{1}H-NMR	Proton nuclear magnetic resonance
ADF	Acid detergent fibre
ADL	Acid detergent lignin
ALL	All metabolites combined as selection variables
BCS	Body condition score
BHG	Biohydrogenation
BW	Body weight
$BW^{0.75}$	Metabolic body weight
CCC	Concordance correlation coefficient
C_b	Bias correction factor
CH_4	Methane
CON	Control diet
CO_2	Carbon dioxide
CRC	Climate respiration chamber
Cr_2O_3	Chromium oxide
CP	Crude protein
CS	Corn silage
DHA	Docosahexaenoic acid
DGAT1	K232A polymorphism of the acyl CoA:diacylglycerol acyltransferase 1 gene
DM	Dry matter
DIM	Days in milk
DMI	Dry matter intake
DVE	Intestinal digestible protein
ER	Energy retention
FA	Fatty acid
FAME	Fatty acid methyl ester
FFA	Free fatty acid
FID	Flame ionization detector
FPCM	Fat- and protein-corrected milk
FTIR	Fourier-transform infrared spectroscopy
F:C	Forage to concentrate ratio
GC	Gas chromatography
GC-MS	Gas chromatography - mass spectroscopy
GE	Gross energy
GEI	Gross energy intake
GHG	Greenhouse gas
g-NA	Non-assigned genus
GS	Grass silage
GS0	0% of the roughage consisting of grass silage

GS100	100% of the roughage consisting of grass silage
GS33	33% of the roughage consisting of grass silage
GS67	67% of the roughage consisting of grass silage
GWAS	Genome wide association study
H_2	Hydrogen
LN(SCC)	Natural logarithm of somatic cell count
LSO	Linseed oil diet
LV	Latent variable
MEI	Metabolizable energy intake
MFA	Milk fatty acid
MIR	Mid-infrared spectroscopy
MS	Maize silage
MSEP	Mean square error of prediction
MUFA	Mono unsaturated fatty acid
MUN	Milk urea nitrogen
N	Nitrogen
NAD	Nicotinamide adenine dinucleotide
NADPH	Nicotinamide adenine dinucleotide phosphate
NDF	Neutral detergent fibre
NE_L	Net energy for lactation
NV	Non-volatile metabolites
OBCFA	Odd- and branched-chain fatty acid
OEB	Rumen degradable protein balance
OM	Organic matter
OST	Oral stomach tube technique
OTU	Operational taxonomic units
O_2	Oxygen
PERMANOVA	Permutational multivariate analysis of variance
PUFA	Poly unsaturated fatty acid
qPCR	Quatitative polymerase chain reaction
r	Pearson correlation coefficient
RMSEC	Root mean square error of calculation
RMSEP	Root mean square error of prediction
RMSECV	Root mean square error of cross validation
RMSPE	Root mean square prediction error
RPD	Ratio of performance to deviation
RQ	Respiration quotient
rRNA	Ribosomal ribonucleic acid
R^2	Coefficient of determination
R^2CV	Coefficient of determination of cross validation
SFA	Saturated fatty acid
SF_6	Sulfur hexafluoride tracer

SMCFA	Straight short- and medium-chain fatty acid
TCA	Tricarboxylic acid cycle
μ	Location shift measure
UFA	Unsaturated fatty acid
UDP	Uridine diphosphate
V	Volatile metabolites
v	Scale shift measure
VFA	Volatile fatty acids

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My PhD project was part of a large Top Institute Food Nutrition (TIFN) project consisting of different disciplines: Animal Breeding and Genetics, Animal Nutrition, Dairy Science and Technology, and Microbiology. In October 2012 our project started with a meeting in Hof van Wageningen. During that meeting we really got to know each other, did not survive the airplane crash (none of us...), and started with the first deliverables. Johan, Henk, Marleen,

Noelle, Sabine, Hauke, Caroline, Tom, Joan, Jueeli, Kasper, Elsa, Jan and (our) Henk, I enjoyed working with you during the last couple of years. I learned a lot from all of you and appreciated our monthly discussions, social interactions, and our collaboration.

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About the author

Curriculum vitae

Overview of scientific publications

Training and supervision plan

CURRICULUM VITAE

Sanne van Gastelen was born on October 29, 1985 and grew up in Purmerend, the Netherlands. She finished secondary school (Purmerendse Scholen Gemeenschap, location Da Vinci, Purmerend, the Netherlands) in 2005, where after she studied at Utrecht University and obtained her BSc degree in Biology in 2008. During her MSc in Neuroscience and Cognition, Sanne followed the specialization in Behavioural Neuroscience. Her major thesis was concerned with the antipredator behavior of orangutans, which involved field work for a period of 8 months in Batumbelin Quarantine Center (Sumatra, Indonesia). Her minor thesis was concerned with the effect of different types of bedding material on cow comfort and risk for lameness and mastitis. The latter resulted in a scientific publication. In 2010 she graduated for her MSc degree at Utrecht University. Sanne started her PhD at Wageningen University & Research (Wageningen, the Netherlands) in July 2012. During her PhD, Sanne studied whether methane emission of dairy cows can be estimated based on milk composition. The results of her PhD work are presented in this thesis. In November 2016, Sanne joined Wageningen Livestock Research (Wageningen, the Netherlands) as a researcher in dairy nutrition.

OVERVIEW OF SCIENTIFIC PUBLICATIONS

Peer-reviewed scientific publications related to this thesis

Van Gastelen, S., E.C. Antunes-Fernandes, K.A. Hettinga, and J. Dijkstra. 2017. Relationships between methane emission of Holstein Friesian dairy cows and fatty acids, volatile metabolites and non-volatile metabolites in milk. Animal 11:1539-1548.

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Van Gastelen, S., H. Mollenhorst, E.C. Antunes-Fernandes, K.A. Hettinga, G.G. van Burgsteden, J. Dijkstra, and J.L.W. Rademaker. Predicting enteric methane emission of dairy cows with milk Fourier-transform infrared spectra and gas chromatography-based milk fatty acid profiles. Journal of Dairy Science *submitted.*

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Van Gastelen, S., H. Mollenhorst, E.C. Antunes-Fernandes, K.A. Hettinga, G.G. van Burgsteden, J. Dijkstra, and J.L.W. Rademaker. 2017. Using milk FTIR spectra and gas chromatography-based MFA profiles to predict CH_4 emission of dairy cows. Methagene final meeting, Caserta, Italy. (oral)

Van Gastelen, S., E.C. Antunes-Fernandes, K.A. Hettinga, and J. Dijkstra. 2016. Using milk composition to estimate methane emission of dairy cattle. 1st International Animal Nutrition Congress, Antalya, Turkey. (invited speaker)

Van Gastelen, S., E.C. Antunes-Fernandes, K.A. Hettinga, and J. Dijkstra. 2016. Using milk metabolome to predict methane emission of dairy cows. Joint Livestock Research Group Network meeting, Melbourne, Australia. (oral)

Van Gastelen, S., Negussie, E., Y. de Haas, F. Dehareng, R. Dewhurst, J. Dijkstra, N. Gengler, D. P. Morgavi, H. Soyeurt, T. Yan, and F. Biscarini. 2016. Making combinations of proxies. The Animal Selection, Genetics and Genomics Network (ASGGN) meeting, Melbourne, Australia. (invited speaker)

Van Gastelen, S., S. van Engelen, J. Dijkstra, and M.H.P.W. Visker. 2016. The effect of *DGAT1* K232A polymorphism and linseed oil supplementation on methane emission of dairy cows. Book of abstracts of 6th Greenhouse Gas and Animal Agriculture (GGAA) Conference, Melbourne, Australia, p128. (poster)

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Edwards, J.E., H.J. van Lingen, J.D. Vaidya, S. van Gastelen, B. van den Bogert, A. Bannink, C.M. Plugge, J. Dijkstra, and H. Smidt. 2016. Diurnal dynamics of metabolites and microbes in the bovine rumen: implications for the control of fermentation pathways. The 10th joint symposium INRA-Rowett on gut microbiology, Clermont-Ferrand, France. (poster)

Van Lingen, H.J., J.D. Vaidya, J.E. Edwards, S. van Gastelen, B. van den Bogert, A. Bannink, H. Smidt, C.M. Plugge, and J. Dijkstra. Metabolic sequences of fermentation products in and from the bovine rumen. 41th Animal Nutrition Research Forum, Wageningen, the Netherlands. (oral)

Van Gastelen, S., E.C. Antunes-Fernandes, K.A. Hettinga, and J. Dijkstra. 2015. Predicting methane emission of dairy cows using fatty acids and volatile and non-volatile metabolites in milk. Book of abstracts of 2015 Joint Annual Meeting of ADSA-ASAS, Orlando, Florida, USA, 98:600. (oral)

Van Lingen, H.J., J.D. Vaidya, S van Gastelen, B. van den Bogert, A. Bannink, C.M. Plugge, H. Smidt, and J. Dijkstra. 2015. Daily patterns of hydrogen and volatile fatty acid concentrations in relation to thermodynamic control on fermentation in the bovine rumen. Book of abstracts of 2015 Joint Annual Meeting of ADSA-ASAS, Orlando, Florida, USA, 98:867-868. (oral)

Van Gastelen, S., E.C. Antunes-Fernandes, K.A. Hettinga, G. Klop, S.J.J. Alferink, and J. Dijkstra. 2014. Replacing grass silage with maize silage affects rumen fermentation characteristics and enteric methane production in dairy cattle. Animal Nutrition Research Forum, Utrecht, The Netherlands. (oral)

Van Gastelen, S., E.C. Antunes-Fernandes, K.A. Hettinga, and J. Dijkstra. 2013. Reduced methane emission in dairy cows. Indicator for methane emission in milk. WIAS science day, Wageningen, The Netherlands. (poster)

Van Gastelen, S., D.J. Houwers, B. Westerlaan, and F.J.C.M. Van Eerdenburg. 2011. Zand slaagt voor ligbedexamen. Ligbedonderzoek: paardenmest comfortabel maar risico voor uiergezondheid. Veeteelt 28:46-47.

TRAINING AND SUPERVISION PLAN

Completed in fulfillment of the requirements for the Education Certificate of the Wageningen Institute of Animal Sciences

The Basic Package (3 ETCS[1])	**Year**
WIAS Introduction Course	2013
Philosophy and Ethics of Science	2013
Scientific Exposure (14 ECTS)	
International conferences	
Greenhouse Gases and Animal Agriculture (GGAA), Dublin, Ireland	2013
Joint Annual Meeting (JAM) of the American Dairy Science Association (ADSA) and American Society of Animal Science (ASAS), Orlando, Florida, USA	2015
6th Greenhouse Gas and Animal Agriculture (GGAA) Conference, Melbourne, Australia	2016
1st International Animal Nutrition Congress, Antalya, Turkey	2016
Seminars and Workshops	
Nutritional Management in Early Lactation, Wageningen, the Netherlands	2012
Annual WIAS Science Day, Wageningen, the Netherlands	2013, 2014
Annual Animal Nutrition Research (ANR) Forum, Utrecht, the Netherlands	2014
Symposium Solutions for Climate Change and Animal Production	2014
Presentations	
Annual WIAS science day (poster)	2013
Annual ANR Forum (oral presentation)	2014
JAM ADSA-ASAS (oral presentation)	2015
GGAA (2x oral presentation, 1x poster)	2016
1st International Animal Nutrition Congress (oral presentation)	2016
In-depth Studies (7 ECTS)	
Disciplinary and Interdisciplinary courses	
Fatty acids in dairy cattle in relation to product quality and health, Ghent, Belgium	2012
SummerSchool Ruminomics, Piacenza, Italy	2014
Methagene workshop (Granada, Spain; Catania, Italy; Jokioinen, Finland)	2014, 2015, 2016
Preconference workshops: SF_6 and climate respiration chambers (Melbourne, Australia)	2016
Methagene training school on Rumen Microbiol Ecosystem (Porto, Portugal)	2016
Advanced Statistic courses	
Advanced statistics of Experimental Design, WIAS	2012
Professional Skills Support Courses (6 ECTS)	
Scientific Publishing	2013
Basic IP for TIFN researchers	2013

[1] One ECTS credit equals a study load of approximately 28 hours.

Professional Skills Support Courses (continued)	
Techniques for Writing and Presenting a Scientific Paper	2014
Presentation Skills	2014
Efficient Writing Techniques	2014
Writing Grant Proposals	2016
Research Skills Training (3 ECTS)	
Preparing PhD Research Proposal	2012
Didactic Skills (12 ECTS)	
Supervising Practicals and Excursions	
Animal Nutrition and Physiology	2015, 2017
Supervising theses	
MSc Thesis, 4x	2015
BSc Thesis, 2x	2013, 2015
Tutorship	
Inleiding in de Dierwetenschappen	2014, 2016
Management Skills Training (3 ECTS)	
Membership of Boards and Committees	
WAPS Council Member - Education Committee	2015
TOTAL 48 ECTS	

Colophon

The studies presented in this thesis were performed within the framework of Top Institute Food and Nutrition.

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