Identification of milk fatty acids as proxies of the enteric methane emissions in dairy cows

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Identification des acides gras du lait comme proxies des émissions de méthane entérique chez la vache laitière

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Résumé

Le méthane (CH_4) est un puissant gaz à effet de serre produit lors de la fermentation microbienne anaérobie des aliments dans le rumen. L'un des enjeux majeurs pour le secteur de l'élevage est de trouver des stratégies (alimentaires, génétique) pour réduire les émissions de CH_4 tout en maintenant les performances animales. Les techniques de mesure de ces émissions sont coûteuses et difficilement utilisables à grande échelle sur le terrain, d'où la nécessité de trouver des alternatives de mesure ou biomarqueurs pour prédire ces émissions. Les acides gras (AG) du lait ont déjà été identifiés comme indicateurs intéressants de la méthanogenèse chez la vache laitière, mais il convient d'améliorer la précision des équations de prédiction du CH_4 existantes ainsi que d'élargir leur domaine d'application à tous types de rations. L'objectif de mon travail de thèse a été de confirmer la pertinence des AG du lait comme indicateurs périphériques de la méthanogénèse chez la vache laitière avec diverses conditions nutritionnelles. Deux bases de données regroupant des données individuelles (issues d'une collaboration scientifique internationale) et moyennes (issues de la littérature) de CH_4, de composition en AG du lait et d'autres performances et caractéristiques de l'animal, ainsi que des données de composition chimique des rations, ont été créées. Parallèlement, l'acquisition *in vivo* de données en conditions expérimentales contrôlées pour des rations mal connues ont permis d'incrémenter la base de données individuelles. Des équations de prédiction des émissions de CH_4 [en g/jour, g/kg de matière sèche ingérée (MSI), et g/kg de lait] ont été développées à partir de certains AG du lait, utilisés seuls ou combinés à d'autres variables d'ingestion et de performances laitières, représentant alors des modèles complexes. Des relations entre les émissions de CH_4 et la teneur de différents AG du lait (C10:0, iso C17:0 + *trans*-9 C16:1, *iso* C16:0, *cis*-11 C18:1, *cis*-15 C18:1, *cis*-9,*cis*-12 C18:2, et *trans*-11,*cis*-15 C18 :2) ont été mises en évidence, confirmant des voies métaboliques communes dans le rumen entre méthanogenèse et métabolisme lipidique. Les équations sont également liées aux types de régimes à partir desquels elles ont été développées. Les équations simples (AG du lait uniquement) sont moins précises que les complexes (erreurs résiduelles de prédiction, respectivement, de 58.6 g/jour, 2.8 g/kg MSI et 3.7 g/kg lait vs. 42.8 g/jour, 2.5 g/kg MSI et 3.3 g/kg lait). Une différence minimum de 16% de CH_4 entre stratégies de réduction pourra être mise en évidence par la meilleure équation de prédiction développée. Des équations basées sur des AG bien déterminés par les méthodes infrarouges devront être testées pour évaluer, en routine et à grande échelle, de nouvelles stratégies de réduction des émissions de CH_4 entérique chez la vache laitière.

Mots clés : Acides Gras du Lait, Base de données, Equation de Prédiction, Méthane, Vache.

Abstract

Methane (CH_4) is a potent greenhouse gas coming from the anaerobic microbial fermentation of the diet in the rumen. One of the main current challenge for the dairy sector is to find CH_4 mitigation strategies (diets or genetics) without altering animal performance. Enteric methane measurement methods are costly and very difficult to apply on a large scale on field. Thus, there is a need to develop alternative measurement methods, such as equations based on proxies to predict CH_4 emissions. Milk fatty acids (FA) have been identified as potential predictors of the methanogenesis in dairy cattle, but the prediction ability of extant published CH_4 equations must be improved, and their domain of applicability must be enlarged to a wide range of diets. The objective of this PhD thesis was to confirm the potential of milk FA as proxies to predict enteric CH_4 emissions in dairy cows fed a wide range of diets. Two databases (based on individual and mean data, respectively) were built thanks to an international collaboration, and gathered data on CH_4, milk FA composition, dairy performances, diet and animal characteristics. Two *in vivo* experiments were conducted with the aim to study the effect of dietary strategies poorly documented, on methanogenesis and milk FA. The data from these experiments were included in the created database. Firstly, simple CH_4 prediction equations were developed [g/d, g/kg of DMI (DMI), and g/kg of milk] based only on milk FA, and secondly other variables related to cow intake or characteristics, and dairy performance were added and constituted complex equations. Relationships between CH_4 and several milk FA (C10:0, *iso* C17:0 + *trans*-9 C16:1, *iso* C16:0, *cis*-11 C18:1, *cis*-15 C18:1, *cis*-9,*cis*-12 C18:2, and *trans*-11,*cis*-15 C18 :2) were found, confirming common rumen metabolic pathways between methanogenesis and lipid metabolism. Equations were also closely related to the diets included in the database used for their development. Simple equations were less accurate than complex ones (prediction error of 58.6 g/d, 2.8 g/kg DMI and 3.7 g/kg milk vs 42.8 g/d, 2.5 g/kg DMI and 3.3 g/kg milk, respectively). A minimum difference of 16% in CH_4 emissions between mitigating strategies can be evidenced with the best prediction equation developed in this PhD. Methane prediction equations based on milk FA well determined by infrared spectrometry methods need to be developed in order to be used on a routine basis and on a large scale. These prediction equations would allow studying the effect of novel mitigation strategies of enteric CH_4 emissions in dairy cows.

Keywords: Milk Fatty acids, Database, Prediction Equation, Methane, Dairy cow.

List of publications

Peer-reviewed international scientific journals:

Bougouin, A., A. Ferlay, M. Doreau, and C. Martin. 2018. Effects of carbohydrate type or bicarbonate addition to grass silage-based diets on enteric methane emissions, milk production, and fatty acid composition in dairy cows. Journal of Dairy Science, 101 (7), 6085-6097.

Bougouin, A., C. Martin, M. Doreau, and A. Ferlay. 2018. Effects of starch-rich or lipid-supplemented diets inducing milk fat depression on lipid metabolism and digestive process in lactating dairy cows. Animal. (Published online: 29 November 2018).

Bougouin, A., J. A. D. Ranga Niroshan Appuhamy, A. Ferlay, E. Kebreab, C. Martin, P. J. Moate, C. Benchaar, P. Lund, and M. Eugène. Individual milk fatty acids are potential predictors of enteric CH_4 emissions from dairy cows fed a wide range of diets: approach by meta-analysis. Global Change in Biology. (Submitted to Journal of Dairy Science: 02 November 2018).

Scientific communications in international congresses:

Bougouin, A., A. Ferlay, M. Doreau, and C. Martin. 2016. Effect of concentrate type (starch vs. fiber) and bicarbonate addition in grass silage-based diets on performance, diet digestibility and enteric methane emissions in lactating dairy cows. Joint Annual Meeting 2016 proceedings, Salt Lake City, Utah, United States of America (oral communication).

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International Symposium on the Nutrition of Herbivores (ISNH10). Cambridge (GBR): Cambridge University Press (Advances in Animal Biosciences, 9 (3)), 2018). (poster).

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Scientific communications in national congresses:

Bougouin, A., C. Martin, M. Eugène, A. Ferlay. Pertinence des acides gras du lait pour prédire les émissions de méthane entérique chez le bovin laitier : état de l'art. Journée d'animation transversale Inra « Journée Glande Mammaire Lait », 17/11/2016 Theix, France (Oral communication).

Bougouin, A., A. Ferlay, M. Doreau, Y. Rochette, S. Rudel, C. Lascoux, C. Martin. Effet du type de glucide (Amidon vs. Fibre) et de l'ajout de bicarbonate dans des rations à base d'ensilage d'herbe sur les performances, la digestibilité de la ration, et la production de méthane entérique chez la vache laitière en lactation. Rencontre ExSybel-Syslait des 26-27 mai 2016, Pin-au-Haras, France (oral communication).

Bougouin, A., Identification et validation de l'utilisation des acides gras du lait comme indicateurs des émissions de méthane entérique chez la vache laitière. Conseil Scientifique Unité Mixte de Recherche sur les Herbivores de l'INRA Theix, 10th of March 2016 & 5th of October 2017 (oral communication).

Bougouin, A., J. A. D. Ranga Niroshan Appuhamy, A. Ferlay, E. Kebreab, C. Martin, P. J. Moate, C. Benchaar, P. Lund, and M. Eugène. Prédiction des émissions de méthane à partir des acides gras du lait chez la vache laitière recevant des rations très diversifiées. Journées de l'Ecole Doctorale SVSAE, Université Blaise Pascal, Clermont-Ferrand, 14 June 2018 (poster and short oral communication).

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List of common abbreviations

AA: Amino acid
ADF: Acid detergent fiber
BW: Body weight
CCC: Concordance correlation coefficient
CH_4: Methane
CO_2 : Carbon dioxide
CP: Crude protein
CS: Corn silage
CV: Coefficient of variation
DHA: Docosahexaenoic acid
DIM: Days in milk
DM: Dry matter
DMI: Dry matter intake
EE: Ether extract
FA: Fatty acid
FAME: Fatty acid methyl ester
FPCM: Fat and protein corrected milk
F:C: Forage to concentrate ratio
GC: Gas chromatography
GE: Gross energy
GEI: Gross energy intake
GHG: Greenhouse gas
GS: Grass silage
GWP: Global warming potential
MFD: Milk fat depression
MUFA: Monounsaturated fatty acid
NDF: Neutral detergent fiber
OM: Organic matter
PCA: Principal Component Analysis
PUFA: Polyunsaturated fatty acid
RBH: Rumen biohydrogenation
RMSPE: Root mean square prediction error
RSR: RMSPE-Observations Standard Deviation Ratio
R^2: Coefficient of determination
SD: Standard deviation
SFA: Saturated fatty acid
SF_6: Sulfur hexafluoride tracer
UFA: Unsaturated fatty acid
VFA: Volatile fatty acid

General introduction

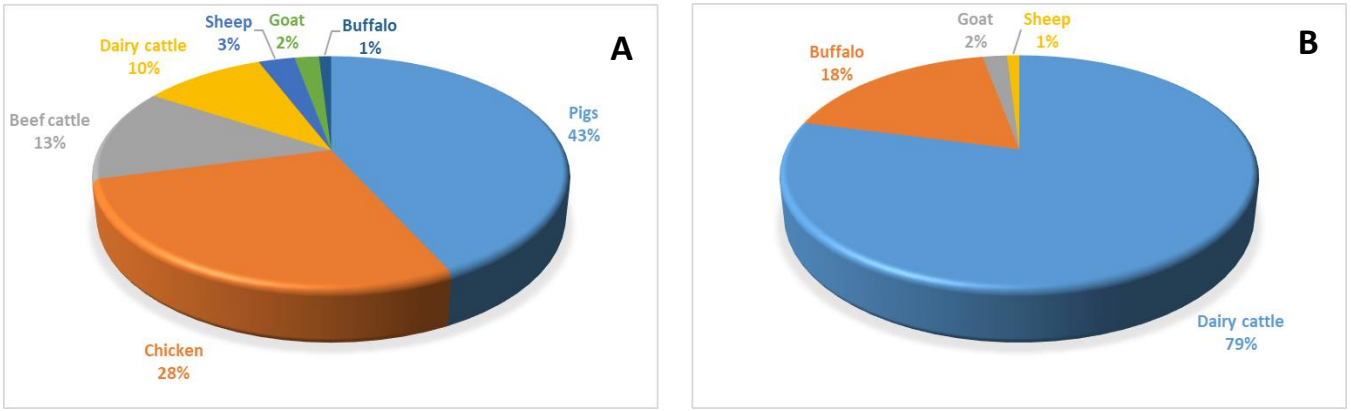

Figure 1 Contribution of ruminants to the overall world meat (A) and milk production (B) (Gerber et al., 2013a)

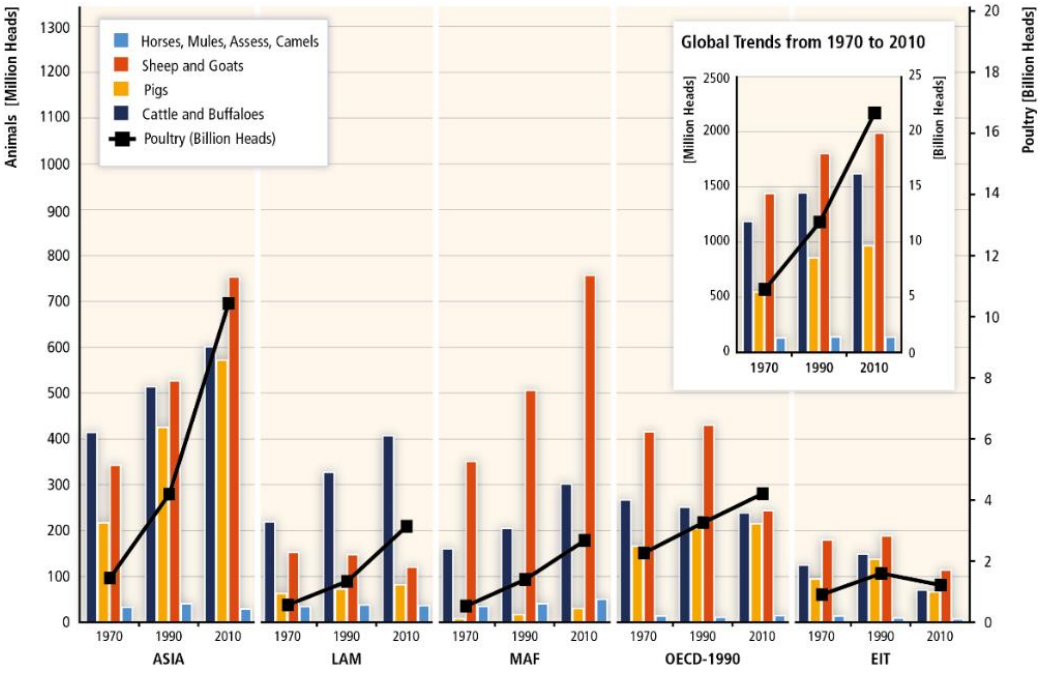

Figure 2 Animals counts (Million heads) and global trends from 1970 to 2010 (Smith et al., 2014) *EIT: Economies in Transition; LAM: Latin and Central America; MAF: Middle East and North Africa.*

The world population has been dramatically increasing for the past decades and it is expected to reach 8.5 billion inhabitants in 2030, according to the latest report of the United Nation (United Nations, 2017). Furthermore, world population is forecasted to pass to more than 9.8 billion in 2050. Nearly all this population growth will be expected to take place in developing countries (FAO, 2009). In addition, the projections show that food level production will have to be raised by 70% from 2007 to 2050, in order to face the increasing world population food demand. In this context, demand for animal products has increased over the past decade, and the total demand for animal products in developing countries is expected to more than double by 2030, and the demand growth for livestock products is projected to be +70% between 2005 and 2050 (Gerber et al., 2013b). Ruminants provide a large part of the animal products with 29% of the overall world meat production (Figure 1A) (Gerber et al., 2013a). Ruminants are almost the sole source of milk for humans, providing 644 million tons of fat-protein corrected milk, among which dairy cattle contribute to 80% (Figure 1B).

Demand for livestock products (dairy and meat products) will increase at a progressively slower pace (Food and Agriculture Organization of the United Nations, 2017). Indeed, dairy and meat products have, nowadays, opposite trends, with increasing of consumption growth of dairy products and decreasing of that of meat products (European Union, 2015). In the last three decades, world milk production has increased by more than 50%, from 500 million tons in 1983 to 769 million tons in 2013. According to (Food and Agriculture Organization of the United Nations, 2017), the increasing demand for dairy products can be mainly attributed to an increasing per capita consumption in developing countries such as India.

In this global world context, ruminants play a major role in the human food supply chain, and cattle number has been constantly increasing over the past decades (Figure 2) and is expected to rise in the future. However, there is an increase in public concern over environmental damage originating from animal feeding operations on the local scale (air, water, and soil pollution by the manure and slurry, with the nitrogen and phosphorus being directly released in the environment), and ruminant livestock productions are more particularly criticized for their high contribution to greenhouse gas (GHG) emissions on the global scale. **Thus, the impact of livestock on climate change and global warming is a major concern worldwide** (Steinfeld et al., 2006; Gerber et al., 2013b).

Global warming is a result of the natural GHG release in the atmosphere, such as carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O), and halogenated compounds.

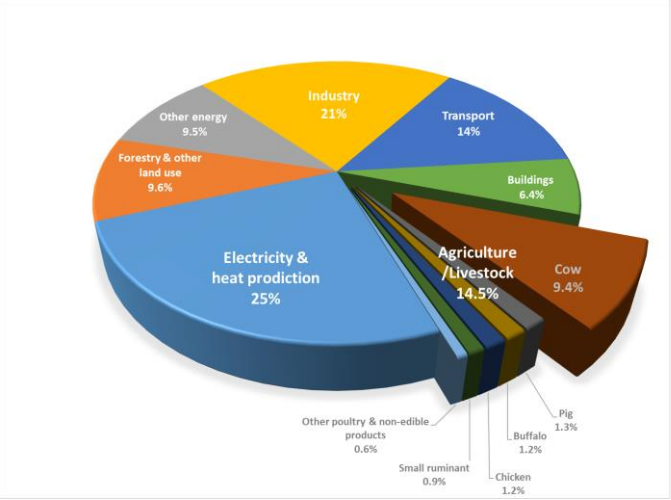

Figure 3 Greenhouse gas emissions by economic sector in the world (US EPA, 2016)

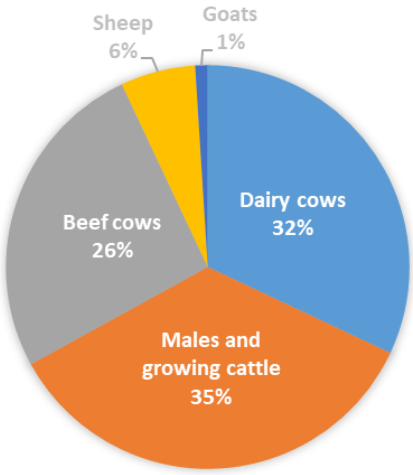

Figure 4 Contribution of cattle, sheep and goats to total methane emissions from ruminants in France (Vermorel et al., 2008)

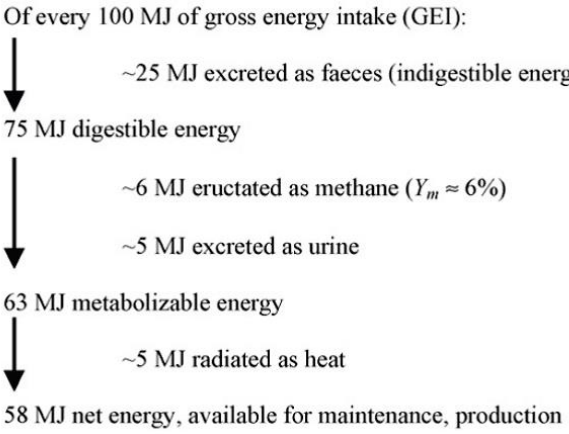

Figure 5 Energy loss during ruminant digestion of a high-quality forage diet (Lassey, 2007)

Humans and anthropogenic activities generate these pollutants and the livestock sector is considered as a major contributor because of emissions of CO_2, CH_4, and N_2O into the atmosphere (Figure 3). Globally, the livestock supply chain contributes to 7.1 billion tons of CO_2-equivalent and 14.5% of total anthropogenic GHG emissions with CH_4, N_2O and CO_2 emissions representing 44, 29, and 27%, respectively (expressed as CO_2-equivalent) (Gerber et al., 2013b).

The CH_4 is not the most abundant GHG, since CO_2 accounts for around 77% of the total anthropogenic GHG emissions and CH_4 for only 14%, but CH_4 is a more potent GHG with a global warming potential of 25 times that of CO_2 over a 100 years' time horizon (IPCC, 2007). However, CH_4 resides in the atmosphere for a shorter period than CO_2 and N_2O (12 ± 3 years *vs.* 100 and 120 years, respectively), which opens opportunities to achieve an impact on GHG atmospheric concentrations by mitigating CH_4 emissions in a relatively short-term periods.

Ruminants are the major producers of enteric CH_4 emissions, which represent 80% of CH_4 emissions from the livestock supply chain, the remaining 20% coming from manure management (Gill et al., 2010). Dairy cows are responsible of 32% of total enteric CH_4 emissions in France (Figure 4). Enteric CH_4 emissions represent 1.1 Gt per year, 46% of the total GHG emissions in dairy supply chain (Gerber et al., 2013b), and are among the main targets of GHG mitigation practices for the dairy industries (Hristov et al., 2013). Furthermore, in addition to be the main GHG emitted at the farm level, CH_4 released by ruminants constitutes an energy loss for the animals, ranging from 2% of gross energy intake (**GEI**) in case of feedlots to 12% of GEI with ruminants fed poor-quality forages (Johnson and Johnson, 1995). On average, CH_4 losses represent 6.5% of the GEI (IPCC, 2007) or 8% of digestible energy (Figure 4; Lassey, 2007).

Consequently, lowering enteric CH_4 emissions in dairy cows is a desirable strategy for reducing energy loss by ameliorating the energy partitioning, with increasing availability of metabolizable energy (Figure 5). Theoretically, this would allow more milk production and enhance animal efficiency in converting more energy nutrients into milk.

Over the past 15 years, several strategies have been tested worldwide in different experiments in order to reduce enteric CH_4 emissions (reviews of Martin et al., 2010; Grainger and Beauchemin, 2011; Gerber et al., 2013a; Knapp et al., 2014). Increasing the productivity of animals by genetic selection and thus decreasing the number of animals is one effective way to decrease CH_4 emissions per unit of product, but most of the strategies consist of manipulating

rumen parameters *via* feeding practices (modification of diet ingredients and composition, supplementation with dietary lipids or additives) or by using biotechnologies (defaunation, use of probiotics, exogenous microbial products or vaccines). However, the main limiting factor in lowering enteric CH_4 production is that these strategies are likely to increase farm production costs without any direct benefits for the farmer. Therefore, governments should encourage farmers to adopt strategies that reduce methane production by offering financial supports. In that sense, a project "Tax Carbon" has been created by the Institute for Climate Economics, following the Paris Agreement in 2015, in order to give an economic value to non-emitted carbon activity and thus to encourage the development of agricultural systems with low carbon emissions. However, to properly evaluate mitigation strategies and the reduction in carbon emitted, it must be possible to estimate cattle emissions under diverse situations in order to highlight CH_4 reduction. Thus, governments would need a simple, routine-based, and reliable estimation methodology to assess CH_4 emissions at the farm level as well as their decrease when mitigation strategies are applied. Routine measurements – along with accuracy – are key factors for a methodology to be adopted as the tool for monitoring CH_4 emissions from herds. However, the accuracy of empirical models (or prediction equations) currently used to predict CH_4 production for inventory or mitigation purposes is low (Ellis et al., 2010), and suffers from narrow spatial focus, limited observations, and limitations of the statistical technique used (Hristov et al., 2018). As stated in their symposium review, Hristov et al. (2018) reported that there is a need to build a dataset encompassing a wide range of diets, within regions and globally, to achieve high prediction accuracy. Furthermore, prediction equations can be complex and require inputs that are not commonly measured on farm (feed dry matter intake, diet ingredients and chemical composition, digestibility of nutrients, etc…). Therefore, there is a need for simple, non-invasive and practical measurements to estimate CH_4 emissions from dairy cows in research experiments (in controlled conditions) and under field conditions. In dairy cattle, milk is easy to sample on a routine basis either from individual cows or from bulk milk. Because the precursors for the synthesis of CH_4 and *de novo* synthesis of milk fatty acids (**FA**) arise in the rumen, the first relationships between CH_4 emissions and milk FA were proposed by Chilliard et al. (2009). Nevertheless, these CH_4 predictive equations were developed from milk FA composition in dairy cows fed linseed under different forms (crude and extruded seeds, and oil). More recently, other authors (Dijkstra et al., 2011; Mohammed et al., 2011; van Gastelen et al., 2018) developed CH_4 predictive equations from milk FA using data from different experimental conditions. In these studies, the predictor variables

contributing to CH_4 were not the same, suggesting that the applicability of these equations through changing feeding conditions may be limited.

In this context, INRA is co-funding a 4 years research program (2015-2018) called IVAMEME for « Identification and VAlidation of Markers of Enteric MEthanogenesis for use in CH_4 mitigation strategies in ruminants », with 11 industrials partners (Text box 1). As part of this program, the primary objective of this PhD research was to study the potential of the milk fatty acids to predict CH_4 emissions in dairy cows fed a wide range of diet. The research hypothesis is that milk FA have the potential to predict CH_4 emissions whatever the diet considered, with an average accuracy as low as possible, in order to highlighting differences in CH_4 mitigation strategies.

OUTLINE OF THE THESIS

This PhD thesis is composed of 6 chapters. **Chapter 1** is a literature review on the biological processes of enteric CH_4 emissions in ruminants and milk FA secretion. This chapter gives insight on the potential links between CH_4 emissions and milk FA composition according to diets, as well as a review of the published studies reporting CH_4 prediction equations from milk FA. **Chapters 2 and 3** report results from our *in vivo* experimental approach, which aimed at studying the effects of different CH_4 mitigation strategies (carbohydrate type +/- bicarbonate addition; starch-rich or lipid-supplemented diets) on digestion processes, including CH_4 emissions, and determination of milk FA composition in dairy cows fed diets based on different forages (grass or corn silage). The choice of dietary treatments has been made regarding the lack of knowledge for some dietary situations in dairy cows. Since, these dietary situations are commonly found in some countries such as France, it is important to explore their impact on methanogenesis and milk FA composition in the context of this PhD work. The *in silico* approach of the PhD is reported in **Chapter 4**. The development of prediction equations of CH_4 emissions from milk FA contents has been done by meta-analysis approach using two datasets (individual data and mean data from published studies) created thanks to an international collaboration. Finally, **Chapter 5** comprises a general discussion of the results obtained in this PhD, including perspective for further research and conclusion on the applicability and development of CH_4 prediction equations based on milk FA.

Text box 1. The research consortium around methanogenesis in ruminants, a collaborative innovation between INRA and eleven private partners

A consortium among INRA and eleven private partners was created to design and conduct a common research project aiming at identifying and validating indicators of enteric methane produced by ruminants. This organization shall help quickly overcome bottlenecks both for research and the ruminant production sector.

Livestock systems face a major worldwide challenge: to meet the growing demand for animal products and to reduce at the same time the environmental impacts and the competition for food resources. Ruminant livestock systems are major producers of greenhouse gases (**GHG**) emissions including methane and ruminants are less efficient transformers of food resources they consume than monogastric animals. By contrast, they can use land and forage areas which could not been used for human nutrition. Producing knowledge to allow reducing the environmental impacts of ruminants, while producing animal products both safe and socially accepted by the consumer and the citizen, is one of the core objectives of the INRA Phase scientific division, and especially the UMR1213 Herbivores. Many operators involved at various steps of the animal production sector (suppliers of additives, genetics, food manufacturers, service companies, and processors) are also seeking for operational solutions to reduce GHG emissions, while maintaining an optimal level of production and improving the competitiveness of farms.

An innovative approach for the co-construction of a research program between INRA and eleven private partners (Adisseo France SAS, Agrial, APIS-GENE, Deltavit, DSM Nutritional Products AG, IDELE, Lallemand, Moy Park Beef Orléans, Neovia, Techna France Nutrition, Valorex) was followed to build a common research project on the identification and validation of indicators of enteric methane production in ruminants. A consortium agreement was signed between the partners that agreed to self-finance the project over the period 2014-2018. The topic of this project presents a hot scientific level and at the same time has a high potential to result in intellectual property rights.

We will follow two approaches to investigate indicators of methanogenesis: (1) an approach focused on milk fatty acids previously identified as interesting in dairy cows. We plan to validate the relationships among methane emissions and different milk fatty acids under various nutritional and physiological conditions of the cow. (2) an exploratory approach (without a priori on markers) that can be applied to any type of ruminants, dairy or meat, productive or not. We will explore simultaneously the metabolomic profiles of different body matrices (rumen fluid, urine, milk, plasma, and feces). The metabolic origin of the relevant indicators will be investigated thanks to genomics analyzes of the rumen microbiota.

This ambitious project requires the implementation and articulation of studies of different nature (methodological development, *in vivo* experimentation, data integration by meta-analysis) and at different levels (rumen microbiota, animal, and herd). It is carried out by UMR1213 Herbivores in collaboration with UMRs Pegase and SAS (INRA Rennes), Mosar (INRA Paris), and Gabi (INRA Jouy-en-Josas). The research program includes 5 work-packages (WP), with the following topics:

WP1- Methane measurement methods to be used in field assays

WP2- Milk Fatty acid targeted approach on dairy cows

WP3- Metabolomics non-targeted approach on dairy or beef cattle

WP4- Rumen microbiota diversity & metagenomic function

WP5- Integration of data from all WPs

I. CHAPTER I

Literature review

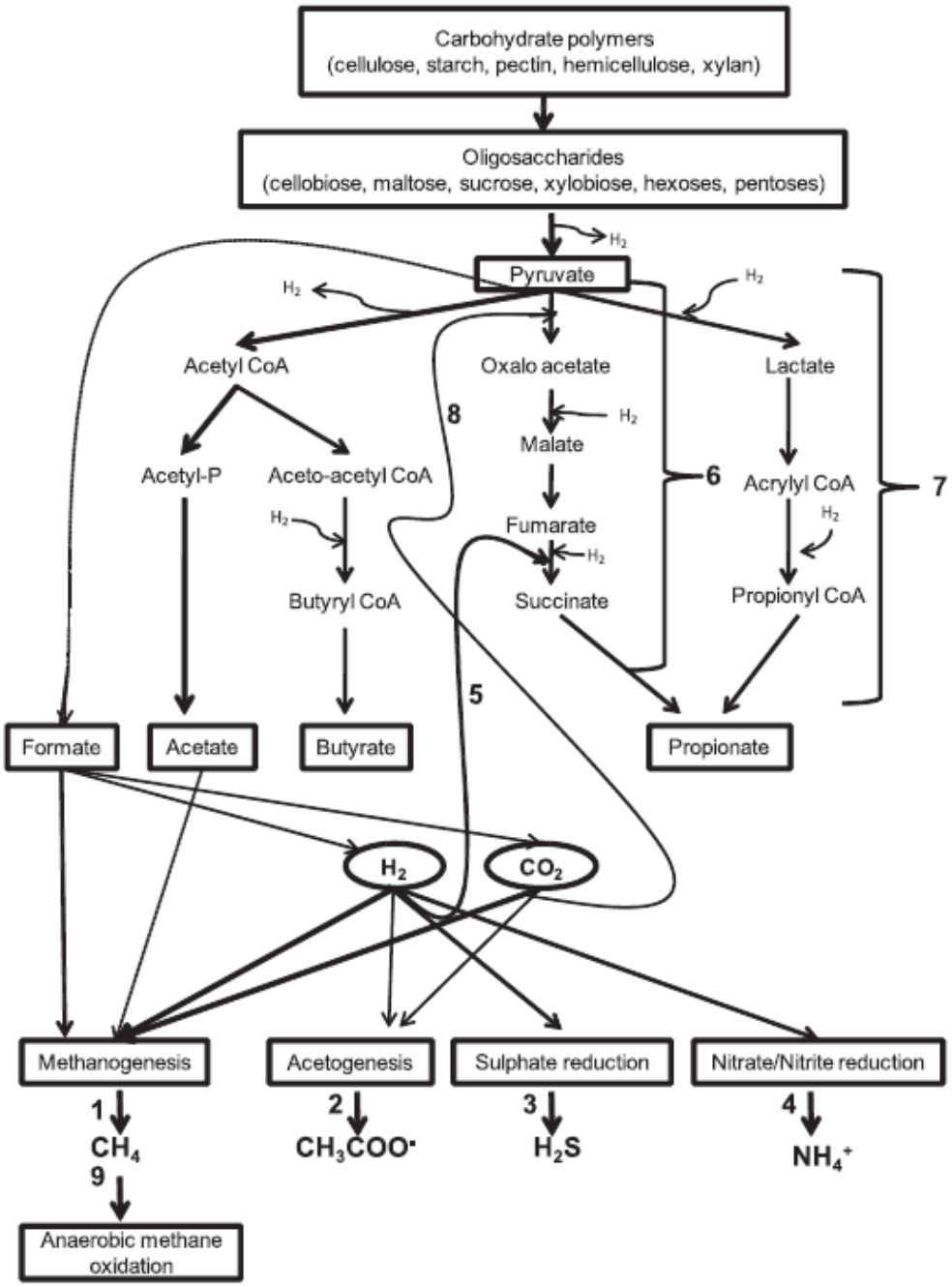

Figure 6 Rumen carbohydrates fermentation. 1. methanogenesis, 2. homoacetogenesis, 3. sulphate reduction, 4. nitrate/nitrite reduction, 5. fumarate reduction, 6. propionate production (succinate/randomizing pathway), 7. propionate production (acrylate pathway) 8. capnophily (CO_2 fixation), 9. methane oxidation (methanotrophy). Adapted from (Jeyanathan et al., 2014)

1. Enteric methane emissions

1.1. Rumen environment and digestion

In ruminants, enteric fermentation mainly occurs within the rumen (on average 87%; from 65 to 70%) and, to a lesser extent, within the large intestine (on average 13%) (Murray et al., 1976). The rumen is characterized by a complex anaerobic ecosystem (T°, pH, …) containing various and numerous microorganisms (bacteria, protozoa and fungi) all interacting together to digest complex feed components and to provide energy and proteins to ruminants. There are two steps of the digestion in the rumen: hydrolysis by enzymes and then fermentation.

Proteins. A proportion of the AA escaping from deamination is either incorporated in microbial proteins, which serve as protein source for the animal (Calsamiglia et al., 2007), or further degraded to ammonia, which is in turn, used by the bacteria for microbial protein synthesis.

Lipids. Dietary lipids and free long-chain FA are hardly hydrolyzed by rumen microorganisms, the end-products are the glycerol and free FA (Harfoot and Hazlewood, 1997), among them, unsaturated FA undergoing rumen biohydrogenation (**RBH**) process. *Further information on rumen lipid metabolism is available in section 2.2 of this chapter.*

Carbohydrates (cellulose, hemicellulose, and pectin found in forage cell walls, and starch and soluble sugars from inner vegetal cells) are hydrolyzed in simple sugars (e.g. glucose), which are in turn fermented into, among others, VFA and some gases, hydrogen (**H_2**), and carbon dioxide (**CO_2**) (Figure 6). Some of the carbohydrates may escape rumen fermentation and be directly digested in the intestine.

VFA are the main energy supply required for maintenance and productive functions for the ruminants (Van Soest, 1994; Boadi et al., 2004) by covering up to 70% of cow energy requirements (Hvelplund, 1991). Major VFA produced during microbial fermentation are the acetate (CH_3COOH, **C2**), propionate (C_2H_5COOH, **C3**) and butyrate (C_3H_7COOH, **C4**), and other minor VFA such as valeric acid (**C5**), caproic acid (**C6**), *iso*-butyrate or *iso*-valerate (iso-C4 or *iso*-C5).

However, VFA composition varies according to the diet composition. For instance, with a forage-based diet (rich in plant cell walls), C2, C3 and C4 would represent on average 70%, 20% and 10% of total VFA, respectively. With concentrate-rich diets (e.i. rich in starch), C3 would rise to 30% of total VFA at the expense of C2. When soluble sugars (saccharose, lactose; for instance molasses) are added, C4 proportion can go up to 20% of total VFA at the expense of C2. Some other VFA such as C5, C6, *iso*-C4 or *iso*-C5 come from the rumen microbial deamination and decarboxylation of valine, leucine or *iso*-leucine (Annison, 1954). Rumen

VFA are mostly (70 to 90%; Bergman, 1990) absorbed throughout the rumen wall into the plasma. Butyric acid is converted to beta-hydroxybutyrate in the rumen wall, and together with C2 and C3, these VFA are absorbed into the blood and used, but not only, by the mammary gland as precursors of the *de novo* synthesis of fatty acids (Cuvelier et al., 2005).

Along with the VFA production, the carbohydrates' fermentation leads to the production of **H_2**. Indeed, acetyl CoA, crotonyl-CoA and butyryl CoA synthesize C2 and C4 from pyruvate, which in turn comes from simple sugar (Figure 6), and these production pathways lead to production of two molecules of H_2. In contrast, acrylate pathway (N°7; Figure 6) leads to the production of C3 from pyruvate and consumes of H_2. From 19% to 33% of H_2 is used in VFA production pathways (Czerkawski, 1986; Mills et al., 2001) and only the C3 and C5 fermentation pathways use H_2, with one H_2 mole required per C3 or C5 mole produced.

High H_2 concentration in the rumen could be toxic and leads to an inhibition of fermentation by blocking oxidation of cofactors involved (Wolin et al., 1997). Thus, as final step, methanogenesis is the main pathway using the H_2 that results from the microbial fermentation (N°1; Figure 6). Archaea methanogens 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). Three methanogenesis pathways have been described according to the final electron acceptor (Pelmont, 2005; Liu and Whitman, 2008):

- Hydrogenotropic: methanogenesis from CO_2 and H_2 : $CO_2 + 4\ H_2 \rightarrow CH_4 + 2\ H_2O$
- Methylotropic: methanogenesis from methanol and by-products
- Acetoclastic: methanogenesis from acetate and by-products

In the rumen, the predominant pathway is the hydrogenotrophic pathway using CO_2 as the carbon source and H_2 as the main electron donor (Hungate, 1967) to form CH_4. In the rumen, CO_2 accounts up to 65% of the total produced gas (Ellis et al., 1991), and is not a limiting substrate for methanogenesis. Therefore, H_2 is a key compound for controlling CH_4 production. Mills et al. (2001) estimated with mechanistic models, that 80% of the H_2 produced end up being used in the methanogenesis process as energy source for growth, but these authors indicated that some other hydrogen sinks were not accounted for in the model. Nonetheless, methanogenesis is using the majority of rumen H_2, and the VFA synthesis (propionate and valerate) would be responsible for 19 to 33% of the H_2 uptake (Czerkawski, 1986; Mills et al., 2001).

Table 1 Most widely used *in vivo* methods to measure enteric CH_4 emissions

Method	Scale	Unit measured	Scope of application
Respiratory Chambers	Individual	CH_4 flow	Research
SF_6 tracer	Individual	CH_4 flow	Research
GreenFeed	Individual	CH_4 flow	Research, Field
Laser Methane Detector, Sniffer	Individual	CH_4 concentration	Research, Field
Micrometeorogical	Herd	CH_4 flow	Research

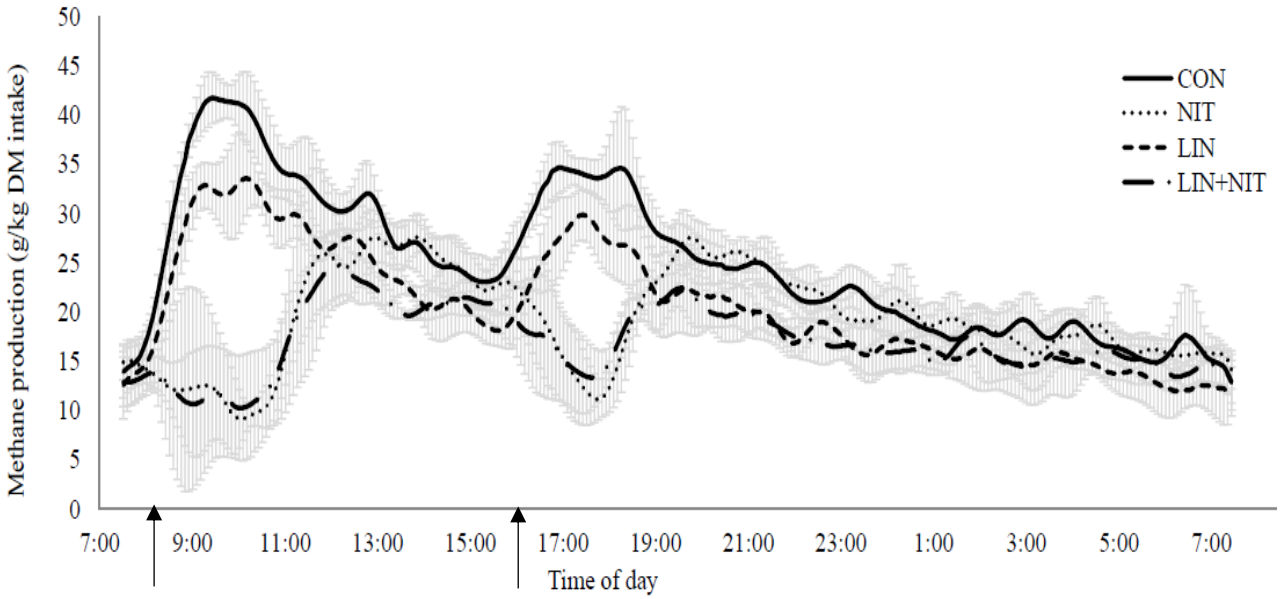

Figure 7 Daily methane production pattern of non-lactating cows fed four different diets containing linseed oil and calcium nitrate alone or in association (n = 4). Treatments consisted of control diet (CON), CON plus 3% calcium nitrate (NIT), CON plus 4% linseed oil (LIN) and CON plus 4% linseed oil and 3% calcium nitrate (LIN+NIT). The arrows indicate time of feeding. Errors bars indicate SD. Adapted from Guyader et al., 2015

1.2. Techniques to quantify enteric CH_4 emissions: advantages and limits

Several *in vivo* measurement techniques of enteric CH_4 emissions have been developed from 1970 to the present (Table 1) to quantify CH_4 emissions at the individual level and at the herd level. However, none of them is perfectly adapted to all kinds of situations in research and on farm. Respiration chambers, SF_6 gas tracer technique, and GreenFeed are the most widely used *in vivo* methods in research to quantify enteric CH_4 emissions for individuals (Table 1), whereas Laser Methane Detector and Sniffer methods measure only CH_4 concentrations in exhaled gases from the animal. The micrometeorogical method is able to quantify enteric CH_4 emissions only at a herd scale. Therefore, the three mostly used *in vivo* methods are described in the following section with their advantages and disadvantages. Details of their principles of use based on a review by (Hammond et al., 2016) can be found and in *Appendix 1***.**

Respiration chambers are regarded as the most reliable method (Gold Standard Method) for measurement of CH_4 emissions from ruminants because all the eructed gases are measured. They also allow observing patterns of CH_4 production throughout the day and offer the possibility to explore the mechanism of action of feeding strategies (Figure 7). This method presents several other advantages, such as the stability of the instruments, the measurements in kinetics, the possibility to use all kinds of ruminants, but also some limitations such as movement restriction of the animal, which affects their normal behavior (Hammond et al., 2016). Some studies report that animals are stressed out when they are confined in the respiration chambers, which might represent a drawback of the system, and could influence dry matter (**DM**) and water intake, and consequently milk and CH_4 production might be affected (Storm et al., 2012). Additionally, there is a need to have a dedicated building for the respiration chambers with preferably controllable conditions. In addition, this method cannot be applied to free ranging animals. It is also a very expensive technique, especially when using a large number of animals for long periods (Hammond et al., 2016).

SF_6 tracer gas method is an indirect measurement technique to quantify CH_4 emissions via a gas tracer, which is used with free ranging animals. It could also be used with all kinds of ruminants fed a wide range of diets (e.g. level of feeding, lipid supplementation, different additives, grazing). As compared to respiration chamber, this method has lower cost and allows a larger number of animals to be breath-tested in a single experiment (McNaughton et al., 2005). However, still a limited number of animals can be equipped at a time (up to 15 at INRA). This

technique is labor-intensive, requires lab equipment for gases analysis (CH_4, SF_6) as well as high technical skills to prepare and calibrate the device.

GreenFeed (C-lock Inc., USA) system requires limited human intervention and is able to measure CH_4 emissions on a large number of cows (n = 20 to 25) in a loose housing barn simultaneously over a long-term period (several months) and in "normal" management conditions. However, measurements are not continuous and it requires at least 20 to 40 spot-measurements per day in order to provide reliable CH_4 measurements, and this system is not suitable for animals fed 100% of forage since a minimum amount of concentrate (2 kg) should be provided to the animals for a visit in the automatic feeder. The system is practical and mobile and can be used in experimental or commercial farms.

Other techniques. Novel approaches such as laser CH_4 detector (Chagunda, 2013), or estimation of CH_4 concentrations based on air spot sampling from eructation during milking (Garnsworthy et al., 2012) are new techniques tested in research. Further information on their principle of utilization are available in the review by (Hammond et al., 2016).

2. Milk fatty acid secretion

For the past decades, important interests have been directed toward milk fatty acid (**FA**) content because of potential links with human health. Milk FA (*for more detail on the nomenclature of milk FA, see Appendix 2*) profiles show significant variability and milk FA composition can be optimized for human health, especially through cow feeding strategies.

2.1. Lipid metabolism in dairy cows

Lipid metabolism is divided into two steps in the rumen. First, lipids are hydrolyzed producing free FA and a glycerol molecule that is rapidly fermented in the rumen onto VFA (essentially C3). Then, free unsaturated FA undergo the RBH.

2.1.1. Lipolysis

Lipids from concentrates (triglyceride, **TG**) and forages (phospholipids and galactolipids) are hydrolyzed by the microbial lipases in the rumen. These enzymes produced by the rumen bacteria are different according to their substrates. Hydrolysis of TG is done by the lipase produced by *Anaerovibrio lipolytica*, while phospholipids and galactolipids are hydrolyzed by lipases from *Butyrivibrio*. Complete lipolysis leads to free FA production, such as saturated and unsaturated FA (Ferlay et al., 2017).

2.1.2. Rumen biohydrogenation of unsaturated fatty acids

Substrates for biohydrogenation are the UFA, which are toxic for rumen bacteria. There is no consensus on the mechanism explaining the toxicity of UFA on rumen bacteria. Keweloh and Heipieper (1996) reported that the double bonds alter the shape of the molecule, such that kinked UFA disrupt the lipid bilayer structure of bacteria. Maia et al., (2010) explained an alternative possibility of the UFA toxicity. It could be that the ready diffusion of the free UFA across the membrane causes chemiosmotic difficulties, perhaps uncoupling the proton-motive force, dissipating the membrane potential by facilitating ion leakage or decoupling intramembrane pathways.

Unsaturated FA are biohydrogenated in the rumen to produce SFA, such as C18:0, the end-product, which is less toxic for the bacteria. In ruminants, the most important dietary UFA are *cis*-9 C18:1, C18:2n-6 and C18:3n-3. The RBH is characterized by successive isomerisations and changes in the FA shapes (e.g. *cis* and *trans* double bonds location), and then by the saturation of the double bonds. It has been shown that bacteria implicated in the RBH are specific to the FA nature. Two groups of bacteria have been identified by Kemp and Lander (1984) and Harfoot and Hazlewood (1997), and are involved in the RBH, while protozoa seem to be of minor importance. Group A bacteria (*Butyrivibrio fibrisolvens*) are able to hydrogenate C18:2n-6 and C18:3n-3 to *trans*-11 C18:1, being the major intermediate product. Group B bacteria (*Fusocillus)* utilize this *trans*-11 C18:1 as a substrate for C18:0, the end-product. However, Maia et al. (2007) have shown that the last step of the RBH is due to *Butyrivibrio hungatei* and *Butyrivibrio proteoclasticus* instead of group B bacteria.

The Figure 8 represents the major RBH pathways of *cis*-9 C18:1, C18:2n-6, and C18:3n-3, in normal rumen conditions. The initial step of RBH is the isomerisation of *cis*-12 double bond to *trans*-11 double bond by linoleate isomerase enzyme (EC 5.2.1.5), as first described by Kepler and Tove (1967). This enzyme is involved in the formation of double bonds from the *cis*-9, *cis*-12 double bond structure of C18:2n-6 and C18:3n-3. This step results in the production of *cis*-9, *trans*-11 CLA and *cis*-9, *trans*-11, *cis*-15 C18:3 from C18:2n-6 and C18:3n-3, respectively. The following step in RBH of C18:2n-6 is the saturation of *cis*-9 and then *cis*-15 double bonds yielding in *trans*-11 C18:1. The final step is the saturation of the *trans*-11 double bond from *trans*-11 C18:1 to C18:0. The *trans*-11 C18:1 could be accumulated in the rumen and be further absorbed since the final step of the linoleic acid RBH is limited (Griinari and Bauman, 1999). Aforementioned RBH intermediates arise from the major RBH pathways, but there are a multitude of other RBH intermediates produced in the rumen.

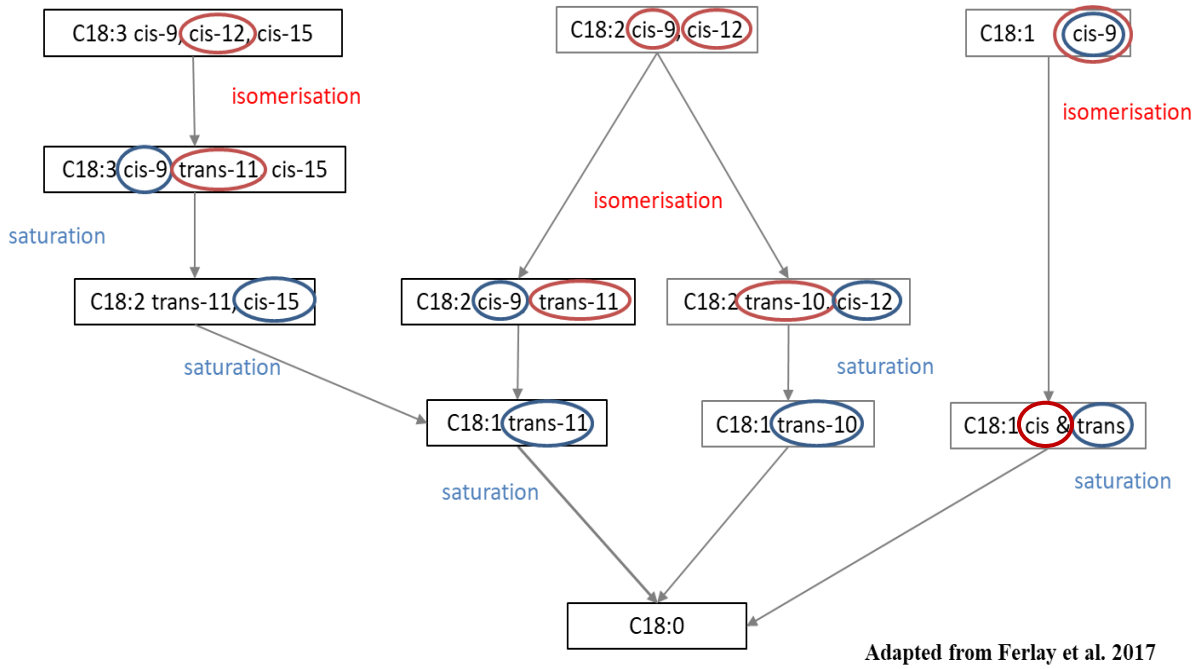

Figure 8 Rumen biohydrogenation pathways of linolenic (*cis*-9,*cis*-12,*cis*-15 C18:3), linoleic (*cis*-9,*cis*-12 C18:2), and oleic (*cis*-9 C18:1) acids and their intermediate fatty acids. In the red and blue circles are represented the isomerization and saturation, respectively.

2.1.3. Mammary fatty acid synthesis and secretion in the milk

Sixty percents (on molar basis) of the milk FA secreted are coming from *de novo* synthesis, while 40% are coming from direct plasma uptake (origins are diet, RBH, or body reserves mobilization). The milk FA composition depends on plasma uptake, *de novo* synthesis, and desaturation (Chilliard et al., 2007).

Plasma fatty acid uptake. Milk long-chain FA originate mainly from dietary lipid absorption from the digestive tract (with the dietary FA undergoing total or partial RBH) and from body reserves mobilization (especially at the beginning of lactation with negative energy balance). Commonly, mobilization of body fat reserves accounts for less than 10% of milk FA, with this proportion increasing when ruminants were in negative energy balance (Bauman and Griinari, 2001).

Mammary *de novo* synthesis. Rumen major VFA (C2, C3, and C4) are taken up from the blood stream by the mammary gland, as well as β-hydroxybutyrate (**BHB**). FA are imported from plasma, where they are either released by the enzyme lipoprotein lipase (**LPL**) (Barber et al., 1997) from TG circulating in chylomicra or Very Low Density Lipoprotein (**VLDL**) or derived from the plasma non-esterified fatty acids (**NEFA**) that circulate bound to albumin (Bernard et al., 2008). This plasma FA come from feedstuffs and/or body reserves mobilization.

From the C2 and BHB, which comes from the plasma C4, and lead to 15% of the *de novo* synthesized FA in the milk, the mammary gland synthesized 40% of the milk FA (on molar basis). Two enzymes are involved in the *de novo* FA synthesis: acetyl-CoA carboxylase (**ACC**) and FA synthase (**FAS**). Both C2 and BHB have their active forms in the mammary epithelial cells, acetyl-CoA and butyryl-CoA, respectively, and are the precursors of the *de novo* synthesis. The enzyme ACC is first activating acetyl-CoA into malonyl-CoA. The FA are then synthesized by repetitive condensations of 2-carbon units derived from malonyl-CoA, upon reaching a carbon chain length of 14 to 16 carbons. Growing FA is released by the cleaving action of a thioesterase enzyme leading to the production of short- and medium-chain saturated FA. The *de novo* synthesis leads to the total production of SFA from C4:0 to C12:0, 95% of the C14:0 and 50% of the C16:0 in the milk fat (Bernard et al., 2008). The inhibitory effect of FA against the *de novo* synthesis is increased as the FA chain is longer, polyunsaturated and contained the *trans*-10 bond (Chilliard et al., 2000; Bauman and Griinari, 2003). Shingfield et al. (2010) reported an inhibitory effect of *trans*-10 C18:1, *trans*-10,*cis*-12, *trans*-9,*cis*-11 and *cis*-10,*trans*-12 CLA on *de novo* synthesis of FA. These FA have an inhibitory effect on ACC enzyme activity that decreases the proportions of *de novo* synthesized FA (8 to 14 carbon) (Chilliard et al., 2000). Furthermore, the mechanisms involved in this inhibition relate to a reduction in the genes expression of several enzymes involved in milk FA synthesis, such as FAS, acetyl-CoA carboxylase, lipoprotein lipase, or Δ9-desaturase (Bauman et al., 2011).

Desaturation. Some of the medium-chain (C10:0, C12:0, C14:0, C16:0) or long-chain FA (C18:0, *trans*-11 C18:1, C20:0 up to C24:0) can be desaturated on the 9th carbon, by the enzyme Δ9-desaturase present in the endoplasmic reticulum of mammary epithelial cell (Palmquist et al., 2005). The enzyme activity depends on the carbon-chain length of the FA (Shingfield et al., 2010) in order to lower the fusion point of the milk fat. Stearic acid is the preferred substrate for the Δ9-desaturase (Bernard et al., 2008) with 49 to 60% of the C18:0 being desaturated to *cis*-9 C18:1in the mammary gland, which represent 60% of the *cis*-9 C18:1 secreted in milk. Furthermore, 90% of the milk *cis*-9 C14:1, 50 to 56% of the milk *cis*-9 C16:1 and more than 60% of the milk *cis*-9,*trans*-11 CLA come from the desaturation of C14:0, C16:0 and *trans*-11 C18:1, respectively (Ferlay et al., 2017). This desaturation is the principal source of *cis*-9 *trans*-11 CLA in milk (Mosley et al., 2006). Polyunsaturated FA, such as C18:2n-6, C20:4n-6, and C20:5n-3 and the *trans*-10,*cis*-12 CLA, have an inhibitory effect on Δ9-desaturase activity (Ntambi and Miyazaki, 2004; Bernard et al., 2008).

Furthermore, endogenous chain elongation of propionyl-CoA as precursor leads to the formation of C5:0, C7:0, C9:0, and C11:0 in milk and these add up to the odd-chain FA C13:0, C15:0 and C17:0 transferred from the duodenum (Fievez et al., 2012). These odd-chain FA can further be desaturated by Δ9-desaturase, but only the conversion of C17:0 to *cis*-9 C17:1 seems quantitatively important as reported by Fievez et al. (2012). These authors also suggested that C15:0 and C17:0 could be synthesized in the mammary gland.

Free FA are esterified in the reticulum of the mammary gland cells, thanks to three specific enzymes (acyl-transferase). Free FA are successively added on a molecule of glycerol-3-phosphate to obtain a TG. New formed TG are transferred into fat globule before being secreted in milk via exocytose.

2.2. General milk fatty acid composition and variations according to nutritional factors

Triglycerides represent on average 98% of milk fat, of which around 95% is FA and more than 400 FA have been identified in milk (Jensen, 2002). Among milk FA, even SFA represent a majority with 69% of total milk FA, ranging from 47 to 78%. Milk C14:0, 16:0 and C18:0 represent 12.0 and 10% of total milk FA, respectively, followed by 29% of MUFA with 19% of *cis*-9 C18:1, and only 3% of PUFA with, notably, 1.3% of C18:2 n-6 and 0.5% of C18:3 n-3. Milk *trans* FA represent 4% of total milk FA with 1.5% of *trans*-11 C18:1, and 0.5% of *cis*-9,*trans*-11 CLA (Ferlay et al., 2008). Milk is composed by 5% of OBCFA (Jensen, 2002; Ferlay et al., 2008; Shingfield et al., 2008). The ruminant diet is an important determinant of milk FA profile. Indeed, changes in feeding practices, with higher proportions of concentrates and corn silages in diets and less grazing (Elgersma et al., 2006), decrease concentrations of MUFA (*cis*-9 C18:1 and *trans*-11 C18:1) and PUFA – (n-3 and *cis*-9,*trans*-11 CLA) and increase concentrations of C12:0, C14:0 and C16:0, when compared with TMR fed (Chilliard et al., 2007). It has been proven that grazing cows have increased milk content of UFA when compared to silage-based diets (Elgersma et al., 2003). Additionally, it has been reported that milk fat from grazing cows had lower C14:0 and C16:0 and higher *cis*-9 C18:1, *trans*-11 C18:1, *cis*-9,*trans*-11 CLA and C18:3n-3 contents in comparison to milk from cows fed preserved forages (hay or silage; Dewhurst et al., 2006; Ferlay et al., 2006, 2008). Feeding oilseed-supplemented diets largely increased PUFA and decreased SFA contents in milk fat (Chilliard and Ferlay, 2004; Glasser et al., 2008). Glasser et al. (2008) carried out a meta-analysis on the effects of the four major dietary oilseed supplements and their form on milk FA composition. They reported that feeding linseed, rapeseed, sunflower, or soybean, whatever the form,

consistently led to an increase in C18 FA content at the expense of SMCFA, and especially C6:0 to C16:0. Milk *trans* C18:1, total CLA and *cis*-9,*trans*-11 CLA contents were also increased by all oilseed supplements, apart from rapeseed when given as seeds or protected or oils (Glasser et al., 2008). Linseed or grazed grass at earlier vegetative stage had more of an effect on milk C18:3n-3 content than other lipid supplement because of their richness in C18:3 content (Ferlay et al., 2013).

2.3. Analytical methods for milk fatty acid determination

Lipids are first extracted and isolated from the other milk components by several methods, most commonly based on the use of organic solvents (Christie, 1993). A mixture of chloroform and methanol (2:1, v:v) is used to extract the lipids fraction from the milk, followed by a washing step with a salt solution (Folch et al., 1957). The gas chromatography (**GC**) technique has revolutionized the study of lipids by allowing a complete FA composition determination in a relatively short time (Christie, 1993). The FA from fat fraction are first converted to methyl esters [*See Appendix 3 for detailed information on fatty acid methyl ester (FAME) preparation*], in order to derivate FA on volatile compounds as described above. The GC with flame ionisation detector is the most widely used method for FA analysis (Juanéda et al., 2007). Flexible fused-silica capillary columns coated with highly polar cyanosilicone stationary phases are required for determining the *cis/trans* FA composition of lipids (Juaneda et al., 2007); with long-length columns (100 and 120 m) recognized to perform better than shorter ones (50 and 60 m). There are other chromatographic techniques, notably high-performance liquid chromatography (**HPLC**), where alternative derivatives, such as those with UV chromophores, are used and show better performances (*for details information on this technique, see Appendix 3).*

The GC analysis is the reference method to quantify the milk FA concentrations but it requires high expertise, and is expensive and time-consuming. Therefore, researchers have developed alternative techniques such as the mid-infrared (**MIR**) spectroscopy, which has the advantages of having very high throughput (up to 500 samples/h; FOSS, 2005), being easy to use, or the near-infrared reflectance (**NIR**) spectroscopy. These 2 methods are non-destructive, rapid, cheap and multiparametric. These infrared methods are alternative techniques to the GC method used for quantification of milk FA (Andueza et al., 2013; Ferrand-Calmels et al., 2014). The infrared spectrum is caused by the absorption of electromagnetic radiations at frequencies that are correlated to the vibrations of specific chemical bonds within a molecule (Coates, 2006). The spectrum therefore illustrates these absorptions at different wavenumbers (cm^{-1}) for a

specific chemical composition (Smith, 2011). The MIR spectroscopy (400 to 4,000 cm^{-1}) is particularly interesting because it is very highly sensitive to the chemical environment, as the fundamental absorptions of molecular vibrations occur in this region (Belton, 1997), and is already implemented in laboratories of Milk Recording Organisation to quantify major milk components used for milk payment. MIR spectroscopy technique can be used to estimate various milk FA based on calibration equations. In the past decades, it has been successfully used to determine the FA composition of oils, butters and margarines (Safar et al., 1994) and to predict the *cis* and *trans* content of fats and oils (van de Voort et al., 1995). More recently, MIR spectroscopy has been successfully used to estimate C12:0, C14:0, C16:0, *cis*-9 C16:1, *cis*-9 C18:1 and SFA and MUFA in cow milk (Soyeurt et al., 2006; Ferrand-Calmels et al., 2014). NIR spectrometry has been successfully used to quantify FA concentrations in foods such as meat products (González-Martín et al., 2005; Pla et al., 2007) or cheese (Lucas et al., 2008). Coppa et al. (2010) and Andueza et al. (2013) have shown that NIR spectrometry can be used to satisfactorily predict milk FA from dairy cows and goats, such as sums (SFA, MUFA, PUFA, total trans FA, total *trans* C18:1 and total *cis* C18:1, total CLA) and some individual milk FA present with medium-to-high concentrations (C4:0 to C18:0, *cis*-9 C18:1, *trans*-11 C18:1 and *cis*-9,*trans*-11 CLA; Coppa et al., 2010). It can also accurately predict milk sums from goat (SFA, MUFA, UFA, total *trans* FA) and *cis*-9,*trans*-11 of CLA, *cis*9-, *trans*-10, and *trans*-11 C18:1 (Andueza et al., 2013). The quality of prediction decreased when FA were present in low to very-low concentrations.

3. Feeding strategies known to reduce CH_4 emissions and potential effects on milk fatty acid composition

3.1. Dietary CH_4 mitigation strategies

Many comprehensive reviews on enteric CH_4 mitigation strategies have been published from the past 15 years (Harris and Kolver, 2001; Boadi et al., 2004; Kebreab et al., 2006; Grainger et al., 2007; Ellis et al., 2008; Martin et al., 2010; Eckard et al., 2010; Cottle et al., 2011; Doreau et al., 2011; Goel and Makkar, 2012; for a full list see Hristov et al., 2013). In this section, firstly we first focused on feeding strategies known to decrease CH_4 emissions, which are the diet composition manipulation and the lipid supplementation, with some information on feed additives addition in dairy cows. Secondly, the effects of these feeding strategies on milk FA profile are reviewed. To finish, the potential links between CH_4 and milk FA are discussed.

Carbohydrate types. Several feeding strategies have been studied in the past decades in order to reduce CH_4 production in dairy cows such as increasing dietary starch level, concentrate proportion or forage nature (Joblin, 1999) stated that the management of H_2 production in the rumen is the most important factor in controlling CH_4 production in ruminants. It has been reported that the nature and rate of fermentation of carbohydrates influence the proportions of individual VFA formed in the rumen and thus the CH_4 emissions because of the varying amounts of H_2 produced or used in the digestive processes. Several studies showed that concentrate rich in cereals, which are rich in starch, lowers CH_4 emissions more than concentrate rich in structural carbohydrates, which are rich in fiber (Moe and Tyrrell, 1979). However, very few direct comparison between different carbohydrate types has been studied on methanogenesis so far. In their review, (Martin et al., 2010) reported that increasing levels of concentrate in the diet or replacing dietary structural carbohydrates from forages (cellulose, hemicellulose) with non-structural carbohydrates (starch and sugars) from energy-rich concentrates reduced CH_4 emissions in dairy cows. Other experiments with lactating dairy cows and beef cattle have shown linear decreases in CH_4 emissions with an increase in the proportion of concentrate (Aguerre et al., 2011; Mc Geough et al., 2010). Nature of forage effect on CH_4 emissions have also been studied, and (Dewhurst, 2013) showed that lower fiber content and higher passage rates of forage legumes appeared to decrease CH_4 production compared with grasses.

Dietary lipid supplementation has also been studied for their potential effect in reducing CH_4 production in dairy cows and there is an extensive number of evidence that lipid supplementation mitigate CH_4 production (Hristov et al., 2013). Meta-analyses by Moate et al. (2011) and Grainger and Beauchemin (2011) reported consistent results with decrease in CH_4 yield (g/kg of DMI) by dietary lipid supplementation (the dietary EE content varies from 12 to 114 g/kg DM). Eugène et al. (2008) have also shown, in a meta-analysis gathering data from 25 published studies, that lipid supplementation reduced CH_4 emission (g/kg of DMI) by 9% in dairy cows, but this result was a direct effect of the dietary lipid on CH_4 production, mostly due to a reduction in DMI (-6%) by lipids added. Dietary lipids, and especially medium-chain FA are known to affect methanogens number (Machmüller et al., 2003) and long-chain FA, such as C18:3n-3, contribute to CH_4 decrease through a toxic effect on cellulolytic bacteria (Nagaraja et al., 1997) and protozoa (Doreau and Ferlay, 1995). Furthermore, long-chain lipids are not fermented in the rumen, unlike other feed constituents such as forages and cereals, decreasing the fermented organic matter part of the diet and leading to a decrease in CH_4, but only when

lipids replace carbohydrates. A greater inhibitory effect of UFA vs. SFA on rumen microbial activity has been reported by Palmquist and Jenkins (1980) and Nagaraja et al. (1997). Unsaturated FA are undergoing the RBH, which can also serve as a hydrogen sink, but it has been suggested that only 1 to 2% of the hydrogen produced in the rumen is used (Czerkawski and Clapperton, 1984; Jenkins et al., 2008) for RBH of dietary UFA. Furthermore, there is no consensus on the greater effect of UFA when contrasted results have been obtained from published studies (Beauchemin et al., 2007; Sauvant et al., 2011), although a greater mitigating effect of PUFA was reported by Doreau et al. (2011).

Feed additives have been widely studied for their impact on microbial methanogenic community and subsequent CH_4 emissions in dairy cows, such as plant extracts (condensed tannins, saponins, and essential oils), probiotics (yeast), ionophore antibiotics (monensin), organic acids or electron receptors (fumarate, nitrates, sulfates), and inhibitors chemical compound (bromochloromethane, 3-NOP). Their depressing effects on CH_4 production, which are not consistent among studies, are due to their action on different metabolic pathways (Hristov et al., 2013). For instance, tannin-containing plants have shown *in vitro* direct effect on ruminal methanogens (antimethanogenic activity) and indirect effect on H_2 production due to lower feed degradation. Saponins have shown anti-protozoal effects (reviewed by Newbold and Rode, 2006), while Martin et al. (2010) have reported antimicrobial proprieties of molecules present in essential oils, which affect rumen fermentation. The effect of ionophores (monensin especially) on methanogenesis is linked to their effect on VFA production towards propionogenesis via their inhibitory effect on gram-positive over gram-negative bacteria that reduce succinate to propionate (McGuffey et al., 2001). Russell (1987) also reported an inhibitory effect of monensin on protozoa-generating hydrogen in the rumen, which lead to lower CH_4 emissions. Organic acids (malate, fumarate and acrylate) are converted by rumen bacteria in succinate and then propionate, thus up taking H_2 (Doreau et al., 2011). Some CH_4 inhibitors or electron receptor molecules, such as bromochloromethane or nitrate, respectively, are effective feed additives to reduce CH_4 production in dairy cows, but they cannot be recommended because of their ozone-depleting effect or nitrite toxicity (Hristov et al., 2013).

3.2. Methanogenesis and links with milk fatty acids

Methanogenesis is the main pathway to expel H_2 produced during microbial fermentation process in the rumen (Moss et al., 2000). Decrease in CH_4 emissions with various feeding strategies is partly explained because of changes in total VFA production with decrease of C2

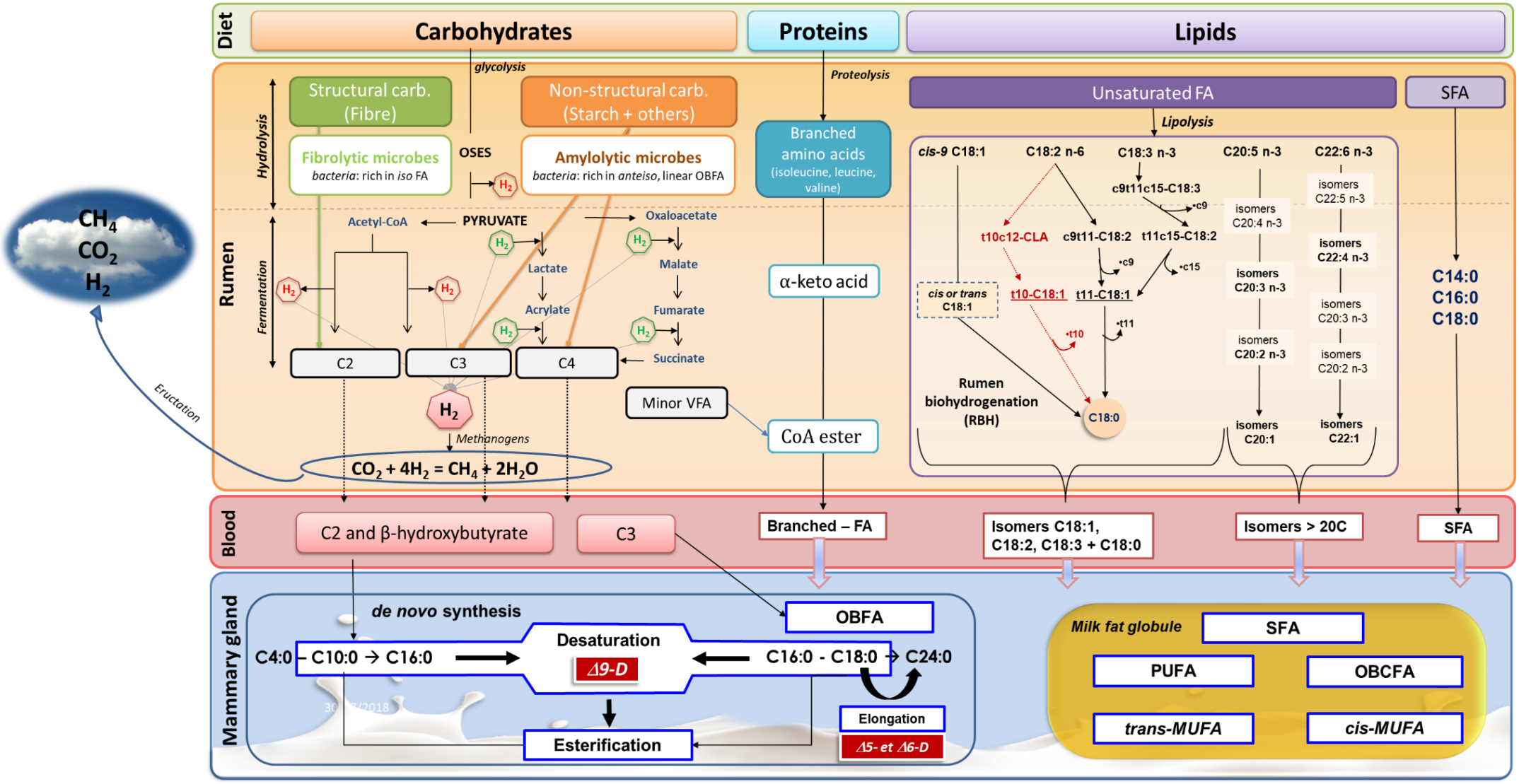

Figure 9 Relationships between diet composition, rumen methanogenesis and FA metabolism in the rumen and mammary gland. C2: acetate; C3:propionate; C4: butyrate; FA: fatty acid; MUFA: monounsaturated FA; OBFA: odd- branched chain FA; SFA: saturated FA.

towards increase in C3 (increasing starch-fermenting microbes) resulting in reduction in H_2 production. Reduction in rumen pH, which is known to inhibit cellulolytic bacteria activity and methanogens growth, reduces activity of these microorganisms and in turn decreases CH_4 emissions. Because feeding strategies have direct effects on rumen fermentation, subsequently on VFA production and methanogenesis, they also have direct effects on lipid metabolism in the rumen. Thus, relationships could be expected among CH_4, RBH pathways and precursors of *de novo* synthesized FA and thus milk FA concentrations (Figure 9). In fact, the *de novo* FA are synthesized in mammary gland from VFA precursors absorbed in the blood stream from the rumen, mostly acetate (85% of *de novo* synthesized FA) but also β-hydroxybutyrate (10 to 15%). Therefore, variations in VFA production modify the *de novo* mammary FA synthesis. In addition, OBCFA might also be related to CH_4 emissions because of their microbial origin (Figure 9). Indeed, OBCFA originate from the outer membrane of fibrolytic and amylolytic bacteria (Vlaeminck et al., 2006), which are linked to the diet

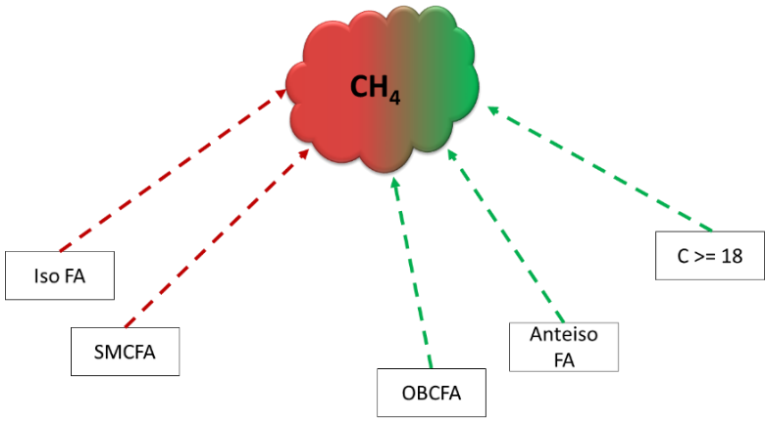

Figure 10 Potential links among milk fatty acids and methane emissions (Red dashed line=positive links; green dashed lines=negative links). FA: fatty acids; OBCFA: odd and branched chain fatty acids; SMCFA: small and medium chain fatty acid

composition and rumen digestion processes, as well as from the *de novo* mammary synthesis of C15:0 and C17:0 with propionate as substrate (Fievez et al., 2012).The milk unsaturated FA originate either from the dietary UFA or from desaturation in the mammary gland. Thus, UFA are indirectly associated with enteric CH_4 emissions (Grainger and Beauchemin, 2011) because their RBH is modulated by the nature of the diet. Relationships are also expected between CH_4 emissions and long-chain UFA, which are arising from the RBH (van Lingen et al., 2014). High starch intake in dairy cows is known to reduce CH_4 emissions because of more H_2 up taken by the amylolitic bacteria for the production of C3, and a limited methanogens' activity due to low pH. In addition, according to Kalač and Samkovà (2010), this dietary strategy is also associated

with more *de novo* mammary FA synthesis resulting in greater milk SFA concentration. Positive relationships are expected between CH_4 emissions and milk SFA (Figure 10). Furthermore, Vlaeminck et al. (2006) and Cabrita et al. (2007) reported a positive relationship between odd-chain FA (C15:0 and C17:0) and dietary starch content. Thus, modifying starch intake in dairy cows by increasing concentrate percentage or directly starch content in the diet could have a direct decreasing effect on rumen pH values and thus could modify the RBH conditions and the production of RBH intermediates (Chilliard et al., 2007; Shingfield et al., 2010) as well as on rumen VFA profile and thus on milk *de novo* synthesized FA.

Several review studies (Chilliard and Ferlay, 2004; Dewhurst et al., 2006; Chilliard et al., 2007; Glasser et al., 2008; Shingfield et al., 2008; Ferlay et al., 2017) have reported effects of dietary lipid supplementation on milk FA composition. Dietary lipids are not digested in the rumen, thus offering less substrate for methanogenesis as opposed to the carbohydrate fermentation. In addition, lipids have an inhibitory effect on bacteria and protozoa, which are known to produce great amount of H_2 (Grainger and Beauchemin, 2011; Guyader et al., 2014). Lipid effects on CH_4 depend on the dietary lipid supplementation level and nature, the form of lipid supplement (oil, seed heat-treated or not) and the interaction with the other dietary ingredients in the diet (especially dietary starch content). Dietary lipids have also a direct impact on milk FA composition. For instance, dietary supplementation with lipids rich in *cis*-9 C18:1 (e.g. rapeseed), or C18:2n-6 (e.g. sunflower and soybean), or C18:3n-3 (e.g. linseed) decrease milk SFA content. Linseed supplementation lead to greater milk C18:3n-3, while corn silage-based diets supplemented with sunflower lead to greater *cis*-9,*trans*-11 CLA and C18:2n-6 (Chilliard et al., 2007). Lipids rich in C18:2n-6 and C18:3n-3 further reduce the OBCFA (Vlaeminck et al., 2006). Conversely, the milk C18:0 is usually increased with these lipid supplements because it is the end-product of RBH. Milk *cis*-9 C18:1 content is also increased because of greater dietary content of this FA, which also depends on the dietary basal forage and on the Δ9-desaturase activity. Preferred substrate of Δ9-desaturase is C18:0, leading to the synthesis of ***cis***-9 18:1, which is the major unsaturated FA found in milk (Ferlay et al., 2017). Potential positive and negative links among milk FA families and CH_4 emissions arising from hypothetical common metabolic pathways described previously are summarized in Figure 10.

Table 2 Published equations to estimate CH_4 emissions (non exhaustive list)

References	Animals (*n*)	CH_4 techniques (*n*)	Forage (%)	Equation
Moe and Tyrrell, 1979	Dairy cows (*n* = 404)	Chambers (*n* = 404)	NA	CH_4 (Mcal/d) = 0.439 + 0.273 SR + 0.512 Hem + 1.393 Cel
Blaxter and Clapperton, 1965	Sheep and cattle[2]	Chambers (20 studies)	NA	CH_4 (kcal/100 kcal feed) = 1.30 + 0.112 D - L (2.37 -0.05D)
Mills et al. (2003)	Dairy cows (*n* = 159)	Chambers (*n* = 159)	55	CH_4 (g/day) = (5.93 + 0.92 × DMI) × Z
Ellis et al., 2007	Dairy (*n* = 89)	Chambers (*n* = 57) SF6 (*n* = 5) Others (*n* = 27)	70	CH_4 (g/day) = (3.23 + 0.809 × DMI) × Z
IPCC, 2007	NA	NA	NA	CH_4= [Intake (MJ/d) × Ym × (365 days/yr)] / [55.65 MJ/kg of CH_4]
Sauvant et al., 2011	Dairy, Beef cattle, Sheep, goat[2]	Chambers (*n* = 976)	60	CH_4 (g/day) = (7.14 + 0.22 × DOM) / DMI
Ramin and Huhtanen, 2013	Dairy (*n* =145) and beef (*n* = 62) cattle	Chambers (*n* = 207)	70	CH_4 (g/day) = (20 + 35.8 × DMI − 0.50 × DMI^2) × 0.71427
Sauvant and Nozière, 2013	Dairy and beef cattle, sheep, goat[2]	Chambers (*n* = 450)	60	CH_4 (g/day) = (45.42 – 6.66 × (DMI:BW) + 0.75 × $(DMI:BW)^2$ + 19.65 × PC – 35.0 × PC^2 - 2.69 × (DMI:BW) × PC) × DOMI
Moraes et al., 2014	Dairy cows (*n* = 1,111)	Chambers (*n* = 1,111)	NA	CH_4 (MJ/day)= -9.311 + 0.042 × gross energy intake + 0.094 × NDF - 0.381 × EE + 0.008 × BW + 1.621 × milk fat
Niu et al., 2018	Dairy cattle (*n* = 2,566)	Chambers, SF6, GreenFeed	NA	CH_4 = -60.5 +12.4 ×DMI -8.78 × EE +2.10 × NDF +16.1 × milk fat + 0.148×BW CH_4 = 15.4 - 0.291 × EE + 0.144 × NDF - 0.104 × ECM + 1.34 × milk fat -1.12 × milk protein + 0.00330 × BW

n: number of treatments; NA: non-available; SR = digestible soluble residue (kg/d), calculated by subtracting crude protein and ether extract from the neutral-detergent solubles; Hem = digestible hemicelluloses (kg/d); Cel = digestible cellulose (kg/d) ; D = digestibility of energy at the maintenance level of feeding (%); L = level of feeding as a multiple of the maintenance level; DMI (kg/day) = dry matter intake;

[1] Z = conversion factor between CH_4 expressed in MJ/day to CH_4 expressed in g/day = 20.0638; DOM (% of DM) = digestible OM in diet = OM content of the diet (% of DM) × OM digestibility (0-1); PC = concentrate proportion (0-1); DOMI = digestible OM intake (kg/day) = DOM × DMI

[2] Proportions not available

4. Existing empirical models to predict CH_4 emissions

4.1. Mathematical models to estimate CH_4 emissions

Governments are nowadays using the IPCC Tier II (IPCC, 2007) to make inventories of CH_4 emissions (kg/year). The IPCC prediction equation is based on the daily gross energy intake [(**GEI**); in MJ/day] and a CH_4 conversion rate according to the species of ruminants and physiological categories:

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

Where *Ym* (%) is the CH_4 conversion rate expressed as a fraction of the GEI (i.e., the fractional loss of GEI as combustible CH_4 in %), for example :

- Feedlot receiving 90% or more concentrate: 3.0 ± 1.0%
- Dairy cows and their youngs: 6.5 ± 1.0%
- Other cattle and buffalos fed low-quality crop residues: 6.5 ± 1.0%
- Other grazing cattle and buffalos: 6.5 ± 1.0%

Other mathematical models have been developed in the past decades (Table 2) because of a lack of easy, practical and cheap CH_4 measurement methods to use on a large scale. These models are either empirical models based on the nutrient intake or mechanistic models estimating CH_4 emissions according to detailed rumen fermentation processes that have been modeled (e.g. rumen feed degradation and formation of VFA). Empirical models are for instance the linear equations developed by Moe and Tyrrell (1979) or Blaxter and Clapperton (1965), based on intake or digestibility of certain nutrients, respectively. Nonlinear empirical models have also been developed by Mills et al. (2003) or Ellis et al. (2007), based on nutrient intake and dietary composition, such as DMI, metabolizable energy intake, dietary NDF or non-fat carbohydrates content. Moraes et al. (2014) and Appuhamy et al. (2016) confirmed the strong relationships between feed intake (either DM or GE intakes) and CH_4 production by developing equations or assessing developed equations from extensive datasets, respectively. These authors have also shown that dietary NDF and EE contents improved the prediction.

Overall, predictions from these broadly applicable models were poor (based on RMSPE) as reported in Hristov et al. (2018). According to Moraes et al. (2014), the poor predictive ability of current models can be due in part to the relatively small datasets used for model parameterization and the modeling techniques. In addition, models based on dietary contents, digestibility and/or intakes are convenient tools to estimate CH_4 emissions but they need to have precise measurements of animal feed consumption, diet composition and digestibility. In

addition, these models might not be applicable when CH_4 mitigating feed additives (e.g. monensin, 3NOP) are added to the diet since DMI or the diet chemical composition are not influenced.

4.2. Prediction equations based on proxies

Several techniques have been developed in the past decade to measure CH_4 emissions from dairy cows in experimental conditions, with varying degrees of accuracy as shown in the previous section, but routine individual measurements on a large scale have shown limits and are difficult and expensive to obtain (Pickering et al., 2015; Negussie et al., 2017). Therefore, identifying proxies (i.e., indicators or indirect traits), which have the potential to predict CH_4 emissions with relatively low costs, and good accuracy on a large scale is a challenge. Mathematical models have been developed based on proxies, to overcome the disadvantages of reference methods. Up to now, no single proxy was found to accurately predict CH_4, and combinations of 2 or more proxies are likely to be a better solution (Negussie et al., 2017). Indeed, combining proxies can increase the accuracy of predictions by 15 to 35% (Negussie et al., 2017), mainly because different proxies describe independent sources of variation in CH_4 and one proxy can correct for shortcomings in the other(s).

In the recent years, scientists have developed proxies approach targeting milk biomarkers, such as urea, acetone or FA, because milk is an easy-to-take/handle sample that can be routinely analyzed by infrared methods. Milk FA gained interest as milk fat contains a large variety of FA originating from several metabolic pathways: rumen (lipolysis and RBH) and mammary gland (uptake from plasma, *de novo* synthesis, Δ9-desaturation) (*See previous section 3.2*). Some authors have shown that dietary strategies have an effect on both milk FA and CH_4 emissions (Sauer et al., 1998; Odongo et al., 2007). Therefore, Chilliard et al. (2009), who developed prediction equation of CH_4 emissions based on milk FA, have first evidenced a relationship between CH_4 and milk FA and several different authors have then developed other prediction equations (van Gastelen and Dijkstra, 2016). Currently, models to predict CH_4 emissions based on milk FA only are available from 7 studies, with milk FA

Table 3 Description of the study design and treatments from the models developed in the literature (milk FA identified by GC)

References	Obs	CH_4 technique	Design	n cow	DIM (days)	Treatments	
Weill et al., 2009	NA	SF6 & RC	Randomised block + LS	NA	NA	CON	Linseed supplementation (*n* = 74)
Chilliard et al., 2009	32	SF6	LS 4x4	8	213	CON (60% of CS) vs.	(1) whole crude linseed; (2) extruded linseed; (3) Linseed oil;
Mohammed et al. 2011	32	RC	LS 4x4	16	96	CON (including Ca salt palm oil; 45% of BS) vs.	(1) sunflower seeds; (2) linseed; (3) rapeseed
Rico et al. 2016	81	RC	LS 3x3	27	96	No CON (60% of forage)	(1) 100% CS, (2) 100% AS, (3) 100% BS, (4) 100% timothy silage, (5) 50:50 of CS and AS, (6) 50:50 of BS and CS, (7) 50:50 of timothy silage and AS
Dijkstra et al. 2011	50	RC	Randomised block + LS	100	176 to 216	CON (53 to 76% of GS or CS) vs.	(1) extruded LS; (2) caprylic+caproïc acids; (3) yucca ; (4) diallyldisulfide; (5) calcium fumarate
van Lingen et al. 2014	146	RC	Randomised block + LS	146	176 to 216	CON (45 to 80% of GS or CS) vs.	(1) extruded linseed; (2) milled rapeseed; (3) palm oil; (4) caprylic and capric acids; (5) coconut oil; (6) glycerol; (7) naked oat; (8) forage:concentrate ratio; (9) CS:GS ratio; (10) yucca; (11) diallyldisulfide; (12) calcium fumarate; (13) DHA (%)
Williams et al. 2014	278	SF6 & Calorimetric chamber	Randomised block + LS	246	57 to 245	CON (70% of AS or pasture) vs.	(1) fat + tannin; (2) tannins (%); (3) grape marc (dried, ensiled); (4) corn concentrate (%); (5) wheat concentrate (%); (6) almond hulls; (7) citrus pulp; (8) red grape marc; (9) white grape marc
van Gastelen et al. 2017	29	RC	Randomised block	32	192	No CON (80% of forage)	(1) 100% GS; (2) 67% GS and 33% CS; (3) 33% GS and 67% CS; (4) 100% CS

RC=Respiratory chamber; SF6= Sulfur hexafluoride gar tracer; LS= Latin square design; CON= control treatment; GS= Grass silage; CS= corn silage; BS= barley silage; AS= Alfalfa silage.

contents measured thanks to gas chromatography: Chilliard et al. (2009), Dijkstra et al. (2011), Mohammed et al. (2011), van Lingen et al. (2014), Williams et al. (2014), Rico et al. (2016) and van Gastelen et al. (2017). Another equation was developed by Weill et al. (2009) and has obtained a patent from EU – ONU. All prediction equations had R^2 ranging from 47 to 95%, and the milk FA included in the models differed considerably (*See following section 4.3*). These differences might be linked to the different units for CH_4 emissions used (g/d or g/kg of DMI), different measurement techniques used (respiration chambers, or SF_6), or the different feeding strategies reported in the studies and thus the applicability domain (*See following section 4.4*).

4.3. Existing CH_4 emissions prediction equations based on milk fatty acid concentrations

The Table 3 summarizes the studies that have investigated the predictive possibility of milk FA composition for CH_4 emissions based on different type of dietary strategies, and developed models to predict CH_4 emissions.

In the study from **Chilliard et al. (2009),** cows received 4 dietary treatments (Table 3), which consisted of a corn silage-based diet and the same diet supplemented with 3 different forms of linseed (whole crude or extruded seeds and oil). Predictive equations (Table 4) included individual milk FA concentrations (*cis*-9 C14:1, C16:0, *trans*-16+*cis*-14 C18:1, and C18:2n-6) and forage intake. The forage intake estimates the part of the organic matter fermented in the rumen that are used in the acetate-CH_4 pathway. The milk C16:0 is partly *de novo* synthesized FA, as explained before, positive relationships are expected between SFA and CH_4. Moreover, *trans*-16+*cis*-14 C18:1 is an intermediate of C18:2n-6 RBH, and C18:2n-6 is the main dietary FA present in corn silage. The R^2 value slightly decreased compared to their first equation (0.953 to 0.931). Chilliard et al. (2009) concluded that the predictive equations established in their study are valid only for corn silage-based diets supplemented with lipids from linseed (rich in C18:3n-3).

Mohammed et al. (2011) also studied the relationships among CH_4 emissions and milk FA concentrations using a 4 x 4 Latin square with 16 lactating Holstein cows fed either a diet with calcium salts of palm oil, or diets supplemented with sunflower seed, or linseed or rapeseed (Table 3). Authors reported negative relationships between CH_4 and *cis*-9 C17:1, *cis*-11 C18:1 and sum of *trans* C18:1 but positive relationships with *trans, trans* CLA and *anteiso* C15:0. Milk C17:0 (microbial origin) is formed from rumen propionate and it is well known that propionogenesis is negatively related to CH_4 production (Fievez et al., 2012) because of

Table 4 Description of the models developed in the literature and relationships among individual CH_4 emissions and milk FA concentrations (Pearson correlation coefficients)

References	Statistics	Equations (R^2)
Chilliard et al., 2009	General Linear Model	CH_4 (g/d) = 9.46 × C16:0 – 97.6 × *trans*-16+*cis*-14 C18:1 + 13.3 × forage intake (kg of DM/d) – 78.3 × *cis*-9 C14:1 + 77.4 × 18:2 n-6 – 21.2 (R^2 = 0.95)
Mohammed et al., 2011	Mixed-effect models: random (cow and period), fixes (treatments) + stepwise regression	CH_4 (g/d)= 272.4 – 486.2 × *cis*-9 C17:1 – 122.7 × *cis*-11 C18:1 + 2.22 × *trans*-CLA – 11.76 × ∑*trans*-C18:1 + 260.1 × *anteiso* C15:0 (R^2 = 0.74)
Rico et al., 2015	Mixed-effect models: random (cow and period), fixes (treatments) + stepwise regression	CH_4 (g/d) = 669.1 + 838.7 × *cis*-11 C14:1 – 493.2 × *cis*-9 C17:1 – 44.2 × *cis*-11 C18:1 - 963.7 × *trans*-8, *cis*-13 C18:2 (R^2 = 0.84)
Dijkstra et al., 2011	Mixed-effect models: random (experiments), fixes (variables) + stepwise regression	CH_4 (g/kg DMI) = 24.6 + 8.74 × *anteiso*-C17:0 − 1.97 × *trans*-10+11 C18:1 − 9.09 × *cis*-11 C18:1 + 5.07 × *cis*-13 C18:1 (R^2 = 0.73)
van Lingen et al., 2014	Mixed-effect models: random (experiments), fixes (variables) + stepwise regression	CH_4 (g/kg DMI) = 23.39 + 9.74 × *iso*-C16:0 − 1.06 × *trans*-10+11 C18:1 − 1.75 × C18:2 n-6 (R^2 = 0.58) CH_4 (g/kg FPCM) = 21.13 – 1.38 × C4:0 + 8.53 × C16:0-*iso* – 0.22 × *cis*-9 C18:1 – 0.59 × *trans*-10+11 C18:1 (R^2 = 0.47)
Williams et al., 2014	General Linear Model	CH_4 (g/d) = 539 + 50.8 × C8:0 – 5.26 × ∑C18 (R^2 = 0.37)
van Gastelen et al., 2017	General Linear Model + stepwise regression	CH_4 (g/d) = 211.2 + 50.4 × C4:0 + 77.7 × *cis*-9 C14:1 - 82.0 × *trans*-11 C18:1 (R^2 = 0.63) CH_4 (g/kg DMI) = 27.2 – 7.0 × *cis*-9,*trans*-11 C18:2 (R^2 = 0.54) CH_4 (g/kg $FPCM^{-1}$) = 16.5 + 24.6 × *iso*-C15:0 – 15.5 × C17:0 + 52.4 × C22:0 (R^2 = 0.47)

[2]CH_4 emissions reported with the same unit as the one used in the model

[3]FPCM (kg/day) = [0.337+0.116 × fat (g/100 g milk)+ 0.06 × protein (g/100 g milk)] × milk yield (kg/day) (CVB, 2012).

consumption of H_2. Milk *cis*-9 C17:1 is produced in the mammary gland from the Δ9-desaturase of C17:0, thus explaining the negative relationship between *cis*-9 C17:1 and CH_4. The other included FA (*cis*-11 C18:1, sum of *trans*-C18:1 and *trans*, *trans* CLA) in the equation are RBH intermediates of PUFA.

Rico et al. (2016) developed prediction equation based on 81 observations from 3 Latin Square-design experiments using 27 cows. The milk *cis*-9 C17:1 and *cis*-11 C18:1 were also negatively associated with CH_4 production (g/d) as shown by Mohammed et al. (2011). Rico et al. (2016) highlighted positive and negative associations of *cis*-11 C14:1 and *trans*-8,*cis*-13 C18:2, respectively, with CH_4 production, which had never been described before. The diet consisted of 40% of concentrate and 60% of forages (timothy, alfalfa, barley or corn silages; Table 3). These types of diets have not extensively been studied before and could explain the link between these two milk FA and CH_4 production found only in this study.

Dijkstra et al. (2011) developed multivariate models using data from several experiments reporting 50 observations from 3 experiments. Diverse dietary treatments were tested (Table 3). These authors reported positive relationships among *anteiso* C17:0, *cis*-13 C18:1 and CH_4 yield (g/kg DMI, equation 4; Table 4), and strong negative relations with certain *trans* C18:1 FA (e.g. C18:1 *trans*-10 or sum of C18:1 *trans*-10+*trans*-11) and *cis*-11 C18:1, but these relationships were not observed for CH_4 intensity (g/kg FPCM). Relationship with *anteiso* C17:0 was attributed to the negative and positive correlations with dietary crude protein (**CP**) and fiber contents, respectively (Cabrita et al., 2003). The degradation of proteins is associated with lower CH_4 production (Bannink et al., 2008), whereas fiber fermentation increases CH_4 emissions. Consequently, a higher milk *anteiso* C17:0 concentration could be linked to higher CH_4 emissions. The milk *trans*-10 C18:1 is provided from another pathway of RBH of C18:2n-6 when diets are rich in starch or/and supplemented with UFA (Griinari and Bauman, 1999). The negative relationship among RBH intermediates, such as *trans*-10 C18:1 and *cis*-11 C18:1, and CH_4 emissions could be due to high dietary starch content or corn silage-based diets (Bougouin et al., 2018) or diets supplemented with dietary PUFA (Chilliard et al., 2007).

van Lingen et al. (2014) presented the most extensive study with the greatest number of studies and observations in comparison to previous work. Their meta-analysis aimed at exploring the potential of milk FA as indicators for CH_4 emissions. The experiments covered a wide variety of diets (Table 3). Equation from these authors included *iso* C16:0 (positive predictor), *trans*-10+*trans*-11 C18:1 and C18:2n-6 (negative predictors). The positive relationships between branched FA and CH_4 emissions have been reported in several other studies (Mohammed et al.,

2011 for CH_4 production in g/d; Dijkstra et al., 2011 for CH_4 yield in g/kg DMI; van Gastelen et al., 2017 for CH_4 intensity in g/kg FPCM). Indeed, outer membrane of fibrolytic bacteria is rich in branched-chain FA, and more specifically in *iso* FA (Vlaeminck et al., 2006), and these bacteria are in great number with fiber-rich diets (Nozière et al., 1996), which are known to be linked to higher CH_4 emissions. Vlaeminck et al. (2006) also reported increasing odd-*iso* FA content in milk from cows fed increasing proportions of forage. Van Lingen et al. (2014) concluded that milk FA have moderate potential to predict CH_4 emissions, because the predictive power (e.g. R^2) of the best CH_4 predictive equation was 0.47 (Table 4) for CH_4 intensity (g/kg FPCM) and 0.54 for CH_4 yield (g/kg DMI; Table 4), while with other published prediction equations, predictive power ranged from 0.73 to 0.95 (Table 4). Because these prediction equations were constructed from 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 accurate and realistic.

Williams et al. (2014) studied the relationships among milk FA and CH_4 production with cows fed pasture or alfalfa silage-based diets and several CH_4 mitigation strategies (Table 3). With the atypical diets used in the study, Williams et al. (2014) were the only authors showing that C8:0 was positively associated with CH_4 production, while the sum of milk C18 FA was negatively related to it. Milk C8:0 originate from the *de novo* synthesis, and as explained above, SFA are positively related to CH_4 because. However, the equation had poor R^2 as compared to the previously quoted ones (Table 3; $R^2 = 0.37$).

van Gastelen et al. (2017) recently published sets of equations using milk FA and CH_4 emissions expressed in different units (g/day; d/kg of DMI; and g/kg of FPCM). These equations were developed from an experiment using 32 multiparous Holstein dairy cows fed either grass silage or corn silage with a forage to concentrate ratio of 80:20 (% of DM basis). These authors reported relationships among C4:0 and *cis*-9 C14:1 (positive), *trans*-11 C18:1 (negative) and CH_4 emissions in g/day as well as negative relationship between *cis*-9,*trans*-11 CLA and CH_4 yield (g/kg of DMI). The model performance were poorer than in the other published equations (Table 4) with an adjusted R^2 varying between 0.47 and 0.63 for CH_4 expressed in g/kg of FPCM and g/kg of DMI, respectively (Table 4).

Weill et al. (2009) also developed a predictive equation for CH_4 emissions that was developed based on milk FA, determined by mid-infrared (**MIR**) spectrometry, and observations from commercial farms. In the equation, the sum of FA with less than 16 carbons (FA ≤ C16) was positively related to CH_4 intensity (g/kg of milk). Milk FA ≤ C16 are *de novo* synthesized FA

in the mammary gland from acetate and β-hydroxybutyrate (Bauman and Griinari, 2003). It is well known that acetate is positively related to enteric CH_4 production, thus milk *de novo* synthesized FA seem to be good predictor.

4.4. Units, range of diets and domain of applicability of the existing predictive equations

For the past decades, CH_4 emissions have been reported in g/day, g/kg DMI also called CH_4 yield, or in g/kg of milk (or ECM or FPCM) also called CH_4 intensity. Government inventories have been using CH_4 production (in g per year per animal; IPCC Tier II, 2007) to quantify the emissions regarding the type of animals. Nowadays, there is no consensus on which unit of CH_4 emissions must be used when evaluating mitigation potential of feeding strategies in dairy cows (Negussie et al., 2017). Methane yield (expressed in g/kg of DMI) has been used in order to exclude DMI effect since DMI is one of the main driver of CH_4 production (Dijkstra et al., 2011). However, DMI is difficult to measure "on farm" and its estimation presents uncertainty, which induces to lower accuracy in CH_4 prediction equations (Bannink et al., 2011). Additionally, DMI could remain steady, while nutritional value of feedstuffs can decrease and modify the dairy performance. Thus, another unit could be used to catch the effects linked to values and characteristics of gross energy intake by dairy cows: CH_4 intensity in g/kg of milk. In a context of global food supply and efficient use of resources, it is important to consider CH_4 yield and intensity. Furthermore, several authors have evaluated the applicability of their developed predictive equations within their study. For instance, Mohammed et al. (2011) observed for dairy cows fed barley silage-based diets supplemented with calcium-salt of palm oil, flaxseed, sunflower seed, or canola seed, an over-prediction of CH_4 emissions (g/d) of 12 to 41% and 48 to 79% with the equations 1 and 2 from Chilliard et al. (2009), respectively. They also reported over-prediction for CH_4 yield (from 2 to 35%) when using equations from Dijkstra et al. (2011). These over-predictions were attributed to the lack of correlations in their study among CH_4 and variables used by Chilliard et al. (2009) or Dijkstra et al. (2011). Williams et al. (2014) combined their data with those reported by Chilliard et al. (2009) in order to developed new predictive equations. They concluded that relationships developed by Chilliard et al. (2009) were not applicable with forage-based diets with various supplements and that the applicability of the equations depended on the diets for which they were developed.

Overall scientific strategy of the PhD

Table 5 Steps and associated experiments conducted during the PhD thesis and justification for selection dietary treatments

Approach	Associated experiment/step	Animal type Number Experimental design	Dietary treatments	Collaboration
in vivo	Step 1, *in vivo* experiment	Lactating Holstein dairy cows (n = 4) Latin square 2 x 2 factorial design	1/ Starch-rich diet 2/ Starch-rich diet + bicarbonate 3/ Fiber-rich diet 4/ Fiber-rich diet + bicarbonate	INRA – UMRH Consortium Methane partners*
in vivo	Step 2, *in vivo* experiment	Lactating Holstein dairy cows (n = 4) Latin square design 4 x 4	1/ Starch-rich 2/ Ca-salt of palm oil 3/ Rapeseed 4/ Sunflower	INRA – UMRH Consortium Methane partners*
in silico	Step 3, model development and evaluation	Lactating Holstein dairy cows 825 individual data (individual database)	cf Chapter IV	INRA – UMRH Consortium Methane partners* International university and research centers
in silico	Step 4, literature model comparison	Lactating Holstein dairy cows 85 mean data (literature database)	cf Chapter IV	INRA – UMRH Consortium Methane patners* International universities

*(Text box 1)

The primary objective of this PhD thesis is to explore by meta-analysis the potential of milk FA as proxy of CH_4 emissions in dairy cows. The research hypothesis is that milk FA have the potential to accurately predict CH_4 emissions whatever the diets fed to the animals.

The literature review showed that multiple dietary strategies have been tested worldwide in order to reduce methanogenesis in dairy cows. This review also highlighted some lacks of information, especially regarding direct comparison between different dietary carbohydrate types (fiber *versus* starch) more particularly with grass silage-based diets, or lipid supplement types (rapeseed and sunflower) with corn silage-based diets. In addition, the created databases in this PhD thesis pointed out the lack of data with these dietary strategies, which are widely used on farm. Therefore, understanding their effects on CH_4 emissions is needed. Furthermore, several researchers have demonstrated potential links among individual milk FA concentrations and CH_4 emissions by developing predicting models, but based on different predictors and units of CH_4 and using narrow range of diets. Moreover, it appears that the developed prediction equations showed good performance when applied within their domain of applicability.

The originality of our experimental *in vivo* approach consisted of studying the effects of nutritional strategies that have not been explored so far on both CH_4 emissions and milk FA composition. These data will be added to a database reporting individual CH_4 emissions and milk FA concentrations data from dairy cows and collected from national (private companies, publics institutes) and international collaborators (institutes and universities). This representative dataset from several countries was then used to develop a set of linear models to predict CH_4 emissions from milk FA concentrations. In addition, another dataset based on published literature data was constructed in order to be used as an external validation dataset for the developed models in this PhD thesis.

The scientific strategy of this PhD thesis was thus based on two complementary *in vivo* and *in silico* approaches (Table 5), from which the two objectives were:

***In vivo* approach: Gather individual data on CH_4 emissions and milk FA concentrations from dairy cows fed diets that have been poorly studied so far. These diets have to mitigate CH_4 emissions and to modulate milk FA composition. To do so, two different *in vivo* experiments were conducted with dairy cows in order to study:**

Step 1. Effects of carbohydrate type (starch *versus* fiber) or bicarbonate addition to grass-silage based diets on enteric methane emissions and milk FA composition in dairy cows.

Step 2. Effects of energy type (starch vs different source of lipid) in corn-silage based diets on methanogenesis and milk FA composition in dairy cows.

In silico **approach: Build two datasets based on mean data from the literature and individual data from the project partners in order to develop prediction equations of CH_4 emissions based on milk FA. To do so, experiments were chosen according to availability of the following criteria: 1) CH_4 emissions measured using one of the 3 techniques most used, i.e. the respiration chambers, the SF_6 tracer gas, or the GreenFeed, 2) milk FA profile analyzed by gas chromatography, 3) dietary composition, 4) daily dry matter intake (DMI), milk production and composition , and 5) cow characteristics such as body weight (BW) and days in milk (DIM).**

Step 3. Construction of the 2 databases for development and validation of prediction equations of enteric CH_4 emissions based on milk FA in dairy cows fed a wide range of diets.

Step 4. Comparison of the performance of the developed prediction equations with the models published in the literature.

II. CHAPTER II

Effects of carbohydrate type or bicarbonate addition to grass silage-based diets on enteric methane emissions and milk fatty acid composition in dairy cows

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Effects of carbohydrate type or bicarbonate addition to grass silage-based diets on enteric methane emissions and milk fatty acid composition in dairy cows

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ABSTRACT

The aim of the study was to compare the effect of fiber- or starch-rich diets based on grass silage, supplemented or not with bicarbonate, on CH_4 emissions and milk fatty acid (FA) profile in dairy cows. The experiment was conducted as a 4 × 4 Latin square design with a 2 × 2 factorial arrangement: carbohydrate type [starch- or fiber-rich diets with dietary starch level of 23.1 and 5.9% on a dry matter basis, respectively], without or with bicarbonate addition [0 and 1% of the dry matter intake, respectively]. Four multiparous lactating Holstein cows were fed 4 diets with 42% grass silage, 8% hay, and 50% concentrate in 4 consecutive 4-wk periods: (1) starch-rich diet, (2) starch-rich diet with bicarbonate, (3) fiber-rich diet, and (4) fiber-rich diet with bicarbonate. Intake and milk production were measured daily and milk composition was measured weekly; CH_4 emission and total-tract digestibility were measured simultaneously (5 d, wk 4) when animals were in open-circuit respiration chambers. Sensors continuously monitored rumen pH (3 d, wk 4), and fermentation parameters were analyzed from rumen fluid samples taken before feeding (1 d, wk 3). Cows fed starch-rich diets had less CH_4 emissions (on average, −18% in g/d; −15% in g/kg of dry matter intake; −19% in g/kg of milk) compared with fiber-rich diets. Carbohydrate type did not affect digestion of nutrients, except starch, which increased with starch-rich diets. The decrease in rumen protozoa number (−36%) and the shift in rumen fermentation toward propionate at the expense of butyrate for cows fed the starch-rich diets may be the main factor in reducing CH_4 emissions. Milk of cows fed starch-rich diets had lower concentrations in *trans*-11 C18:1, sum of *cis*-C18, *cis*-9,*trans*-11 conjugated linoleic acid (CLA), and sum of CLA, along with greater concentration of some minor isomers of CLA and saturated FA in comparison to the fiber-rich diet. Bicarbonate addition did not influence CH_4 emissions or nutrient digestibility regardless of the carbohydrate type in the diet. Rumen pH increased with bicarbonate addition, whereas other rumen parameters and milk FA composition were almost comparable between diets. Feeding dairy cows a starch-rich diet based on grass silage helps to limit the negative environmental effect of ruminants, but does not lead to greater milk nutritional value because milk saturated FA content is increased.
Key words: bicarbonate, carbohydrate type, dairy cow, methane emission, milk fatty acid

INTRODUCTION

Livestock farming is a great contributor to total greenhouse gas emissions via CH_4 production by cattle. Dairy cow CH_4 emissions account for 46% of the total greenhouse gas emissions in dairy supply chains, when expressed as CO_2-equivalents (Gerber et al., 2013), and also lead to significant energy losses ranging between 2 to 12% of the gross energy (**GE**) intake by animals (Johnson and Johnson, 1995). Livestock competitive- ness needs to reduce enteric CH_4 emissions without altering animal performance to improve feed efficiency and to reduce the carbon footprint of the dairy cattle sector.

Among the different dietary strategies tested worldwide, increasing the proportion of concentrate to above 30 to 40% in the diet is known to mitigate CH_4 emissions in ruminants (Martin et al., 2010). Limited information is available on the effect of carbohydrate type in the diet on methanogenesis, though Hindrichsen et al. (2005) studied the effects of concentrates (50% of the diet) providing different carbohydrates type on enteric CH_4 emissions in dairy cows. These authors reported similar CH_4 emissions (g/d and g/kg of DMI) with fiber-rich concentrate (containing soybean hulls) as compared with starch-rich concentrate (containing wheat).

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Moe and Tyrrell (1979) reported that CH_4 production in dairy cows is reduced further as the carbohydrate digestion rate is high. In addition, diets rich in readily fermentable carbohydrates such as starch are known to modify the rumen environment greatly through a decrease in pH and, consequently, to increase the risk of SARA (Krause and Oetzel, 2006). To limit these rumen disorders associated with high-starch diets, fiber can replace starch in carbohydrate diets because it is fermented more slowly, or sodium bicarbonate can be added to diets as a digestive regulator to reduce the risk of SARA (Solorzano et al., 1989). To the best of our knowledge, the effect of buffer addition to the diet of dairy cows on methanogenesis has been studied by very few authors. Hellwing et al. (2012) reported no effect of bicarbonate addition (9.5 g/kg of DM) to a grass-clover silage-based diet rich in molasses on CH_4 emissions (g/d and g/kg of DMI) in dairy cows when compared with a diet rich in wheat.

In addition to CH_4 mitigation, high-starch diets may decrease milk fat content and modify milk fatty acid (**FA**) composition in dairy cows (Cabrita et al., 2007; Shingfield et al., 2008). Such diets may influence rumen biohydrogenation (**RBH**) of PUFA (Bauman and Griinari, 2003), resulting in a shift from the *trans*-11 C18:1 to the *trans*-10 C18:1 pathway. High-starch diets also modify the activity or number of bacteria implicated in the synthesis of odd- and branched-chain FA (Vlaeminck et al., 2006; Pirondini et al., 2015). Nevertheless, to the best of our knowledge, no authors have studied the effect of carbohydrate type on milk FA composition in dairy cows fed grass silage-based diets.

The aim with the study was to test the effects of (1) the carbohydrate type in diets [fiber-rich diets (**F**) or starch-rich diets (**S**)], (2) the addition of bicarbonate to diets, and (3) the interaction between the carbohydrate type and bicarbonate addition on digestive process, more particularly on CH_4 emissions, and on milk FA composition in lactating dairy cows fed grass silage-based diets.

MATERIALS AND METHODS

The experiment was conducted at the animal experimental facilities of INRA Theix (Saint-Genès-Champanelle, France) from February to June 2015. Procedures involving animals were performed in accordance with the French Ministry of Agriculture guidelines for animal research and the applicable European Union guidelines and regulations on animal experiments. The Auvergne Regional Ethics Committee on Animal Experimentation C2EA-02 approved the experiment with the reference number 821–2015060811534198.

Cows, Diets, and Experimental Design

Four multiparous lactating Holstein cows (mean ± SD, average BW of 639 ± 62 kg, DIM of 61 ± 12.5, lactation rank of 2.8 ± 0.4, and milk yield of 31.5 ± 4.6 kg/d at the start of the experiment) were used in the experiment which was conducted as a 4 × 4 Latin square design with a 2 × 2 factorial arrangement. Each experimental period lasted 4 wk (28 d). From d 1 to 20, cows were housed together in a free-stall barn and received the experimental ad libitum concentrates and forages. From d 21 to 26, cows were moved to individual open-circuit respiration chambers for measurement of CH_4 emissions and total-tract digestibility, and were fed 95% of individual voluntary feed intake (determined during d 1 to 20) to ensure complete consumption of the feed. Changes from one diet to another were achieved with 6-d transition at the beginning of each 28-d period. From d 27 to 28, cows returned to the freestall barn and were fed the same diet than from d 1 to 26.

The 4 dietary treatments aimed at evaluating the main effects of the type of carbohydrates (fiber vs. starch), addition of bicarbonate, and their interaction and were (1) high-fiber diet (F), (2) high-fiber diet with bicarbonate addition (**F+b**), (3) high-starch diet (S), and (4) high-starch diet with bicarbonate addition (**S+b**). Diets contained a 50:50 forage-to-concentrate ratio, on a DM basis, 45% grass silage (natural grass- land, first cut), 5% hay (natural grassland, first cut), and 50% pelleted concentrates and were formulated to meet individual energy and protein requirements for lactation and maintenance (INRA, 2007). In the F+b and S+b diets, bicarbonate was weighed and mixed every day with the concentrate and given all together with the grass silage at the level of 1% of the DMI. The chemical composition of the different dietary ingredients and diets are reported in Table 1. Diets were iso-energy and iso-protein and were adjusted daily to maintain the forage-to-concentrate ratio as close as possible to the 50:50 ratio targeted. Hay was given once daily (0730 h); the grass silage and concentrates were mixed together by hand as a partial mixed ration (**PMR**) and were given twice a day (66% at 0900 h and 34% at 1600 h). Cows had free access to water throughout the experiment.

Measurements, Sampling, and Chemical Analyses

Feed Intake and Composition. Feed intake was weighted and recorded on 4 d in wk 1, 2, and 3 and on 5 d in wk 4 of each experimental period to estimate DMI as the difference between DM offered and refused. The DM content of feed was determined (103°C for 24 h)

Table 1. Chemical composition of the dietary ingredients and diets

		Ingredient			Diet[2]	
Item[1]	Grass silage[3]	Hay	Fiber-rich concentrate[4]	Starch-rich concentrate[5]	F	S
OM	890	924	919	944	906	918
CP	140	73	153	158	141	144
NDF	395	636	404	160	419	297
ADF	232	365	223	55	238	154
Starch	—	—	117	462	58.5	231
Ether extract	21.0	15.3	36.3	36.5	28.0	28.5
Gross energy, MJ/kg of DM	19.0	17.9	18.2	18.3	18.5	18.6
Fatty acids (FA), mg/g of DM						
C14:0	0.11	0.07	0.03	0.03	0.07	0.07
C16:0	3.11	1.58	5.38	4.88	4.17	3.92
C16:1 *cis*-9	0.04	0.06	0.08	0.06	0.06	0.05
C18:0	0.29	0.16	0.84	0.45	0.56	0.37
C18:1 *cis*-9	0.65	0.34	8.11	6.16	4.37	3.39
C18:1 *cis*-11	0.08	0.06	0.40	0.30	0.23	0.19
C18:2n-6	4.19	1.21	17.41	16.71	0.02	0.02
C18:3n-3	9.51	1.98	1.23	1.04	4.99	4.90
Other[6]	2.65	1.93	0.96	0.76	6.80	6.60

[1]Chemical composition expressed as grams per kilogram of DM unless stated otherwise.

[2]F = fiber-rich diets; S = starch-rich diets.

[3]Fermentation characteristics of fresh silage juice: pH = 4.3; lactic acid = 53.1 g/kg of DM; N-NH_3 = 8.0% of total N.

[4]Composition (% on DM basis) of the fiber-rich concentrate: beet pulp (22.3), soybean hull (22.2), distillers dried corn grains (15.5), wheat bran (10.9), corn grain (7.5), hay (5.4), corn gluten 60 (0.9), molasses (1.5), and vitamins and minerals (3.6).

[5]Composition (% on DM basis) of the starch-rich concentrate: wheat (30.1), corn grain (25.5), wheat middling (9.7), wheat starch (6.8), beet pulp (6.3), corn gluten 60 (4.8), molasses (1.5), and vitamins and minerals (4.2).

[6]Sum of all the other FA analyzed.

on samples (100 g) taken twice a week for grass silage and once a week for hay and concentrates (on wk 1, 2, and 3), and for 5 consecutive days in wk 4. If there were refusals in wk 4, the DM content of each refused feedstuffs was measured (103°C for 24 h). In addition, samples of each feedstuff (100 g) were taken twice in wk 4, pooled to provide 1 sample per period, and stored at 4°C (concentrates, hay) or at −20°C (grass silage). At the end of the experiment, all feedstuff samples were freeze-dried and ground (1-mm screen, ZM 200 Retsch Mill) for chemical composition determination (In Vivo Labs, Chierry, France) including fermentation parameters from fresh grass silage. Organic matter was determined by ashing at 550°C for 6 h (method 942.05; AOAC International, 2005). Total N was analyzed by combustion according to the Dumas method (method 968.06; AOAC International, 2005), and CP content was calculated as N content × 6.25. Fiber (NDF and ADF) was determined by sequential procedures (Van Soest et al., 1991) after pretreatment with amylase, and expressed exclusive of residual ash. Starch was analyzed using an enzymatic method (Faisant et al., 1995). Gross energy was analyzed by isoperibolic calorimetry (model C200, IKA, Staufen, Germany). Ether extract content was determined after acid hydrolysis (method 954.02; AOAC International, 2005). The pH of fresh grass silage juice as well as lactate, VFA, and ammonia-N concentrations were determined as described in Guyader et al. (2016). Fatty acid profile was analyzed according to Sukhija and Palmquist (1988) in all samples for grass silage (1 sample per period) and in a pooled sample for hay and for concentrates for the whole experiment. Total lipids from each diet ingredient were extracted with chloroform:methanol (1:3) along with an internal standard (C23:0, tricosanoic acid). For each ingredient, lipids were methylated with methanolic HCl and each individual FA amount and concentration was determined relative to the response factors for a known amount of the internal standard. Because refusals were negligible (<1%, data not shown), chemical composition of refusals was considered similar to that of the composition of the diet offered.

Digestibility. Total feces and urine collection was performed in individual boxes for 5 consecutive days on wk 4 when cows were housed in the open-circuit respiration chambers. Each morning, after weighing and mixing of feces and urine, one aliquot (1%) was used for DM determination (103°C for 24 h) and another aliquot (0.5%) was pooled per week and per animal before being frozen (−20°C). At the end of the experiment, samples were thawed, freeze-dried, and ground (1-mm screen, ZM 200 Retsch mill) for chemical composition

determination of DM, OM, NDF, ADF, starch, and GE as previously described.

Body Weight, Energy Balance, and Milk Production and Composition. Cows were weighted at the beginning of the experiment and on wk 3 for each period. The energy balance (**EB**) was calculated as the difference between the energy intake and the energy requirement for lactation and maintenance (INRA, 2007). Cows were milked twice daily at 0730 and 1530 h, and milk yield was individually recorded at each milking. Milk samples (30 mL) were collected for each cow on d 22 to 24 (wk 4) before storage at +4°C with Bronopol (2–2-nitropropane-1,3-diol) as a preservative for milk component analysis. Fat, protein, lactose, and urea nitrogen contents were determined using MilkoScan 4000 (Foss Electric A/S, Hillerod, Denmark; Lial, Aurillac, France). Fat- and protein-corrected milk yield was calculated according to Gerber et al. (2011). For milk FA composition, another individual milk sample (3 mL) was taken on d 24 (wk 4) and frozen (−20°C) without preservative after each milking. All samples were freeze-dried and then composited per day based on am and pm milk yields. The milk FA com- position was determined as described by Ferlay et al. (2013). The composition of FAME of CLA isomers in milk fat was analyzed according to Lerch et al. (2012), with some modifications. Briefly, the FAME of CLA isomers were determined using an HPLC system (Agilent, 1200 series) equipped with 3 silver-impregnated silica columns (ChromSpher 5 Lipids, 250 × 4.6 mm, 5-μm particle size; Chromoptic, Courtaboeuf, France) coupled in series. Methyl esters of CLA were separated under isocratic conditions at 22°C using 0.1% (vol/ vol) acetonitrile in n-heptane at a flow rate of 1 mL/ min and monitoring effluent at 233 and 210 nm. The CLA isomers were identified based on retention time comparisons with a mixture of authentic standards (O5632, Sigma-Aldrich, St.-Quentin-Fallavier, France). Concentrations of CLA isomers were calculated from the proportionate peak area responses determined by HPLC and the sum of concentrations of *trans*-7,*cis*-9 CLA, *trans*-8,*cis*-10 CLA, and *cis*-9,*trans*-11 CLA [with the following minor CLA isomers also taken into account in the sum: *trans*-11,*cis*-13 CLA (coeluted with *cis*-9,*cis*-11), *trans*-11,*trans*-13 CLA, *trans*-10,*trans*-12 CLA, *trans*-9,*trans*-11 CLA, and *trans*-8,*trans*-10 CLA] weight percentage determined by GC analysis.

Methane Emissions. Cows were housed in open- circuit respiration chambers (16.6 m^3 each) from d 21 (0730 h) to d 26 (0730 h) in wk 4, during which the total CH_4 emissions of each cow were measured continuously. The design and associated analytical equipment of the open-circuit respiration chambers are detailed in Guyader et al. (2015). Open-circuit respiration chambers operated at a slight negative pressure, with an airflow of 421 ± 12 m^3/h on average (approximately 45 air changes per h). The open-circuit respiration chambers was flushed with ambient air for 3 d before each measurement period.

The front and rear doors were never opened simultaneously to avoid an air stream into the open-circuit respiration chambers. Rear doors were opened twice daily: in the morning for milking and to remove recovery boxes for feces and urine collection, and in the afternoon for milking. When the rear doors were closed, the front doors were opened for morning feeding (0830 h for hay and 0900 h for the PMR) and afternoon feeding (1600 h for the PMR). Missing data were estimated as being similar to the last measurement data before open-circuit respiration chambers disturbance. Methane emissions were calculated as the difference between open-circuit respiration chambers and ambient CH_4 concentrations multiplied by the airflow corrected for environmental parameters (temperature, relative humidity, and pressure) according to Pinares-Patiño et al. (2012).

Plasma Parameters, Rumen pH, and Fermen- tation Parameters. Blood samples were collected from the coccygeal vein using tubes containing EDTA (2.1 mg/mL) after morning milking and before feeding on d 18 (wk 3). Blood samples were kept on ice after sampling and plasma was separated within 1 h, by centrifugation at 1,700 × *g* for 20 min at 4°C, and frozen at −20°C until it was analyzed for nonesterified FA (**NEFA**), BHB, acetate (**C2**), and glucose concentrations. Plasma NEFA, glucose, and acetate concentrations were determined by spectrophotometry using glucose dehydrogenase (Glucose RTU kit; BioMérieux, Lyon, France), acyl-CoA synthetase (Wako NEFA HR2 kit, Oxoid, Dardilly, France), and l-malate dehydrogenasecitrate synthase-acyl-CoA synthetase methods (Enzyplus EZA 811 + kit, Biocontrol Systems, Lyon, France), respectively. The BHB concentration was determined as described by Brashear and Cook (1983).

Rumen pH was monitored continuously using a commercial sensor (eBolus, eCow, Exeter, UK) over 3 d in wk 4 when animals were in open-circuit respiration chambers. At the start of the experiment, 1 calibrated sensor per cow was introduced permanently in the rumen through the esophagus by using a dedicated balling gun. Each sensor was set up to record mean pH over 15 min (96 data points per day) with an accuracy of ±0.1. Data were downloaded every 15 d using an eCow handset (smartphone + antenna) with an eCow Android application.

Rumen fluid samples (500 mL) were collected by stomach tubing (Shen et al., 2012) before the morning feeding on d 18 (wk 3) of each experimental period. Samples were strained through a polyester monofilament fabric (250 μm pore size) and the filtrate was sub- sampled for VFA concentration and protozoa counting. For VFA, 0.8 mL of filtrate was mixed for 2 h at 4°C with 0.5 mL of a 0.5 *M* HCl solution containing 2% (wt/vol) metaphosphoric acid and 0.4% (wt/vol), and then stored at −20°C. The VFA concentration was analyzed by GC with a flame ionization detector (Morgavi et al., 2008). For protozoa counting, 1-mL aliquot of rumen filtrate was mixed with 1 mL of methyl green formalin-saline solution, and stored at room tempera- ture in darkness. Protozoa counts were done by mi- croscopy and categorized as either small (<100 μm) or large (>100 μm) entodiniomorphs, or as holotrichs (*Dasytricha* or *Isotricha*; Williams and Coleman, 1992). Data for protozoa were $\log_{10}$-transformed for statistical analysis.

Statistical Analysis

Data were analyzed using mixed effect models with the lme4 package (version 1.1–10) in R statistical soft- ware (version 0.98.1102, R Foundation for Statistical Computing, Vienna, Austria). Fixed effects of period, carbohydrate type (F and F+b vs. S and S+b), bicarbonate addition (F+b and S+b vs. F and S), and the random effect of cow were tested with the following model:

$$Y_{ijk} = \mu + P_i + A_j + B_k + C_l + (C_l \times B_k) + \varepsilon_{ijkl},$$ where

Y_{ijk} is the dependent variable, μ is the overall mean, P_i is the period (i = 1 to 4), A_j is the animal (j = 1 to 4), B_k is the bicarbonate addition (k = 1 and 2), C_l is the type of carbohydrate (l = 1 and 2), $C_l \times B_k$ is the interaction between carbohydrate type and bicarbonate addition, and ε_{ijkl} is the random residual error.

Data measured for several days in wk 4 (intake, milk production and composition, CH_4 emissions, rumen pH, total-tract digestibility) were averaged before being included in the statistical analyses. In period 1, the cow fed the diet S was taken out of the experiment because of health problems. It was replaced by another cow, fed diet S+b in period 1, and was used for the following 3 periods. In addition, it was not possible to download the pH data for one cow during the experiment because the sensor was not responding.

Least squares means were reported with the pooled standard error of the mean derived from the model. Effects were considered significant at a probability of $P \leq 0.05$.

RESULTS

Total DMI, Milk Production and Composition, and Plasma Metabolites

Total DMI was significantly reduced for the starch- rich compared with the fiber-rich diets (−750 g; $P = 0.02$) because of a reduced intake of grass silage and concentrates (Table 2). The type of carbohydrate had no effect on milk production and composition, as well as on feed efficiency, BW, and EB (Table 2). Plasma NEFA, glucose, and acetate concentrations were not influenced by the carbohydrate type (Table 3). However, plasma BHB concentration was reduced ($P < 0.001$) and urea concentration was greater ($P = 0.003$) for starch-rich diets than for fiber-rich diets, respectively.

Bicarbonate addition did not change total DMI, BW, EB, milk yield, milk content and yields of fat, protein and lactose, milk urea content, and feed efficiency. Bicarbonate addition to both starch- and fiber-rich diets reduced plasma NEFA concentration ($P = 0.03$; Table 3), whereas the other plasma metabolites were unchanged.

Nutrients Intake and Diet Digestibility

Intakes of OM did not differ among diets (Table 4). Due to the experimental design, fiber intake was greater (on average +2.9 and +2.0 kg/d for NDF and ADF, respectively; $P < 0.01$), and starch intake was lesser (on average −3.7 kg/d; $P < 0.01$) for fiber- than for starch-rich diets, respectively. Gross energy intake was also increased in the fiber-rich diet as compared with the starch-rich diets (on average +12.4 MJ/d; $P = 0.03$). Bicarbonate addition had no effect on the different nutrient intakes for the F and S diets.

Total-tract apparent digestibility of DM and OM was significantly greater for the starch-rich than for the fiber-rich diets (on average +5.4 and +5.0%, respectively; $P < 0.001$; Table 4). Fiber (NDF and ADF) digestibility did not differ among diets, whereas starch and GE digestibility was also greater for starch-rich than for fiber-rich diets (on average +2.7 and +3.9%, respectively; $P < 0.01$). Bicarbonate addition did not change total-tract diet digestibility of nutrients for F and S diets.

Methane Emissions

The carbohydrate type affected CH_4 emissions (Table 5). Compared with fiber- rich diets, CH_4 emissions (g/d) decreased by −18% with starch-rich diets ($P = 0.007$), by roughly −15% when expressed per kilogram of DMI, kg of OM intake, or % of GE intake ($P = 0.02$).

Table 2. Feed intake, milk yield, and composition in dairy cows fed diets containing concentrates rich in fiber or starch, with or without bicarbonate addition

	Diet[2]					*P*-value[3]		
Item[1]	F	F+b	S	S+b	SEM	C	b	C × b
Feed intake, kg of DM/d								
Grass silage + concentrate	21.5	21.9	20.9	21.0	1.57	0.02	0.31	0.55
Hay	1.51	1.50	1.49	1.50	0.007	0.23	0.96	0.79
Total DMI	23.0	23.4	22.4	22.4	1.57	0.02	0.09	0.53
Forage:concentrate ratio	1.04	1.07	1.06	1.04	0.271	0.91	0.83	0.29
Milk production, kg/d								
Milk yield	29.6	30.4	29.6	29.5	3.34	0.22	0.57	0.24
FPCM[4]	30.2	29.8	29.1	30.7	2.60	0.94	0.72	0.50
Milk composition, %								
Fat	4.41	4.34	4.08	4.55	0.322	0.72	0.27	0.16
Protein	3.13	3.25	3.29	3.27	0.191	0.15	0.24	0.12
Lactose	5.28	5.30	5.22	5.19	0.059	0.10	0.26	0.54
Milk urea, mg/L	185	164	181	176	10.7	0.71	0.12	0.24
Milk yield, kg/d								
Fat yield	1.28	1.32	1.20	1.29	0.113	0.410	0.30	0.66
Protein yield	0.88	0.97	0.95	0.96	0.076	0.36	0.21	0.27
Lactose yield	1.56	1.61	1.56	1.54	0.182	0.48	0.66	0.42
Feed efficiency[5]	1.29	1.32	1.31	1.37	0.042	0.41	0.26	0.61
Energy balance,[6] kJ/d	−5.14	−0.46	−1.06	0.97	4.570	0.50	0.41	0.74
BW, kg	645	657	663	648	40.2	0.58	0.86	0.13

[1]Means of measurements on wk 4: 5 d for feed intake and milk production and 3 d for milk composition.

[2]F = diet rich in fiber; S = diet rich in starch; F+b and S+b = F and S diets supplemented with bicarbonate at 1% of DMI.

[3]C = carbohydrate type effect (F and F+b vs. S and S+b); b = bicarbonate supplementation effect (F and S vs. F+b and S+b), and their interaction (C × b).

[4]FPCM, fat- and protein-corrected milk = milk yield (kg/d) × [0.337 + 0.116 × fat (%) + 0.06 × protein (%)] according to Gerber et al. (2011).

[5]Feed efficiency = FPCM/DMI.

[6]Energy balance was estimated as the difference between energy intake and total energy requirements for lactation and maintenance (INRA, 2007).

Expressed per kilogram of NDF intake or kilogram of NDF digested, CH_4 emissions were greater for starch than for fiber diets by +19% ($P < 0.05$). Emissions of CH_4 per kilogram of milk were significantly reduced with starch-rich diets in comparison to fiber-rich diets (−19%, $P < 0.001$). Irrespective of the unit used, bicarbonate addition in the diets did not affect CH_4 emissions in dairy cows.

Rumen pH and Fermentation Parameters

Mean rumen pH was not affected by the carbohydrate type, but minimum pH was reduced with starch-rich diets as compared with fiber-rich diets (on average −0.14 pH unit, $P < 0.01$; Table 6). Bicarbonate addition in F and S diets increased the mean pH (+0.14 pH unit; $P = 0.006$) and minimum pH (+0.06 pH unit, $P \leq 0.05$).

Table 3. Plasma metabolites concentration in dairy cows fed diets containing concentrates rich in fiber or starch, with or without bicarbonate addition

	Diet[2]					*P*-value[3]		
Item[1]	F	F+b	S	S+b	SEM	C	b	C × b
NEFA, mmol/L	0.26	0.09	0.25	0.17	0.045	0.48	0.03	0.40
BHB, mmol/L	0.72	0.74	0.51	0.42	0.042	<0.001	0.26	0.12
Glucose, g/L	0.72	0.71	0.74	0.74	0.024	0.23	0.96	0.73
Acetate, mmol/L	1.0	0.85	0.72	0.64	0.148	0.16	0.49	0.82
Urea, g/L	0.13	0.14	0.18	0.17	0.016	0.003	0.76	0.22

[1]Measurements taken on wk 3. NEFA = nonesterified fatty acids.

[2]F = diet rich in fiber; S = diet rich in starch; F+b and S+b = F and S diets supplemented with bicarbonate at 1% of DMI.

[3]C = carbohydrate type effect (F and F+b vs. S and S+b); b = bicarbonate supplementation effect (F and S vs. F+b and S+b), and their interaction (C × b).

Table 4. Daily nutrient intake and total-tract apparent digestibility in dairy cows fed diets containing concentrates rich in fiber or starch, with or without bicarbonate addition

	Diet[2]					*P*-value[3]		
Item[1]	F	F+b	S	S+b	SEM	C	b	C × b
Daily nutrient intake, kg/d (unless stated otherwise)								
OM	20.9	21.2	20.6	20.7	1.43	0.12	0.32	0.61
NDF	9.6	9.7	6.8	6.7	0.53	<0.001	0.81	0.13
ADF	5.4	5.5	3.6	3.5	0.30	<0.001	0.97	0.15
Starch	1.3	1.3	4.9	5.1	0.28	<0.001	0.48	0.49
Gross energy, MJ/d	426.6	433.8	416.8	418.8	29.1	0.03	0.31	0.55
Apparent digestibility, %								
DM	62.2	62.4	68.4	67.0	0.78	<0.001	0.48	0.35
OM	68.7	69.2	74.4	73.4	0.82	<0.001	0.74	0.38
NDF	63.5	62.7	61.6	61.7	1.37	0.31	0.81	0.76
ADF	62.1	61.0	60.3	61.3	2.02	0.66	0.96	0.54
Starch	95.7	95.3	98.1	98.2	0.42	<0.001	0.68	0.51
Gross energy	63.5	64.9	68.1	68.1	0.75	0.003	0.41	0.41

[1]Average data of the 5-d measurement period in wk 4.

[2]F = diet rich in fiber; S = diet rich in starch; F+b and S+b = F and S diets supplemented with bicarbonate at 1% of DMI.

[3]C = carbohydrate type effect (F and F+b vs. S and S+b); b = bicarbonate supplementation effect (F and S vs. F+b and S+b), and their interaction (C × b).

Total VFA concentration was reduced with starch- rich than with fiber-rich diets (−13.5 m*M*; $P = 0.02$; Table 6). The proportion of C2 in total VFA was unaf- fected, whereas the proportions of propionate (**C3**) and valerate were increased ($P = 0.003$) and that of butyrate (**C4**) was decreased ($P = 0.002$), as were C2:C3 and (C2+C4):C3 ratios ($P < 0.002$), by starch- compared with fiber-rich diets. Bicarbonate addition increased the proportion of C3 in total VFA and decreased the C2:C3 and (C2+C4):C3 ratios ($P \leq 0.05$), whatever the diet.

Total protozoa concentration was on average 2.8 times greater for fiber-rich compared with starch-rich diets ($P = 0.01$, Table 6). Type of carbohydrate and bicarbonate addition did not modify the rumen concen- trations of the total and different species of protozoa, irrespective of diet.

Milk Fatty Acid Composition

The starch-rich diets induced a greater concentra- tion of SFA (72.1 vs. 67.6% of total FA for starch- and fiber-rich diets, respectively; Table 7, $P \leq 0.05$) and of short- and medium-chain FA (sum FA <16 C, 30.0 vs. 26.1% of total FA for starch- and fiber-rich diets, respectively; Table 7, $P \leq 0.05$) than with the fiber-rich diet. However, starch-rich diets induced less MUFA con- centration as compared with fiber diets (21.3 vs. 25.5% of total FA, respectively; $P = 0.03$). The C5:0, C6:0, C7:0, C8:0, C9:0, C10:0, C11:0, C12:0, C13:0, C14:0, C15:0, C16:0, C17:0, *cis*-9 C12:1, *cis*-9 C14:1, *trans*-9 C14:1 concentrations were greater for starch-rich than

Table 5. Methane emissions in dairy cows fed diets containing concentrates rich in fiber or starch, with or without bicarbonate addition

	Diet[2]					*P*-value[3]		
Item[1]	F	F+b	S	S+b	SEM	C	b	C × b
CH_4, g/d	470	495	408	381	34.8	0.007	0.82	0.36
CH_4, g/kg of DMI	20.9	21.3	18.5	17.3	1.55	0.02	0.73	0.44
CH_4, g/kg of OM intake	23.0	23.5	20.1	18.8	1.68	0.01	0.73	0.44
CH_4, g/kg of NDF intake	50.2	51.3	61.8	58.6	4.99	0.04	0.76	0.55
CH_4, % of gross energy intake	6.2	6.3	5.5	5.2	0.46	0.02	0.63	0.45
CH_4, g/kg of OM digested	33.5	34.0	27.2	25.8	2.39	0.009	0.80	0.63
CH_4, g/kg of NDF digested	79.1	81.8	100.1	96.2	8.71	0.06	0.93	0.67
CH_4, g/kg of milk	16.9	17.0	14.0	13.2	1.91	<0.001	0.49	0.38
CH_4,[4] g/kg of FPCM	16.4	18.7	14.2	12.7	2.08	0.06	0.83	0.31

[1]Average data of 5-d measurement period in wk 4.

[2]F = diet rich in fiber; S = diet rich in starch; F+b and S+b = F and S diets supplemented with bicarbonate at 1% of DMI.

[3]C = carbohydrate type effect (F and F+b vs. S and S+b); b = bicarbonate supplementation effect (F and S vs. F+b and S+b), and their interaction (C × b).

[4]FPCM, fat- and protein-corrected milk = milk yield (kg/d) × [0.337 + 0.116 × fat (%) + 0.06 × protein (%)] according to Gerber et al. (2011).

for fiber-rich diets ($P \leq 0.05$; Table 7 and Supplemental Table S1; https://doi.org/10.3168/jds.2017-14041).

Cows fed starch-rich diets had less milk concentration of total C18 FA than those fed fiber-rich diets (31.0 vs. 37.2% of total FA, respectively; $P \leq 0.05$; Table 6), but greater concentration of several isomers of CLA (i.e., *trans*-10,*trans*-12, *trans*-7,*cis*-9 + *trans*-8,*cis*-10, and *cis*-11 *trans*-13; Table 8) and *trans*-9,*cis*-12 C18:2, *cis*-11 C18:1, and *cis*-13 C18:1 (Table 8). However, concentrations of the sum of *cis* isomers of C18:1, *cis*-9,*trans*-11 CLA, and the sum of CLA were reduced with starch-rich than with fiber-rich diets ($P \leq 0.05$; Table 8).

Bicarbonate addition had a slight effect on milk FA composition. Milk concentrations of C4:0, *iso* C18:0, and some isomers of CLA (*trans*-10,*trans*-12, *trans*-7,*trans*-9, and *trans*-12,*cis*-14) were reduced by bicarbonate addition, whereas milk concentrations of *cis*-9,*trans*-11 CLA, *cis*-9 20:1 ($P \leq 0.05$; Table 8), total PUFA, and total CLA were increased ($P \leq 0.05$; Table 7).

DISCUSSION

Methane Emissions and Other Digestive Processes

Effect of Carbohydrate Type. In our study, cows fed the starch-rich diets had less CH_4 emissions (on average, −18% in g/d; −15% in g/kg of DMI; −14% in % of GE intake) than fiber-rich diets. Using a modeling approach, Benchaar et al. (2001) also observed a 14% decrease in CH_4 emissions (g/d) in cows when 10% NDF from beet pulp was replaced by starch from barley in a 70% alfalfa hay-based diet. To the best of our knowledge, very few authors have compared the concentrate carbohydrate type with grass silage based-diet on CH_4 emissions. Hindrichsen et al. (2005) studied the effects of concentrates (50% of the diet) containing different carbohydrates types on enteric CH_4 emissions in dairy cows fed a mixture of forages (22% corn silage, 45% grass silage, 33% hay on a DM basis). Contrary to our results, these authors did not report effect of the carbohydrate types on CH_4 emissions (g/d and g/kg of DMI) between fiber-rich (containing soybean hulls) and starch-rich diets (containing wheat). However, less CH_4

Table 6. Rumen pH[1] and fermentation characteristics[2] in dairy cows fed diets containing concentrates rich in fiber or starch, with or without bicarbonate addition

	Diet[3]					*P*-value[4]		
Item	F	F+b	S	S+b	SEM	C	b	C × b
Rumen pH								
Mean pH	6.38	6.52	6.40	6.54	0.071	0.75	0.006	0.47
Minimum pH	5.93	5.99	5.79	5.85	0.082	<0.001	0.05	0.89
Total VFA, m*M*	61.3	60.6	41.8	53.1	4.38	0.02	0.29	0.23
VFA composition, % of total VFA								
Acetate (C2)	70.4	69.5	70.2	70.1	0.55	0.65	0.25	0.43
Propionate (C3)	15.1	15.8	17.1	18.6	0.59	0.003	0.05	0.44
Butyrate (C4)	11.5	11.8	9.3	8.1	0.92	0.002	0.38	0.20
Valerate	1.0	1.0	1.4	1.5	0.07	0.002	0.55	0.66
Caproate	0.4	0.4	0.4	0.3	0.03	0.55	0.11	0.05
Branched-VFA[5]	1.6	1.6	1.8	1.6	0.17	0.57	0.41	0.67
C2:C3	4.7	4.4	4.1	3.8	0.13	0.002	0.03	0.80
(C2+C4):C3	5.5	5.2	4.7	4.3	0.19	0.001	0.04	0.62
Total protozoa,[6] 10^3 cells/mL	227.1	218.9	101.1	57.9	54.1	0.04	0.66	0.77
Entodiniomorphs, 10^3 cells/mL	221.6	216.7	101.0	57.1	52.6	0.04	0.67	0.74
Small (<100 μm)	214.7	212.1	98.3	54.3	51.0	0.03	0.68	0.71
Large (>100 μm)	6.95	4.58	2.64	2.85	3.51	0.44	0.78	0.74
Holotrichs, 10^3 cells/mL	5.48	2.22	1.25	0.77	1.855	0.13	0.28	0.41
Isotricha spp.	0.50	0.24	0.32	0.15	0.208	0.53	0.33	0.82
Dasytricha spp.	4.98	1.98	0.87	0.62	1.734	0.12	0.32	0.40

[1]Average data of 3 d when animals were in open-circuit respiration chambers (wk 4). Data were not recovered for 1 cow throughout the duration of the experiment and for another cow during 1 experimental period ($n = 11$).

[2]Data from rumen juice sampled before morning feeding on d 18.

[3]F = diet rich in fiber; S = diet rich in starch; F+b and S+b = F and S diets supplemented with bicarbonate at 1% of DMI.

[4]C = carbohydrate type effect (F and F+b vs. S and S+b); b = bicarbonate supplementation effect (F and S vs. F+b and S+b), and their interaction (C × b).

[5]Sum of isobutyrate and isovalerate.

[6]For protozoa data, statistical analyses were done with $\log_{10}$ values.

Table 7. Milk fatty acid (FA) composition[1] in dairy cows fed diets containing concentrates rich in fiber or starch, with or without bicarbonate addition

	Diet[2]					*P*-value[3]		
Item, % of total FA	F	F+b	S	S+b	SEM	C	b	C × b
C4:0	3.48	3.38	3.32	3.26	0.053	0.006	0.05	0.59
C6:0	2.52	2.52	2.73	2.58	0.048	0.03	0.16	0.15
C8:0	1.45	1.48	1.77	1.60	0.058	0.008	0.28	0.14
C10:0	3.10	3.20	4.31	3.72	0.215	0.006	0.25	0.12
C12:0	3.25	3.38	4.82	4.02	0.296	0.008	0.26	0.13
C13:0	0.08	0.09	0.16	0.13	0.013	0.003	0.25	0.13
C14:0	10.9	11.0	12.9	11.9	0.252	<0.001	0.14	0.09
cis-9 14:1	0.80	0.82	1.01	0.99	0.078	0.03	0.99	0.74
C16:0	29.7	29.9	32.8	31.7	0.562	0.003	0.49	0.35
cis-9 16:1	1.18	1.11	1.29	1.42	0.107	0.01	0.58	0.11
C18:0	11.0	10.8	7.4	7.7	0.912	0.002	0.98	0.69
C18:3n-6	0.03	0.03	0.03	0.03	0.005	0.31	0.50	0.80
C18:3n-3	0.67	0.67	0.63	0.68	0.042	0.29	0.20	0.24
ΣSFA[4]	69.0	69.4	73.5	70.1	1.24	0.05	0.19	0.12
ΣMUFA[5]	24.3	23.9	20.1	22.9	1.16	0.04	0.25	0.14
Σ *cis* MUFA[6]	21.0	20.6	18.0	18.6	0.777	0.02	0.91	0.52
Σ *trans* MUFA[7]	2.72	2.78	2.20	3.75	0.774	0.77	0.31	0.35
ΣPUFA[8]	5.03	5.13	4.76	5.22	0.301	0.34	0.02	0.08
ΣFA *trans*	7.63	7.72	6.63	8.46	0.796	0.87	0.23	0.27
ΣCLA	0.53	0.58	0.39	0.49	0.047	0.005	0.02	0.34
ΣOBFA[9]	3.56	3.63	3.88	3.69	0.137	0.07	0.48	0.16
ΣFA <16C	25.9	26.2	31.3	28.5	0.862	0.006	0.22	0.13
ΣFA 16C	29.7	29.9	32.7	31.7	0.562	0.003	0.49	0.35
ΣFA >16C	40.0	39.5	31.3	34.7	1.12	<0.001	0.26	0.13
ΣC18:1 *cis*	19.3	18.8	15.5	16.3	0.761	0.004	0.85	0.46
ΣC18:1 *trans*	3.09	3.14	2.52	3.89	0.696	0.89	0.31	0.34
ΣFA C18	37.5	36.8	28.8	32.1	1.10	<0.001	0.27	0.14

[1]Measurement taken on d 24 in wk 4.

[2]F = diet rich in fiber; S = diet rich in starch, F+b and S+b = F and S diets supplemented with bicarbonate at 1% of DMI.

[3]C = carbohydrate type effect (F and F+b vs. S and S+b); b = bicarbonate supplementation effect (F and S vs. F+b and S+b), and their interaction (C × b).

[4]Sum SFA = odd-chain fatty acids + branched-chain fatty acids + even fatty acids, including fatty acids from 4 to 26 carbon atoms.

[5]Sum of MUFA from 10 to 22 carbon atoms.

[6]Sum of MUFA from 10 to 22 carbon atoms with the *cis* configuration.

[7]Sum of MUFA from 10 to 22 carbon atoms with the *trans* configuration.

[8]Sum of PUFA from 18 to 26 carbon atoms.

[9]OBFA = sum of odd-chain and branched-chain fatty acids.

emissions were measured for the fiber-rich diet when expressed per unit of NDF, which is in agreement with our results.

The observed CH_4 abatement with starch-rich diets appears to be linked to pre-prandial modifications in rumen fermentation and microbial population. The reduced total VFA concentration in the rumen with starch-rich diets may be due to the more limited DMI. Carbohydrate type in diets also affected the rumen VFA profile, with greater C3 and reduced C4 proportions in starch-rich diets. It is well known that increasing starch level in the diet, at the expense of fiber carbohydrates (NDF and ADF), leads to a shift in rumen fermentation in favor of the propionate pathway (Bannink et al., 2006), creating an alternative H_2 sink to methanogenesis (Martin et al., 2010).

The total count of protozoa, specifically entodinomorphs, was reduced for starch-rich than for fiber-rich diets, leading to less C4 proportion, as protozoa are C4 producers (Morgavi et al., 2012). Hassanat et al. (2013) also reported a decrease in protozoa population and in C4 proportion in the rumen of cows fed high-starch diets based on corn silage. In addition, the observed decrease in protozoa population may have led to a reduction of H_2 transfer from protozoa to methanogens and consequently to reduced methanogenesis. A meta-analysis of 28 experiments and 91 treatments indicated a significant linear relationship between CH_4 emissions and protozoa population (r = 0.96) in the rumen (Guyader et al., 2014). Also, the reduced protozoa population with starch-rich diets led to faster bacterial rumen fermentation and a reduced minimum

Table 8. Milk C18:1, C18:2, and CLA composition[1] in dairy cows fed diets containing concentrates rich in fiber or starch, with or without bicarbonate addition

	Diet[2]					*P*-value[3]		
Item, % of total fatty acids	F	F+b	S	S+b	SEM	C	b	C × b
trans-4 C18:1	0.03	0.03	0.02	0.03	0.002	0.09	0.21	0.76
trans-5 C18:1	0.02	0.02	0.02	0.03	0.002	0.61	0.07	0.19
trans 6–8 C18:1	0.29	0.28	0.23	0.29	0.039	0.52	0.42	0.41
trans-9 C18:1	0.27	0.27	0.23	0.28	0.031	0.44	0.39	0.27
trans-10 C18:1	0.31	0.44	0.64	1.50	0.502	0.19	0.33	0.46
trans-11 C18:1	1.31	1.27	0.75	1.01	0.149	0.03	0.47	0.34
trans-16 C18:1[4]	0.44	0.41	0.31	0.36	0.022	0.005	0.61	0.14
cis-9 C18:1	17.4	17.0	13.8	14.2	0.736	0.004	0.98	0.64
cis-11 C18:1	0.44	0.39	0.45	0.50	0.031	0.05	0.85	0.11
cis-12 C18:1	0.35	0.34	0.28	0.29	0.024	0.03	0.93	0.60
cis-13 C18:1	0.09	0.08	0.09	0.10	0.006	0.04	0.82	0.08
cis-15 C18:1[5]	0.19	0.20	0.20	0.22	0.014	0.43	0.39	0.45
cis-16 C18:1	0.10	0.09	0.08	0.09	0.005	0.10	0.59	0.05
cis-9,*cis*-12 C18:2	2.86	2.88	2.66	2.82	0.257	0.03	0.08	0.16
cis-9,*trans*-13 C18:2	0.22	0.21	0.20	0.23	0.027	0.90	0.18	0.09
trans-9,*trans*-12 C18:2	0.02	0.02	0.02	0.03	0.009	0.19	0.65	0.70
trans-9,*cis*-12 C18:2[6]	0.02	0.02	0.04	0.03	0.004	0.02	0.12	0.68
trans-11,*cis*-15 C18:2	0.12	0.14	0.13	0.20	0.043	0.39	0.27	0.48
cis-9,*cis*-11 CLA	0.02	0.03	0.03	0.02	0.004	0.74	0.68	0.15
trans-9,*trans*-11 CLA	0.02	0.02	0.02	0.01	0.002	0.21	0.05	0.05
trans-12,*trans*-14 CLA	0.01	0.01	0.01	0.01	0.001	0.49	0.23	0.54
trans-11,*trans*-13 CLA	0.01	0.01	0.02	0.01	0.002	0.26	0.51	0.74
trans-10,*trans*-12 CLA	0.004	0.004	0.005	0.004	0.0003	0.01	0.01	0.31
trans-8,*trans*-10 CLA	0.003	0.002	0.003	0.002	0.0001	0.07	0.01	0.18
trans-7,*trans*-9 CLA	0.008	0.008	0.007	0.005	0.001	0.004	0.03	0.37
trans-12,*cis*-14 CLA	0.006	0.004	0.006	0.005	0.0007	0.28	0.02	0.77
trans-11,*cis*-13 CLA	0.01	0.01	0.01	0.01	0.001	0.07	0.73	0.28
cis-11,*trans*-13 CLA	0.0007	0.0006	0.001	0.001	0.0001	0.01	0.19	0.49
cis-9,*trans*-11 CLA[7]	0.38	0.39	0.37	0.38	0.006	0.03	0.40	0.40
trans-7,*cis*-9 CLA[8]	0.04	0.04	0.05	0.05	0.003	0.009	0.34	0.63

[1]Measurement taken on d 24 in wk 4.

[2]F = diet rich in fiber; S = diet rich in starch, F+b and S+b = F and S diets supplemented with bicarbonate at 1% of DMI.

[3]C = carbohydrate type effect (F and F+b vs. S and S+b); b = bicarbonate supplementation effect (F and S vs. F+b and S+b), and their interaction (C × b).

[4]Coelution with *cis*-14 C18:1.

[5]Coelution with C19:0. [6]Coelution with *trans*-10 C19:1.

[7]Coelution with *trans*-10,*cis*-12 CLA and *trans*-9,*cis*-11 CLA.

[8]Coelution with *trans*-8,*cis*-10 CLA.

pH (Guyader et al., 2014), which may have impaired methanogen activity when pH is less than 6.0 (Van Kessel and Russell, 1996).

Effect of Bicarbonate Addition. To the best of our knowledge, the effect of dietary buffer addition on CH_4 production in ruminants has poorly been studied. Our experiment showed that the addition of 1% bicar- bonate had no effect on methanogenesis in dairy cows, regardless of the type of carbohydrate in diets based on grass silage, which is in agreement with Hellwing et al. (2012). Indeed, these authors reported no effect of 0.95% bicarbonate addition to a grass-clover silage- based diet rich in molasses. Furthermore, the addition of bicarbonate to diets did not influence nutrient digestibility, which confirms previous data in dairy cows fed a diet based on alfalfa and corn silage supplemented with 0.8% bicarbonate (Pereira and Armentano, 2000). Meschy et al. (2004) found by a meta-analysis approach (42 diets, 40 studies) that adding buffer at concentrations ranging from 0.5 to 2.5% of DMI had no effect on DM digestibility, but improved fiber digestibility.

Rumen pH measured thanks to commercial sensors positioned permanently in the reticulum of animals, could explain the relative high minimum pH values reported in our study whatever the diet. Reticulum pH has been reported to be higher than in the ventral and dorsal sacs of the rumen of cows fed diets of hay or mixed with 60% barley (Martin et al., 1999). As expected, bicarbonate addition acted as a rumen digestive regulator by increasing rumen pH (on average +0.14 units for mean pH and +0.06 units for minimum pH). Meschy et al. (2004) observed a similar effect of

bicarbonate addition on the mean rumen pH, but to a lesser extent (+0.07 units for pH mean per % added buffer). Other rumen characteristics (VFA and protozoa) measured before the morning feeding were unaffected by addition of bicarbonate (only C3 proportions and ratios were slightly modified) regardless of the type of carbohydrates in diets. This was consistent with the absence of effect of bicarbonate addition on methanogenesis and on nutrient digestibility whatever the diet.

Milk Fatty Acid Composition

Effect of Carbohydrate Type. We reported that short- and medium-chain FA (C6:0, C8:0, C12:0, C14:0, and C16:0), as well as the sum of SFA, were enhanced in milk fat from dairy cows fed starch-rich diets. As described in a review paper (Kalač and Samkova, 2010), high starch intake is associated with more milk SFA arising from a great level of de novo mammary synthesis. The milk C4:0 concentration was reduced with cows fed the starch-rich diets, and this could be explained by the reduced numerical plasma C2 concentration, as well as the reduced rumen C4 concentration because C2 and C4 are precursors of de novo FA synthesis (Chilliard et al., 2007). Another explanation concerning the specific variation of C4:0 compared with other short-chain FA could be due to the fact that this FA is synthesized in part by non-malonyl CoA mechanisms (not involving acetyl-CoA carboxylase; Chilliard et al., 2000).

Cows fed the starch-rich diets produced milk fat with greater C15:0 and C17:0 concentrations than fiber-rich diets. Our results are in agreement with those of Vlaeminck et al. (2006) and Cabrita et al. (2007) who reported a positive relationship between odd-chain FA (C15:0 and C17:0) and dietary starch content. The increased milk concentration of C15:0 and 17:0 with more degradable dietary carbohydrate (i.e., starch-rich diets) could be due to a greater population of amylolytic bacteria, which produce and contain relatively large concentrations of C15:0 and C17:0 (Minato et al., 1988). A lower milk *anteiso* C15:0 concentration was found with cows fed starch-rich diets, which agrees with previous data on cows fed concentrate rich in corn grain compared with concentrate rich in citrus pulp (Cabrita et al., 2007).

Cows fed the starch-rich diets had less milk concentrations of total CLA, several major isomers of CLA, and C18:0. Our results suggest that RBH was reduced when cows received starch-rich diets. The lesser C18:2 n-6 and C18:3 n-3 intakes, due to the limited DMI in starch-rich diets, may explain the reduced RBH because these 2 FA are the main precursors of RBH. Moreover, the lower minimum pH in the rumen observed with starch-rich diets could have modified the RBH in cows fed the starch-rich diets in comparison to fiber-rich diets, by increasing the milk *trans*-10 C18:1 concentration, leading only to a shift from *trans*-11 to *trans*-10 C18:1 for the S+b diet, as shown by Bauman and Griinari (2003). In our study, it seems that the RBH pathway from C18:2 n-6 to C18:0, including the intermediates as *trans*-10,*trans*-12 and *trans*-8,*cis*-10 CLA, and *trans*-10 C18:1, has been favored for starch-rich diets.

Effect of Bicarbonate Addition. Bicarbonate addition decreased the concentration of some RBH intermediates, whereas we observed a greater concentration of the sum of CLA and *cis*-9,*trans*-11 CLA. These variations in milk FA composition suggest modifications in RBH pathways with bicarbonate addition, favoring the production of *cis*-9,*trans*-11 CLA. In contrast, Cabrita et al. (2009) observed a decrease in almost all RBH intermediate concentrations and a greater C18:0 concentration, suggesting a more complete RBH with dietary buffer addition. Kennelly et al. (1999) observed a decrease in *trans*-10 C18:1 when buffer was added to a diet based on 75:25 concentrate-to-forage ratio. These authors suggested that the mechanism by which dietary buffers promote the completeness of RBH is due to an increase in rumen pH, which modifies bacterial activity. In our study, buffer addition stabilized the rumen pH, as hypothesized, and could have increased bacterial activity or number involved in the production of *cis*-9,*trans*-11 CLA. We observed a greater content of some *trans* FA, which was not expected at first. Fur- ther studies are needed to improve understanding of the mechanism by which dietary buffers modify RBH.

CONCLUSIONS

This study shows that for a 50:50 forage:concentrate ratio in grass silage-based diets, the dietary carbohy- drate type had an effect on CH_4 emissions in dairy cows as expected, but not on milk yield or on fat- or protein-corrected milk yield. Replacement of fiber by starch in diets decreased the energetic loss as CH_4 emis- sions without improving feed efficiency of diets. The decrease in rumen protozoa number and the shift in rumen fermentation toward propionate for cows fed the starch-rich diets may be the main factors for re- duced methanogenesis. Furthermore, starch-rich diets increased milk saturated short- and medium-chain FA and decreased the completeness of the biohydrogenation pathways of PUFA in the rumen, resulting in greater CLA content than in fiber-rich diet. The addition of 1% bicarbonate to diets induced rumen environment changes, through the increase of pH in starch-rich diets. Furthermore, no effects on CH_4 emissions or nutrient digestibility were observed with bicarbonate addition, regardless of the type of carbohydrate in diets. Milk FA

composition was slightly different between diets with or without bicarbonate, but bicarbonate addition did not prevent *trans* FA increase in milk as hypothesized. Reducing CH_4 emissions in dairy cows fed starch-rich diets based on grass silage help to limit the negative environmental effect of ruminant livestock. However, milk nutritional value was depressed as saturated milk FA were present at greater concentration with starch-rich diets than with fiber-rich diets.

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III. CHAPTER III

Effects of starch-rich or lipid-supplemented diets that induce milk fat depression on lipid metabolism and methanogenesis in lactating dairy cows

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Effects of starch-rich or lipid-supplemented diets that induce milk fat depression on rumen biohydrogenation of fatty acids and methanogenesis in lactating dairy cows

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Optimizing milk production efficiency implies diets allowing low methane (CH_4) emissions and high dairy performance. We hypothesize that nature of energy (starch v. lipids) and lipid supplement types (monounsaturated fatty acid (MUFA) v. polyunsaturated fatty acid (PUFA) mitigate CH_4 emissions and can induce low milk fat content via different pathways. The main objective of this experiment was to study the effects of starch-rich or lipid-supplemented diets that induce milk fat depression (MFD) on rumen biohydrogenation (RBH) of unsaturated fatty acids (FA) and enteric CH_4 emissions in dairy cows. Four multiparous lactating Holstein cows (days in milk = 61 ± 11 days) were used in a 4 × 4 Latin square design with four periods of 28 days. Four dietary treatments, three of which are likely to induce MFD, were based (dry matter basis) on 56% maize silage, 4% hay and 40% concentrates rich in: (1) saturated fatty acid (SFA) from Ca salts of palm oil (PALM); (2) starch from maize grain and wheat (MFD-Starch); (3) MUFA (cis-9 C18:1) from extruded rapeseeds (MFD-RS); and (4) PUFA (C18:2n-6) from extruded sunflower seeds (MFD-SF). Intake and milk production were measured daily. Milk composition and FA profile, CH_4 emissions and total-tract digestibility were measured simultaneously when animals were in open-circuit respiration chambers. Fermentation parameters were analysed from rumen fluid samples taken before feeding. Dry matter intake, milk production, fat and protein contents, and CH_4 emissions were similar among the four diets. We observed a higher milk SFA concentration with PALM and MFD-Starch, and lower milk MUFA and trans-10 C18:1 concentrations in comparison to MFD-RS and MFD-SF diets, while trans-11 C18:1 remained unchanged among diets. Milk total trans FA concentration was greater for MFD-SF than for PALM and MFD-Starch, with the value for MFD-RS being intermediate. Milk C18:3n-3 content was higher for MFD-RS than MFD-SF. The MFD seems more severe with MFD-SF and MFD-RS than PALM and MFD-Starch diets, because of a decrease in milk SFA concentration and a stronger shift from trans-11 C18:1 to trans-10 C18:1 in milk. The MFD-SF diet increased milk trans FA (+60%), trans-10 C18:1 (+31%), trans-10,cis-12 CLA (+27%) and PUFA (+36%) concentrations more than MFD-RS, which explains the numerically lowest milk fat yield and indicates that RBH pathways of PUFA differ between these two diets. Maize silage-based diets rich in starch or different unsaturated FA induced MFD with changes in milk FA profiles, but did not modify CH_4 emissions.

Keywords: dairy cow, low-fat milk syndrome, rumen lipid digestion, milk fatty acids, methane emissions

Implications

Optimizing milk production efficiency implies diets allowing low methane (CH_4) emission and high dairy performance. Maize silage-based diets supplemented with starch or dif- ferent sources of lipids induced low milk fat content, sugg- esting milk fat depression (MFD) in dairy cows, which represents economic losses for farmers. All of these diets led to low enteric CH_4 emissions, which can limit the dairy cows' impact on the environment.

Introduction

Dairy producers are interested to improve milk yield and composition of their dairy cows, because economic outputs as payments are based on milk fat and protein contents. However, the genetic selection of high-yielding cows redu- ces, in the long term, the milk fat content (Le Mezec and Launay, 2017). Moreover, to meet the energy requirements of high-yielding dairy cows and to increase their milk yield (Jenkins and McGuire, 2006), the use of dietary lipid sup- plementation and/or the distribution of concentrates rich in starch have become common feeding practices for dairy producers. These latter feeding practices could markedly

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modify milk fat content and composition (Chilliard et al., 2007). More precisely, feeding dairy cows with diets rich in starch (due to a high level of concentrate and/or maize silage-based diets), supplemented or not with unsaturated fatty acids (FA), can induce MFD, also called low milk fat syndrome (Bauman and Griinari, 2003). Changes occurring during MFD are characterized by a decrease in milk fat con- tent, ranging from 0.9 to 2.5 g/kg without changes in milk yield (Shingfield et al., 2010). Lower milk FA secretion, due to the reduction in de novo mammary FA synthesis (Bauman and Griinari, 2003), is also observed in the case of MFD, as are changes in ruminal biohydrogenation (RBH) of poly- unsaturated fatty acid (PUFA), with greater production of total trans FA, including trans-10 C18:1 and trans-10,cis-12 CLA, which is known to inhibit de novo mammary FA synthesis (Ferlay et al., 2017). Conversely, maize silage- based diets supplemented with saturated fatty acid (SFA) (e.g. C16:0 from palm oil or Ca salts of palm oil) induced higher milk fat content and yield, and a higher C16:0 concentration in milk than no supplemented diets (Lock et al., 2013). We hypothesize that the different dietary energy substrates would lead to different degrees of MFD as well as changes in RBH pathways and milk FA concentration.

These feeding strategies (starch, lipids) are also known to lower CH_4 emissions in dairy cows, though various mitigating mechanisms are involved (Martin et al., 2010). However, to the best of our knowledge, no direct comparisons of the energy substrate effect (starch v. lipids) on CH_4 emissions have been carried out in the past. Nonetheless, it has been reported that the CH_4-decreasing effect of lipids appears to be dependent on the dietary FA profile. Indeed, PUFA sup- plementation is more effective than SFA (Martin et al., 2010), even though no lipid source effect was reported by Grainger and Beauchemin (2011). Furthermore, it is well known that the short-chain FA (acetate, propionate and butyrate) arising from rumen fermentations are precursors of the de novo synthesis of short- and medium-chain FA in the mammary gland and also are related to CH_4 production because of these common biochemical pathways in the rumen.

Thus, the main objective of this experiment was to study the effects of starch-rich or different lipid-supplemented diets inducing MFD on milk yield, fat yield and FA profile in dairy cows fed maize silage-based diets in comparison to non-MFD diet. At the same time, the effects on methano- genesis and on other digestive processes (rumen fermenta- tion parameters, total tract digestibility), as well as the links between individual milk FA and CH_4 emissions, were investigated.

Material and methods

The experiment was conducted at the animal experimental facilities of Institut National de la Recherche Agronomique (INRA) (UE 1414 Herbipôle, Saint-Genès-Champanelle, France) from February to June 2015.

Cows, experimental design and diets

Four multiparous lactating Holstein cows (BW = 621 ± 12 kg, days in milk = 61 ± 10 days, milk yield = 30.7 ± 1.8 kg/day, and milk fat content = 3.31 ± 6.20% at the start of the experiment) were used in a 4 × 4 Latin square design. Each cow was fed four diets based (dry matter (DM) basis) on 60% forage (56% maize silage and 4% natural grassland hay) and 40% of pelleted concentrates (InVivo NSA, Chierry, France). The four concentrates differed in their energy substrate (starch or lipids) likely to modify milk fat content: (1) a diet rich in SFA with Ca salts of palm oil (PALM), likely to increase milk fat content; (2) a diet able to induce a MFD with high dietary starch content (MFD-Starch); (3) a diet able to induce a MFD containing monounsaturated fatty acid (MUFA)-rich lipid supplement as extruded rapeseeds (MFD-RS); and (4) a diet able to induce a MFD containing PUFA-rich lipid sup- plement as extruded sunflower seeds (MFD-SF) (Table 1). Diets were formulated to be iso-energy and iso-protein and to meet individual energy and protein requirements for lactation and maintenance (INRA, 2007). They were adjusted daily in order to maintain the forage-to-concentrate ratio as close as possible to the 60 : 40 ratio targeted. Ingredients and chemical composition of diets are reported in Table 1.

Each experimental period lasted 28 days. From days 1 to 20, cows were housed in a free stall barn and were fed ad libitum with forage (maize silage and hay). From days 21 to 26, cows were placed individually in open-circuit respiratory chambers and fed an amount limited to 95% of individual voluntary feed intake to ensure complete consumption of the diets. From days 27 to 28, cows returned to the free stall barn and were fed the same diet from days 1 to 26. Changes from one diet to another were achieved with a 6-day transition period during week 1 of each experimental period. Hay was given once daily (0830 h) and the maize silage and con- centrates were mixed together as a partial mixed ration and given twice a day (66% at 0900 h and 34% at 1600 h). Cows had free access to water throughout the experiment.

Measurements

BW, feed intake and diet composition. Cows were weighed at the beginning of the experiment and in week 3 for each period. Dry matter intake (DMI) was determined on 4 con- secutive days in weeks 1 to 3, and on 5 consecutive days in week 4 by individually weighing feed offered and refusals. The DM content of the feed offered and refusals was deter-mined (103°C for 24 h) in samples (100 g) taken twice a week for maize silage and once a week for hay and con- centrates. In addition, samples of each feedstuff (100 g) were taken twice in week 4, pooled to provide one sample per period and stored at 4°C (concentrates, hay) or at −20°C (maize silage). At the end of the experiment, all feedstuff samples were freeze-dried and ground (1-mm screen; ZM 200 Retch Mill, Haan, Germany) for chemical composition determination of organic matter (OM), CP, NDF, ADF, starch, FA profile, gross energy (GE) and fermentative parameters of fresh maize silage juice (pH, lactate, volatile fatty acid (VFA) and ammonia N ($N\text{-}NH_3$)). As refusals were negligible (<1%

Table 1 Ingredients and chemical composition of the four experi- mental diets[1] fed to dairy cows

Items	PALM	MFD-Starch	MFD- RS	MFD- SF
Ingredients (% DM)				
Maize silage[2]	56	56	56	56
Hay	4	4	4	4
Pelleted concentrate (% DM)	40	40	40	40
Maize grains		13.4	5.8	6.3
Ca salts of palm oil	12.3			
Extruded rapeseed			24.5	
Extruded sunflower seeds				24.5
Barley grains	2.6	4.0	9.6	
Wheat starch		12.0		
Wheat middlings	10.7	25.0		2.0
Wheat bran	28.8	20.0	12.6	10.5
Distillers dried maize grains				2.2
Soybeans	24.9		21.3	23.6
Beet pulp	5.1	18.8	20.0	25.0
Hay	10.0			
Molasses	4.0	2.0	2.0	2.0
Premix, vitamins and minerals Composition (%)	1.6	4.8	4.2	3.9
Organic matter	87.0	87.2	86.9	86.9
CP	13.0	12.9	13.3	13.3
NDF	41.6	40.6	40.1	41.2
ADF	19.2	17.9	19.2	19.2
Starch	18.2	22.9	18.5	16.7
Ether extract	6.3	3.3	7 .1	7.4
Gross energy (MJ/kg DM)	18.4	19.1	19.2	19.2
Fatty acids (g/kg DM)				
C14:0	0.54	0.04	0.06	0.06
C16:0	23.87	4.06	4.95	5.20
C18:0	2.31	0.69	0.96	1.55
cis-9 C18:1	19.44	4.29	25.54	12.01
cis-11 C18:1	0.57	0.23	1.63	0.58
C18:2n-6	14.26	12.55	16.04	32.12
C18:3n-3	1.96	1.91	6.43	2.50

[1]PALM = diet with Ca salts of palm oil; MFD-Starch = diet inducing milk fat depression (MFD) rich in starch; MFD-RS = diet inducing MFD with extruded rapeseed,; MFD-SF = diet inducing MFD with extruded sunflower seeds.
[2]Fermentation characteristics of fresh maize silage juice: pH = 3.4; lactic acid = 98.5 g/kg dry matter (DM); N-NH3 = 7.5% of total N.

of total DMI, data not shown), chemical composition of refusals was considered similar to that of the diet offered.

Milk yield and composition. Cows were milked twice daily at 0730 and 1530 h and milk yield was individually recorded at each milking. Milk samples (30 ml) were collected for each cow on days 22 to 24 and stored at +4°C with Bronopol (2-2-nitropropane-1,3-diol) as preservative for milk component analyses. For milk FA composition, another individual milk sample (3 ml) was taken on day 24 and frozen (−20°C) without preservative after each milking. All samples were freeze-dried and then composited per day based on am and pm milk yields, respectively.

Total tract digestibility. Total faeces and urine were collectedin individual boxes for 5 consecutive days during week 4. Each morning, after weighing and mixing the faeces and urine, a 1% fresh aliquot was used for DM determination (103°C for 24 h) and another 0.5% fresh aliquot was pooled per week and per animal before being frozen (−20°C). At the end of the experiment, samples were thawed, freeze-dried, and ground (1-mm screen) for chemical determination (OM, NDF, ADF, starch, GE).

Enteric methane emissions and rumen fermentation parameters. Cows were housed in individual open-circuit respiration chambers (16.6 m^3 each) from days 21 (0730 h) to 26 (0730 h), during which total CH_4 emissions were continuously measured. Open-circuit respiration chambers operated at a slight negative pressure with an airflow of 421 ± 12 m^3/h on average (~45 air changes per hour). System design, associated analytical equipment and procedures are detailed in Bougouin et al. (2018), as is rumen fluid sampling. Briefly, rumen fluid samples (500 ml) were collected by stomach tubing before the morning feeding on day 18 of each experimental period. Samples were strained through a polyester monofilament fabric (250 µm pore size) and the filtrate was subsampled for determination of VFA concentration and protozoa counting.

Chemical analyses

Chemical composition and rumen fermentation parameters. The OM, NDF, ADF, CP and GE content of feed and excreta (faeces + urine), as well as FA composition of all feedstuffs and fermentative parameters of fresh maize silage juice were determined using procedures as described in Bougouin et al. (2018). Concentrations of rumen VFA and N-NH_3 were, respectively, analysed by gas chromatography with a flame ionization detector and by colorimetry (Morgavi et al., 2008). Total protozoa were counted by optical micro- scopy and categorized as either entodiniomorphs or holotrichs (Williams and Coleman, 1992). Data for protozoa were log_{10}-transformed for statistical analyses.

Milk composition. Milk fat, protein, lactose and urea nitro- gen contents were determined using MilkoScan 4000 (Foss Electric A/S, Hillerod, Denmark; Lial, Aurillac, France). The milk FA composition was determined as described by Ferlay et al. (2010). Lipids were directly methylated using 2 ml of 0.5 M sodium methoxide plus 1 ml of hexane at 50°C for 5 min, followed by cooling with the addition of 75 µl of 12 M HCl at room temperature for 10 min. The FA methyl esters from all the samples were separated on a 100 m × 0.25 mm i.d. fused-silica capillary column (CP-Sil 88; Chrompack, Mid- delburg, The Netherlands). The composition of methyl esters of CLA isomers in milk fat was analysed according to Bougouin et al. (2018). Briefly, the methyl esters of CLA isomers were determined using an HPLC system (Agilent, 1200 series, France) equipped with three silver-impregnated silica col- umns (ChromSpher 5 Lipids, 250 × 4.6 mm, 5-µm particle

Table 2 Milk performance in dairy cows fed the four experimental diets[1]

Items	PALM	MFD-Starch	MFD-RS	MFD-SF	SEM	P-value
Total DM intake (kg/day)	18.5	18.4	18.6	18.5	0.65	0.88
Milk production (kg/day)						
Milk yield	25.2	27.3	27.3	27.5	2.19	0.24
FPCM[2]	22.2	23.2	21.7	21.1	1.92	0.62
Milk composition (%)						
Fat	2.99	2.94	2.43	2.00	0.332	0.15
Protein	3.29	2.93	3.13	3.22	0.170	0.25
Lactose	4.94	5.24	5.10	5.28	0.158	0.10
Fat yield	0.75	0.79	0.66	0.55	0.096	0.22
Protein yield	0.81	0.80	0.83	0.88	0.055	0.08
Lactose yield	1.26	1.44	1.38	1.48	0.150	0.11
Energy balance (kJ/day)	−0.27	−1.08	−0.76	−1.20	0.383	0.36

DM = dry matter.

[1]PALM = diet with Ca salts of palm oil; MFD-Starch = diet inducing milk fat depression (MFD) rich in starch; MFD-RS = diet inducing MFD with extruded rapeseed; MFD-SF = diet inducing MFD with extruded sunflower seeds.

[2]FPCM: fat- and protein-corrected milk = milk yield (kg/day) × [0.337 + 0.116 × fat (%) + 0.06 × protein (%)] (Gerber et al., 2011).

size; Chromoptic, F-91971 Courtaboeuf) coupled in series. Methyl esters of CLA were separated under isocratic conditions at 22°C using 0.1% (vol/vol) acetonitrile in n-heptane at a flow rate of 1 ml/min and monitoring effluent at 233 and 210 nm. CLA isomers were identified based on retention time comparison with a mixture of authentic standards (O5632; Sigma-Aldrich, F-38297 St. Quentin Fallavier, USA). Concentrations of CLA isomers were calculated from the proportional peak area responses determined by HPLC and the sum of concentrations of *trans*-7,*cis*-9 CLA, trans-8,cis-10 CLA and cis-9,trans-11 CLA determined by GC analyses (the following minor CLA isomers were also taken into account in the sum: trans-11,cis-13 CLA (coeluted with cis-9,cis-11), trans-11,trans-13 CLA, trans-10,trans-12 CLA, trans-9,trans- 11 CLA, and trans-8,trans-10 CLA).

Statistical analyses

Data were analysed by ANOVA using mixed effect models with the lme4 package (version 1.1–10) in R (Version 0.98.1102, R Foundation for Statistical Computing, Vienna, Austria). Fixed effects of period, diets and the random effect of cow were tested with the following model:

$$Y_{ijk} = \mu + P_i + A_j + B_k + \varepsilon_{ijk}$$

where Y_{ijk} is the dependent variable, μ the overall mean, P_i the period (i = 1 to 4), A_j the diet (j = 1 to 4), B_k the cow (k = 1 to 4) and ε_{ijk} the random residual error. Pairwise comparisons were performed using a Tukey test.

Data measured for several days (intake, milk yield and composition, CH_4 emissions, total tract digestibility) were averaged for the statistical analyses. Least square means are reported with the pooled standard error of the mean derived from the model. Pearson's coefficient was used to calculate correlation coefficients between milk fat content, individual milk FA and CH_4 emissions. Effects were considered significant at a probability of $P < 0.05$.

Results

Total DMI did not differ between diets and nor did milk yield and composition or the energy balance of dairy cows (Table 2). The PALM and MFD-Starch diets induced higher milk SFA concentrations than the two other diets (+30%, P < 0.01; Table 3). The MFD-Starch diet produced milk with the highest concentrations of individual short even-chain SFA (FA with atoms of carbon ⩽ 10; $P < 0.01$). Milk from PALM had the highest milk C16:0 concentration, whereas that from MFD-SF had the lowest C16:0 one ($P < 0.001$).

The MFD-Starch diet produced milk with the highest concentrations of several odd- and branched-chain FA (OBCFA), such as C5:0, C7:0, iso C15:0, anteiso C15:0, anteiso C17:0 and greater total OBCFA than other diets (P < 0.05; Supple- mentary Table S1). Milk from both MFD-RS and MFD-SF had significantly higher concentrations of MUFA and several individual trans and cis MUFA (*trans*-4, *trans*-5, *trans*-6,7,8, *trans*-9, *trans*-10, *trans*-13, *rans*-16 and *cis*-10 C18:1, $P < 0.05$; Table 4) than MFD-Starch and PALM diets. No significant differences were observed for *trans*-11 and *cis*-9 C18:1 between diets. Overall, we observed a higher milk concentration of total *trans* C18:1 with MFD-SF than with PALM and MFD-Starch, the value with MFD-RS being intermediate ($P < 0.001$, Table 4).

The milk C18:2n-6 concentration was higher for MFD-SF (5.3 g/100 g, $P < 0.05$, Table 4) than for PALM and MFD-RS, the value with MFD-Starch being intermediate. The milk C18:3n-3 concentration was higher for MFD-RS (0.47/100 g) and lower for PALM (0.22 g/100 g, $P < 0.05$) than for MFD- Starch and MFD-SF. The milk concentrations of *trans*-7,*cis*-9, *cis*-9,*trans*-11, *trans*-10,*cis*-12 and *trans*-10,*trans*-12 CLA

Table 3 Concentrations of 4- to 24-carbon (not including 18:1, 18:2 or 18:3 isomers) fatty acids (FA) in milk fat in dairy cows fed the four experimental diets[1]

Items (% of total FA)	PALM	MFD-Starch	MFD-RS	MFD-SF	SEM	P-value
C4:0	2.01^{b}	2.93^{a}	1.86^{b}	1.62^{b}	0.169	< 0.001
C6:0	0.93^{b}	1.81^{a}	1.06^{b}	0.84^{b}	0.108	< 0.001
C8:0	0.41^{b}	0.97^{a}	0.55^{b}	0.39^{b}	0.071	< 0.001
C10:0	0.85^{b}	2.11^{a}	1.30^{b}	0.88^{b}	0.197	< 0.01
C12:0	1.13^{b}	2.41^{a}	1.74^{ab}	1.33^{b}	0.228	0.01
iso C14:0	0.05^{c}	0.07^{a}	0.05^{a}	0.06^{b}	0.004	< 0.001
C14:0	6.32^{b}	9.95^{a}	8.06^{ab}	6.43^{b}	0.763	0.03
cis-9 C14:1	0.74^{b}	1.08^{a}	1.16^{b}	0.84^{b}	0.219	0.40
iso C15:0	0.11^{b}	0.18^{a}	0.13^{b}	0.12^{b}	0.009	0.01
anteiso C15:0	0.25^{b}	0.41^{a}	0.31^{b}	0.31^{b}	0.032	0.01
C15:0	0.56	0.83	0.70	0.70	0.083	0.19
iso C16:0	0.14^{b}	0.25^{a}	0.16^{b}	0.19^{b}	0.013	< 0.01
C16:0	37.7^{a}	29.6^{b}	23.4^{bc}	18.3^{a}	2.47	< 0.001
cis-9 C16:1	2.70^{a}	2.08^{b}	1.96^{bc}	1.41^{a}	0.234	0.01
anteiso C17:0	0.29^{b}	0.53^{a}	0.38^{b}	0.37^{b}	0.029	<0.001
C17:0	0.35^{c}	0.62^{a}	0.45^{b}	0.45^{b}	0.019	< 0.001
iso C17:0[2]	0.66^{b}	0.50^{c}	0.62^{b}	0.90^{a}	0.045	0.0002
cis-9 C17:1	0.20^{b}	0.33^{a}	0.24^{b}	0.21^{b}	0.017	< 0.01
iso C18:0	0.03^{c}	0.07^{a}	0.04^{b}	0.04^{b}	0.003	< 0.001
C18:0	6.83	7.46	8.35	10.07	1.02	0.22
C20:4n-6	0.06^{b}	0.14^{a}	0.08^{b}	0.08^{b}	0.013	0.01
C20:5n-3	0.02	0.02	0.04	0.03	0.005	0.11
C22:5n-3	0.04^{b}	0.11^{a}	0.07^{b}	0.06^{b}	0.010	0.01
C22:6n-3	0.01	0.006	0.009	0.013	0.0024	0.14
Σ SFA[3]	58.2^{a}	60.6^{a}	49.0^{b}	42.4^{b}	2.41	< 0.01
Σ MUFA[4]	35.4^{b}	32.1^{b}	42.8^{a}	46.3^{a}	2.05	0.01
Σ PUFA[5]	4.38^{b}	5.21^{b}	5.05^{b}	8.29^{a}	0.507	<0.001
Σ *trans* FA	9.0^{c}	8.4^{c}	13.7^{b}	19.3^{a}	1.24	<0.001
Σ OBCFA[6]	1.89^{b}	3.18^{a}	2.40	2.40^{b}	0.164	<0.001
Σ n-3	0.28^{c}	0.48^{ab}	0.57^{a}	0.40^{b}	0.032	0.001
Σ n-6	2.5^{bc}	3.3^{b}	2.4^{c}	5.5^{a}	0.38	< 0.001
Σ FA < 16 C[7]	13.5^{b}	23.2^{a}	17.2^{b}	13.7^{b}	1.57	< 0.01
Σ FA 16 C[8]	40.8^{a}	32.3^{b}	25.9^{c}	20.1^{c}	2.68	< 0.001
Σ FA > 16 C[9]	44.6^{bc}	43.5^{c}	55.0^{ab}	64.6^{a}	3.66	< 0.01

^{a,b,c}Values within a row with different superscripts differ significantly at P < 0.05.

[1]PALM = diet with Ca salts of palm oil; MFD-Starch = diet inducing milk fat depression (MFD) rich in starch; MFD-RS = diet inducing MFD with extruded rapeseed; MFD-SF = diet inducing MFD with extruded sunflower seeds.

[2]*iso* C17:0 coeluted with *trans*-9 C16:1.

[3]SFA = saturated FA (odd FA + branched-chain FA + even FA; from 4 to 26 carbon atoms).

[4]MUFA = monounsaturated FA from 10 to 22 carbon atoms).

[5]PUFA = polyunsaturated FA from 18 to 26 carbon atoms.

[6]OBCFA = odd- and branched-chain FA.

[7]Sum of C4:0, C5:0, C6:0, C7:0, C8:0, C9:0, C10:0, cis-9 C10:1, C11:0, C12:0, cis-9 C12:1, C13:0, iso C13:0, anteiso C13:0, C14:0, cis-9 C14:1, trans-9 C14:1, iso C14:0, C15:0, *iso* C15:0, *anteiso* C15:0.

[8]Sum of C16:0, *iso* C16:0, *trans*-6,7,8 C16:1, *cis*-6,8 C16:1 + *trans*-11 C16:1, *cis*-9 C16:1, *cis*-11 C16:1.

[9]Sum of FA with more than 16 carbons except iso C17:0 because of coelution with *trans*-9 C16:1.

were lower for MFD-RS than MFD-SF ($P < 0.05$). In addition, milk *cis*-9,*trans*-11 CLA concentration was greater for MFD-Starch than for PALM and *trans*-10,*cis*-12 CLA concentration in milk was greater with PALM than with MFD-RS. The milk *trans*-9,*trans*-11 CLA concentration was higher for PALM and MFD-Starch than for MFD-RS and MFD-SF. Overall, milk concentration of total CLA was lower for MFD-RS than for MFD-SF, PALM and MFD-Starch. Other C18:2 isomer concentrations (*cis*-9,*trans*-12 and *cis*-9,*trans*-13 C18:2) were greater with MFD-RS and MFD-SF than with PALM and MFD-Starch.

MFD-Starch diet induced the highest milk concentra- tions of FA < C16 (Table 3). Milk from MFD-SF had higher a concentration of PUFA than other diets and that from MFD-SF had a higher concentration of trans FA (Table 3) than PALM and MFD-starch, the value from MFD-RS being intermediate.

On the one hand, milk SFA, even SFA and C16:0 concentrations were positively correlated with milk fat content (r = 0.62, 0.64 and 0.65, respectively, $P < 0.05$; Supplementary Table S2 and Figure 1). On the other hand, several milk FA concentrations were negatively correlated (r > 0.60;

Table 4 Concentrations of C18:1, C18:2 and C18:3 isomers (% of total fatty acid (FA)) in milk fat in dairy cows fed the four experimental diets[1]

Items	PALM	MFD-Starch	MFD-RS	MFD-SF	SEM	P-value
trans C18:1						
4	0.06^{b}	0.03^{c}	0.11^{a}	0.10^{a}	0.011	<0.01
5	0.07^{b}	0.04^{b}	0.13^{a}	0.11^{a}	0.010	< 0.001
6,7,8	0.84^{b}	0.46^{c}	1.31^{a}	1.31^{a}	0.109	< 0.001
9	0.69^{b}	0.41^{c}	0.89^{a}	0.90^{a}	0.059	0.01
10	2.37^{c}	2.89^{c}	5.11^{b}	8.63^{a}	0.624	< 0.001
11	1.46	1.34	1.43	2.68	0.541	0.21
12[2]	0.68^{bc}	0.48^{c}	0.83^{b}	1.17^{a}	0.077	<0.01
13	0.72^{b}	0.74^{b}	1.29^{a}	1.48^{a}	0.115	<0.01
16[3]	0.28^{b}	0.30^{b}	0.50^{a}	0.53^{a}	0.054	0.02
Σ *trans* C18:1	7.38^{c}	6.98^{c}	12.05^{b}	17.23^{a}	1.142	< 0.001
cis C18:1						
9	21.9	18.9	23.9	23.2	1.60	0.20
10	0.75^{b}	0.87^{b}	1.19^{a}	1.21^{a}	0.099	0.02
11	0.81^{b}	0.85^{b}	1.18^{a}	0.86^{b}	0.069	0.01
12	0.32^{b}	0.28^{b}	0.27^{b}	0.68^{a}	0.053	<0.01
13	0.13^{c}	0.13^{bc}	0.21^{a}	0.17^{ab}	0.012	<0.01
15[4]	0.21^{b}	0.28^{b}	0.45^{a}	0.33^{ab}	0.046	0.03
16	0.10^{c}	0.09^{c}	0.15^{b}	0.19^{a}	0.010	<0.01
Σ *cis* C18:1	23.9	21.1	26.8	26.1	1.71	0.15
Non-conjugated C18:2						
18:2 n-6	2.35^{b}	2.99^{b}	2.19^{b}	5.25^{a}	0.371	< 0.001
cis-9,*trans*-12[5]	0.13^{b}	0.12^{b}	0.21^{a}	0.22^{a}	0.017	0.01
trans-9,*cis*-12[6]	0.07^{b}	0.09^{a}	0.08^{ab}	0.07^{b}	0.008	0.09
cis-9,*trans*-13	0.33^{b}	0.30^{b}	0.50^{a}	0.54^{a}	0.037	<0.01
trans-9,*trans*-12	0.023^{c}	0.017^{c}	0.067^{a}	0.034^{b}	0.0034	< 0.001
trans-11,*cis*-15	0.08^{b}	0.07^{b}	0.25^{a}	0.11^{b}	0.015	< 0.001
Conjugated C18:2						
trans-7,*trans*-9	0.0035^{a}	0.0038^{a}	0.0038^{a}	0.0025^{b}	0.00030	0.03
trans-7,*cis*-9[7]	0.08^{b}	0.06^{b}	0.12^{a}	0.07^{b}	0.008	<0.01
cis-9,*cis*-11	0.02	0.02	0.03	0.02	0.005	0.20
cis-9,*trans*-11[8]	0.28^{ab}	0.32^{a}	0.24^{b}	0.30^{a}	0.015	0.02
trans-10,*cis*-12	0.011^{ab}	0.007^{bc}	0.004^{c}	0.015^{a}	0.0014	< 0.001
trans-9,*trans*-11	0.006^{a}	0.006^{a}	0.004^{b}	0.004^{b}	0.0003	<0.01
trans-8,*trans*-10	0.006^{a}	0.003^{b}	0.002^{b}	0.003^{b}	0.0003	< 0.001
trans-10,*trans*-12	0.006^{a}	0.004^{bc}	0.002^{c}	0.006^{a}	0.0006	0.01
cis-11,*trans*-13	0.0006^{a}	0.0006^{a}	0.0007^{a}	0.0003^{b}	0.00008	< 0.001
trans-11,*cis*-13	0.004	0.003	0.003	0.002	0.0004	0.07
trans-11,*trans*-13	0.008^{a}	0.004^{bc}	0.005^{b}	0.003^{c}	0.0005	< 0.001
trans-12,*trans*-14	0.003^{ab}	0.002^{bc}	0.004^{a}	0.002^{c}	0.0030	0.01
trans-12,*cis*-14	0.001	0.002	0.002	0.001	0.0003	0.56
Σ CLA	0.40^{a}	0.42^{a}	0.39^{b}	0.41^{a}	0.005	<0.01
C18:3						
18:3 n-3	0.22^{c}	0.33^{b}	0.47^{a}	0.31^{b}	0.032	< 0.001
cis-6,*cis*-9,*cis*-12	0.02	0.03	0.02	0.03	0.003	0.45

[a,b,c]Values within a row with different superscripts differ significantly at P < 0.05.

[1]PALM = diet with Ca salts of palm oil; MFD-Starch = diet inducing milk fat depression (MFD) rich in starch; MFD-RS = diet inducing MFD with extruded rapeseed; MFD-SF = diet inducing MFD with extruded sunflower seeds.

[2]*trans*-12 C18:1 is coeluted with *cis*-6,7,8 C18:1.

[3]*trans*-16 C18:1 is coeluted with *cis*-14 C18:1.

[4]*cis*-15 C18:1 is coeluted with C19:0.

[5]*cis*-9, *trans*-12 C18:2 is coeluted with *cis*-9,*trans*-14 C18:2.

[6]*trans*-9,*cis*-12 C18:2 is coeluted with *trans*-10 C19:1.

[7]*trans*-7,*cis*-9 is coeluted with *trans*-8,*cis*-10 CLA.

[8]*cis*-9,*trans*-11 CLA coeluted with cis-10,trans-12 and trans-9,cis-11 CLA.

$P < 0.05$) with milk fat content, such as *trans*-10 C18:1, *cis*-16 C18:1, *iso* C17:0, *trans* MUFA, *cis*-9,*trans*-13 C18:2 and PUFA (Supplementary Table S2 and Figure 1).

No differences in CH_4 emissions were observed between diets, whatever the unit considered (g/day, g/kg DMI, % GE intake and g/kg milk; Table 5). Rumen VFA concentration and

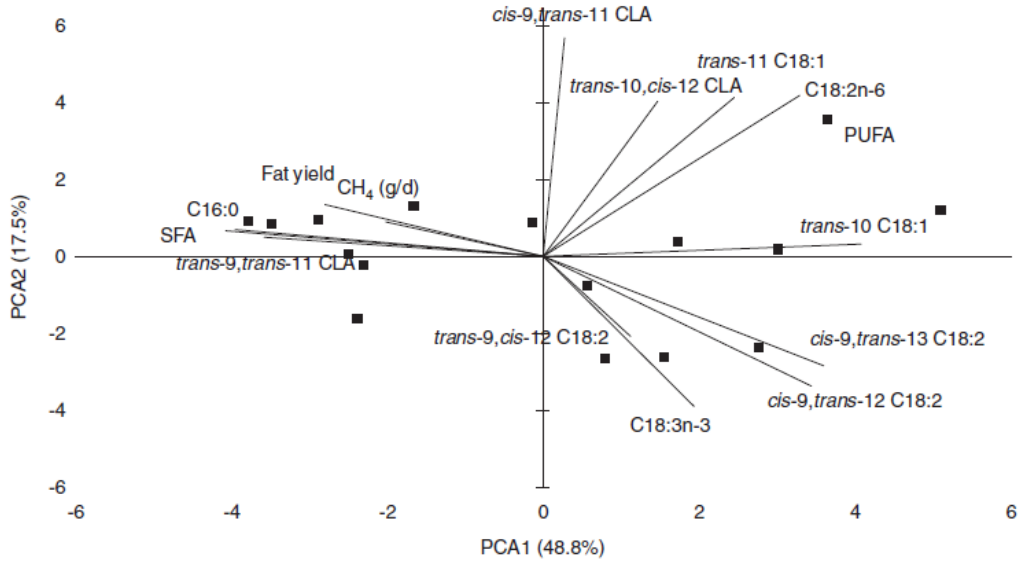

Figure 1 Results of a principal components analysis (PCA) based on the contribution of the different milk fatty acids (FA, % of total FA), milk fat yield (g/day) and methane (CH_4) production (g/day) from dairy cows with the first (PCA1) and second (PCA2) dimensions, and the individual samples (closed squares) (n = 16). SFA = saturated fatty acids; PUFA = Polyunsaturated fatty acids.

Table 5 Enteric methane emissions in dairy cows fed the four experimental diets[1]

Items	PALM	MFD-Starch	MFD-RS	MFD-SF	SEM	P-value
CH_4 (g/day)	374.1	346.3	353.5	349.2	36.0	0.58
CH_4 (g/kg dry matter intake)	20.2	18.7	19.0	18.8	1.61	0.55
CH_4 (% gross energy intake)	5.83	5.58	5.50	5.45	0.473	0.66
CH_4 (g/kg milk)	15.2	12.7	12.9	13.2	1.45	0.09

CH_4 = methane.
[1]PALM = diet with Ca salts of palm oil; MFD-Starch = diet inducing milk fat depression (MFD) rich in starch; MFD-RS = diet inducing MFD with extruded rapeseed; MFD-SF = diet inducing MFD with extruded sunflower seeds.

Table 6 Rumen fermentation parameters and protozoa numbers in dairy cows fed the four experimental diets[1]

Items	PALM	MFD-Starch	MFD-RS	MFD-SF	SEM	P-value
Total VFA (mmol/l)	62.1	59.2	57.6	57.5	12.77	0.98
VFA composition (% of total VFA)						
Acetate	66.1	65.7	64.7	65.1	2.20	0.92
Propionate	19.3	19.4	18.0	19.8	1.87	0.89
Butyrate	11.0	10.4	12.8	11.2	0.71	0.14
Acetate:propionate	3.68	3.48	3.67	3.38	0.453	0.93
NH_3-N (mmol/l)	2.24	0.91	1.18	1.61	0.391	0.15
Total protozoa[2] (10^4 cells/ml)	383.5	206.6	186.8	158.2	87.70	0.32
Entodiniomorphs (10^4 cells/ml)	378.4	205.0	186.0	156.4	86.20	0.32
Holotrichs (10^4 cells/ml)	5.07	0.73	0.85	1.79	2.258	0.46

VFA = volatile fatty acids.
[1]PALM = diet with Ca salts of palm oil; MFD-Starch = diet inducing milk fat depression (MFD) rich in starch; MFD-RS = diet inducing MFD with extruded rapeseed; MFD-SF = diet inducing MFD with extruded sunflower seeds.
[2]For protozoa, statistical analyses have been done with $\log_{10}$ values.

composition, N-NH_3 concentration as well as protozoal counts were unaffected by diets (Table 6). Total tract apparent digestibility of nutrients (OM, CP, GE) was similar among diets, except for fibre (and more particularly for ADF) and starch. Digestibility of ADF was lower for MFD-Starch and PALM (−4.8% unit on average, $P < 0.05$), and starch digestibility was lower for PALM (−1.3% unit on average, $P < 0.05$) compared to the other diets (Supplementary Table S3).

Milk concentrations of C4:0 and *trans*-9,*cis*-12 C18:2 (+ *trans*-10 C19:1) were correlated with daily CH_4 emissions (g/day) (r = 0.52, and −0.62, respectively, < 0.05;

Supplementary Table S4 and Figure 1). We observed negative relationships between iso C17:0 (+ *trans*-9 C16:1), *cis*-11 C18:1 and *trans*-9,*cis*-12 C18:2 (+ *trans*-10 C19:1) and CH_4 emissions (g/kg of DMI) (r = −0.50, −0.50 and −0.70, respectively). Milk concentration of C16:0 and CH_4 emissions (g/kg of milk) were positively related (r = 0.55) while nega- tive relationships were observed with total odd-FA, *iso* C17:0 (+ *trans*-9 C16:1), *trans*-9,*cis*-12 C18:2 (+ *trans*-10 C19:1), C18:3n-3 and the total n-3 FA.

Discussion

Effect of milk fat depression-inducing diets on milk fat content and fatty acid composition

In dairy cows, MFD occurs with particular diets such as starch-rich diets supplemented or not with unsaturated lipids (Bauman and Griinari, 2003). As expected, the three MFD-inducing diets (MFD-Starch, MFD-RS and MFD-SF) led to low milk fat content, below the French national average (3.93%; France Conseil Elevage (FCEL), 2013). Furthermore, milk fat content decreased with MFD-inducing diets in comparison to the pre-experimental milk fat content values (−26 ± 9.7% on average for three diets), and more particularly with MFD-SF (−40%) and MFD-RS (−27%). In addition, the expected shift from trans-11 to trans-10 C18:1 concentrations in milk fat reported in the case of MFD in dairy cows (Bauman and Griinari, 2003) was observed with the three MFD-inducing diets.

Concerning PALM, rich in SFA, we also observed lower milk fat content as compared to the pre-experimental value (−10%) as well as the shift from *trans*-11 to *trans*-10 C18:1 concentrations as observed with three MFD diets, which characterized the MFD situation. Rather, we expected a greater milk fat yield with PALM than with the MFD-inducing diets. Mosley et al. (2007) reported that high-palmitic acid supplements increased the milk fat yield in dairy cows. The low milk fat content observed with PALM diet could be due to the strongest reduction in the sum of milk FA < 16C, because the secretion of de novo synthesized FA is reduced in the case of MFD. The plausible explanation of the low milk fat content observed with the MFD-Starch may be related to a lower rumen pH because of a rapid fermentation of dietary starch (Sauvant et al., 2018). The possible low ruminal pH with this type of diets could induce a shift from *trans*-11 to *trans*-10 C18:1 in milk fat (Zened et al., 2013). In addition, the MFD observed with PALM and MFD-Starch diets could also be explained by the shift from trans-11 to trans-10 C18:1 in milk fat, or by the concentration of other RBH intermediates such as *trans*-10,*cis*-12 CLA. These FA with *trans* 10-double bonds are known to inhibit milk fat secretion (Shingfield et al., 2010).

The milk FA profiles were different between MFD-RS andMFD-SF. The higher intake of *cis*-9 and *cis*-11 C18:1, and C18:3n-3 from cows fed MFD-RS in comparison to MFD-SF produced milk fat with greater concentrations of these FA. Furthermore, milk FA profile with MFD-RS was characterized by higher concentrations of several RBH intermediates of C18:3n-3 (*trans*-9,*trans*-12 and *trans*-11,*cis*-15 C18:2, *trans*-7,*trans*-9, *trans*-7,*cis*-9, *cis*-11,*trans*-13, *trans*-11,*trans*- 13 and *trans*-12,*trans*-14 CLA), as reported by Shingfield et al. (2010) and Ferlay et al. (2017). In agreement with our results, Chilliard et al. (2007) also reported a greater milk concentration of *trans*-7,*cis*-9 CLA along with a high level of *cis*-9 C18:1 intake, and greater milk concentrations of *trans*-11,*cis*-15, *trans*-11,*trans*-13 and *trans*-12,*trans*-14 CLA with a higher level of 18:3n-3 intake.

Cows fed the MFD-SF diet had the greatest milk concentrations of C18:2n-6 and several RBH intermediates of the C18:2n-6 (*trans*-10 C18:1, *trans*-12 C18:1, *cis*-12 C18:1, *cis*-16 C18:1, *cis*-9,*trans*-11 CLA, *trans*-10,*cis*-12 CLA, and *trans*-10,*trans*-12 CLA), and total PUFA than MFD-RS. Our results are in line with those of Chilliard et al. (2007), who found greater concentrations of milk *trans*-10 C18:1, *trans*- 12 C18:1 and *trans*-10,*trans*-12 CLA with dairy cows having a high C18:2n-6 intake. Loor et al. (2005) also reported similar results with dairy cows fed high-concentrate diets based on grass hay (forage : concentrate ratio 35 : 65) and supplemented with 5% of sunflower oil. Indeed, they observed greater milk concentrations of trans-10 C18:1 and C18:2n-6 when compared to the same high-concentrate diet supplemented with C18:3n-3 from linseed oil. Nevertheless, according to an indirect comparison from Glasser et al. (2008), no differences of milk *cis*-11 C18:1, C18:3n-3 and C18:2n-6 concentrations were observed with diets supple- mented with either rapeseeds or sunflower seeds, whatever the nature of forage in the basal diet. The higher milk con- centrations of trans FA and PUFA with MFD-SF than MFD-RS suggested a less complete RBH with the former diet.

The different milk FA profiles between MFD-RS and MFD-SF, and especially the greatest concentration of trans- 10,cis-12 CLA, as well as the most pronounced shift from trans-11 to trans-10 C18:1 concentrations in milk observed with MFD-SF suggest that the diet supplemented with C18:2n-6 resulted in a more severe MFD than the diet sup- plemented with cis-9 C18:1 and C18:3n-3, such as the MFD- RS, as shown by He and Armentano (2011). However, the increase in milk trans-10,cis-12 CLA concentration is not observed in all cases of MFD, as shown by Shingfield and Griinari (2007). This fact could explain the low concentration of this FA in the milk of cows fed the MFD-RS diet, even though this diet led to MFD. The MFD-RS and MFD-SF diets probably modified RBH pathways differently, resulting in different milk FA composition and MFD severity.

Milk concentrations of total PUFA, *cis*-16 C18:1, *trans*-10 C18:1 and C18:2n-6 were negatively correlated with milk fat content in our study. This could also explain the low milk fat content observed with all diets, since they produced milk with a great concentration of these FA, and especially the MFD-SF, which showed the more severe MFD. Indeed, when compared to the average 3.93% milk fat content observed in France (FCEL, 2013), the MFD-SF induced the more severe reduction (−1.9%). In addition, we confirm the inhibitory effect of *trans*-10 C18:1 on milk fat content in cows fed diets

supplemented with unsaturated FA (Bauman and Griinari, 2003; Shingfield et al., 2010), and also in dairy cows fed diets rich in starch. Surprisingly, we observed the same phenom- enon with cows fed diets supplemented with SFA from Ca salts of palm oil, but this needs further investigation.

We could also hypothesize that the main forage of the diet, especially the maize silage, was responsible for the low milk fat contents and the MFD observed for all the diets. Indeed, the particle size of the maize silage was fine with more than 30% of particles <0.4 mm (data not shown). In fact, Sauvant and Peyraud (2010) reported that feeding diets containing forages for which particle size was <3 to 4 mm was likely to decrease milk fat content.

Milk FA profile from cows fed MFD-RS presented the highest nutritional quality for human consumption because of the lowest C16:0 concentration and greatest n-3 FA and lowest n-6 FA concentrations, with intermediate trans FA concentration. Indeed, it has been shown that SFA con- sumption in humans increases the risk of cardiovascular diseases (Ferlay et al., 2017), whereas n-3 FA consumption has positive health outcomes in the areas of cardiovascular diseases, platelet aggregation, hypertension, hyperlipidae- mia, cancer, depression and inflammation (McManus et al., 2011). Regarding the human consumption of some trans FA of ruminant origin, such as *trans*-11 C18:1, the negative effect described in the past decade is nowadays questioned since ruminant trans FA intake is not correlated with cardi- ovascular diseases and may even have beneficial effect in human health (Ganguly and Pierce, 2015; Ferlay et al., 2017).

Effect of milk fat depression-inducing diets on methanogenesis

The four diets led to similar CH_4 emissions whatever the unit considered (on average 356 (± 36.0) g/day, 19.2 (±1.61) kg/DMI, 5.6 (±0.47) % gross energy intake (GEI) and 13.5 (±1.45) g/kg milk). Our results are in agreement with data (mean and standard deviation) reported in a recent meta-analysis (Niu et al., 2018), using a wide range of EU diets and fed to dairy cows with similar DMI and milk yield (on average 18.5 kg and 26.4 kg/day, respectively). Indeed, these authors reported an average CH_4 emission of 392 (±88.8) g/day, 21.4 (±3.39) g/kg DMI and 6.4 (±1.04) % of GEI. To the best of our knowledge, no studies have directly compared starch and lipids as sources of energy in methanogenesis in dairy cows. Our study is the first to report that maize silage-based diets supplemented with starch or lipids have the same CH_4 emissions whatever the unit. This is consistent with the absence of variation between diets in rumen fermentation parameters (VFA concentration and composition) and protozoal population, which is known to be a high H_2-producer (Williams and Coleman, 1992).

Moreover, we did not observe any effect of the nature ofthe lipids with maize silage-based diets (total dietary ether extract content ranging from 3.3% to 7.4%) on methanogenesis, which is in agreement with the meta-analysis of Grainger and Beauchemin (2011), who used a large variety of basal diets and total dietary ether extract content ranging from 1.0% to 8.0%. These authors reported no effect of rapeseed-, sunflower- or palm-supplemented diets on CH_4 emissions in dairy cows. In contrast, Martin et al. (2010) have shown a greater negative effect of unsaturated than SFA supplementation on CH_4 emissions, with lipid additions ranging from 1% to 10% of diet DM (3% in our study). These authors explained this difference by a specific and negative effect of unsaturated FA on the microbiota, especially protozoa, which are strongly positively correlated with CH_4 yield (Guyader et al., 2014). In our study, even though the milk FA profile differed between diets, the energy source (starch vs. lipids) did not influence CH_4 emissions whatever the unit, which is in line with the similar rumen fermentation parameters observed with all diets. This is of interest and raises the question about the accuracy of models based on milk FA profile in predicting CH_4 emissions.

Several authors have shown relationships between CH_4 emissions and individual milk FA concentrations using various dietary strategies in either individual experiments (Chilliard et al., 2009) or meta-analysis (Dijkstra et al., 2011; Williams et al., 2014). A positive relationship between C16:0 and CH_4 emissions (g/kg milk) was observed in our study as well as by Chilliard et al. (2009) for CH_4 emissions (g/day) measured from dairy cows fed maize silage-based diets supplemented or not with lipids from linseed. We also reported a negative relationship between CH_4 emissions (g/kg DMI) and cis-11 C18:1, which was highlighted by Dijkstra et al. (2011) in a meta-analysis using different CH_4 emissions mitigation strategies (linseed, lauric acid, myristic acid, mixture of caprylic and capric acids, yucca plant powder, diallyldisulphide or calcium fumarate) in dairy cows fed a mixture of grass and maize silage. However, we did not observe the same positive relationships between CH_4 emissions (g/day) and C8:0 and total C18 FA reported by Williams et al. (2014). This discrepancy between our study and that of Williams et al. (2014) is probably due to the difference in dietary conditions. In Williams et al. (2014) cows were fed pasture (predominantly perennial ryegrass) or lucerne hay-based diets supplemented with different ingredients (linseed, rapeseed, tannin, DHA, grape marc, wheat, almond hulls or citrus hulls). We also observed a negative relationship between *trans*-9,*cis*-12 C18:2 and CH_4 emissions whatever the unit, which was not reported in the previously quoted studies.

Conclusions

The four maize silage-based diets rich in starch or supple- mented with Ca salts of palm oil or rapeseed or sunflower seeds induced MFD in dairy cows leading to low milk fat contents and a shift of *trans*-11 C18:1 to *trans*-10 C18:1 in milk fat, but did not change milk production, enteric CH_4 emissions or other digestive processes. Neither the energy (starch vs. lipids) nor the lipid sources modified methanogenesis, but the milk FA profiles differed greatly among diets.

Furthermore, we noted positive or negative correlations between some individual milk FA and CH_4 emissions.

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Declaration of interest

No conflicts of interest to declare.

Ethics statement

Procedures involving animals were performed in accordance with the French Ministry of Agriculture guidelines for animal research and with the applicable European Union guidelines and regulations on animal experiments (Directive 2010/63). The Auvergne regional ethics committee on animal experimentation approved the experiment (reference number 821-2015060811534198).

Software and data repository resources

None of the data were deposited in an official repository.

Supplementary Material

To view supplementary material for this article, please visit https://doi.org/10.1017/S1751731118003154

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Table S1 *Concentrations of minor fatty acids in milk fat from dairy cows fed the 4 experimental diets*[1]

Fatty acid[2] (g/100 g of total FA)	PALM	MFD-Starch	MFD-RS	MFD-SF	SEM	*P*-values
C5:0	0.01[b]	0.03[a]	0.01[a]	0.01[ab]	0.002	0.01
C7:0	0.01[b]	0.02[a]	0.01[ab]	0.01[b]	0.002	0.03
C9:0	0.006[b]	0.015[a]	0.008[ab]	0.005[b]	0.002	0.04
cis-9 C10:1	0.07[b]	0.17[a]	0.12[ab]	0.06[b]	0.023	< 0.01
C11:0	0.01	0.03	0.02	0.01	0.005	0.13
cis-9 C12:1	0.007	0.009	0.008	0.008	0.0008	0.07
C13:0	0.03	0.07	0.05	0.05	0.01	0.20
iso C13:0	0.02	0.003	0.02	0.03	0.0053	0.70
anteiso C13:0	0.02	0.06	0.05	0.03	0.011	0.16
trans-9 C14:1	0.010	0.014	0.015	0.011	0.0025	0.33
cis-11 C16:1	0.04	0.05	0.05	0.03	0.009	0.49
trans-6,7,8 C16:1	0.05[b]	0.04[c]	0.06[a]	0.06[a]	0.004	0.002
iso C18:0	0.03[c]	0.07[a]	0.04[b]	0.04[b]	0.003	0.0001
cis-9 C20:1	0.10[b]	0.10[b]	0.14[a]	0.11[ab]	0.010	0.04
cis-11 C20:1	0.08[b]	0.10[b]	0.25[a]	0.10[b]	0.0151	0.0001
C20:0	0.11	0.10	0.16	0.11	0.019	0.20
C21:0	0.02	0.03	0.03	0.04	0.005	0.34
C22:0	0.03	0.03	0.06	0.06	0.009	0.05
C23:0	0.02	0.03	0.03	0.02	0.006	0.75
C24:0	0.02	0.03	0.04	0.02	0.004	0.10
C20:2n-6	0.04	0.04	0.03	0.05	0.004	0.09
C20:3n-6	0.06[b]	0.10[a]	0.06[b]	0.07[b]	0.007	0.009
C22:4n-6	0.01[ab]	0.03[a]	0.02[b]	0.02[b]	0.003	0.02

[1]PALM = diet with Ca salts of palm oil; MFD-Starch = diet rich in starch, MFD-RS = diet with extruded rapeseed, MFD-SF = diet with extruded sunflower seeds.

[a,b,c] Values within a row with different superscripts differ significantly at $P < 0.05$.

Table S2 *Pearson correlation coefficient[1] (r) between milk fatty acid concentrations (g/100 g of total fatty acids) and milk fat content (g/kg) in dairy cows fed the 4 experimental diets[2] (n = 16)*

Item	r
C16 :0	0.65
Even SFA	0.64
SFA[3]	0.62
trans-11,*cis*-13 CLA	0.57
trans-9,*trans*-11 CLA	0.52
trans-13 C18:1	-0.50
cis-13 C18:1	-0.54
trans-5 C18:1	-0.54
C18:2n-6	-0.54
C21:0	-0.54
cis-9,*trans*-12 C18:2 (+*cis*-9,*trans*-14 C18:2)	-0.55
MUFA[4]	-0.55
trans-6,7,8 C18:1	-0.57
trans-6,7,8 C16:1	-0.59
trans-12 C18:1 (+*cis*-6,7,8 C18:1)	-0.59
cis-16 C18:1	-0.62
PUFA[5]	-0.62
cis-9,*trans*-13 C18:2	-0.63
iso 17:0 (+ *trans*-9 C16:1)	-0.73
trans-10 C18:1	-0.77

[1]Only $r \geq 0.50$ or $r \leq -0.50$ ($P < 0.05$) are reported.

[2]PALM = diet with Ca salts of palm oil; MFD-Starch = diet rich in starch, MFD-RS = diet with extruded rapeseed, MFD-SF = diet with extruded sunflower seeds.

[3]SFA: Saturated fatty acids.

[4]MUFA: Monounsaturated fatty acids.

[5]PUFA: Polyunsaturated fatty acids.

Table S3 *Total-tract apparent digestibility of the 4 experimental diets in dairy cows*[1]

Items (%)	PALM	MFD-Starch	MFD-RS	MFD-SF	SEM	*P*-value
Organic matter	68.9	68.9	70.0	70.6	0.72	0.29
Crude protein	49.9	49.4	49.0	51.9	1.71	0.64
NDF	58.0	57.5	60.0	62.1	1.13	0.06
ADF	49.1^{bc}	46.4^{c}	51.0^{ab}	54.0^{a}	1.39	0.02
Starch	96.7^{b}	98.3^{a}	98.1^{a}	97.6^{ab}	0.56	0.02
Gross energy	64.8	64.0	64.8	65.9	1.21	0.75

[1]PALM = diet with Ca salts of palm oil; MFD-Starch = diet rich in starch, MFD-RS = diet with extruded rapeseed, MFD-SF = diet with extruded sunflower seeds.

[a, b, c] Values within a row with different superscripts differ significantly at $P < 0.05$.

Table S4 *Pearson correlation coefficient[1](r) between individual milk fatty acid (g/100 g of total fatty acids) and methane emissions (in different units) in dairy cows fed the 4 experimental diets[2]*

	r		
Item	CH_4 g/d	CH_4 g/kg of DMI	CH_4 g/kg of milk
C4:0	0.52		
C16:0			055
Odd-chain milk FA			-0.50
iso 17:0 (+ *trans*-9 C16:1)		-0.50	-0.53
cis-11 C18:1		-0.51	
trans-9,cis-12 C18:2 (+ *trans*-10 C19:1)	-0.62	-0.70	-0.69
C18:3n-3			-0.58
PUFAn-3			-0.53

[1]Only $r \geq 0.50$ or $r \leq -0.50$ ($P < 0.05$) are reported.

[2]PALM = diet with Ca salts of palm oil; MFD-Starch = diet rich in starch, MFD-RS = diet with extruded rapeseed, MFD-SF = diet with extruded sunflower seeds.

IV. CHAPTER IV

Individual milk fatty acids are potential predictors of enteric CH_4 emissions from dairy cows fed a wide range of diets: approach by meta-analysis

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Individual milk fatty acids are potential predictors of enteric CH_4 emissions from dairy cows fed a wide range of diets: approach by meta-analysis.

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Interpretive summary

Individual milk fatty acids are potential predictors of enteric CH_4 emissions from dairy cows fed a wide range of diets: approach by meta-analysis. Bougouin et al., 2018. Dairy cows contribute to greenhouse gas emissions through enteric CH_4 production. There is a need to quantify CH_4 emissions with low-cost measurement methods to assess CH_4 mitigation strategies on large scale. Equations based on milk fatty acids alone or with dry matter intake, diet composition, and BW lead to an average prediction error of 16% to 24%, which allow the estimation of a difference of 65 g/day of CH_4 emissions.

ABSTRACT

There is a need to quantify CH_4 emissions with alternative measurement methods. For the past decade, milk fatty acids (**MFA**) have been used as proxies to predict CH_4 emissions from dairy cows because of potential common rumen biochemical pathways. However, equations have been developed based on a narrow range of diets and with limited number of data. The study's objectives were to (1) construct a set of empirical models based on individual data of CH_4 emissions and MFA from a large number of lactating dairy cows fed a wide range of diets, (2) to further increase models' level of complexity (from farm to research level), with additional independent variables such as dietary chemical composition [organic matter (**OM**); NDF; crude protein (**CP**); Starch; ether extract (**EE**)], dairy performance (milk yield and composition) and animal characteristics [days in milk (**DIM**) or body weight (**BW**)], and (3) to evaluate the models' performance on independent datasets including measurements from individual animals or average measurements of groups of animals. Prediction equations based only on MFA [C10:0, *iso* C17:0 + *trans*-9 C16:1, *cis*-11 C18:1, and *trans*-11,*cis*-15 C18:2 for CH_4 production (g/d); *iso* C16:0, *cis*-11 C18:1, *trans*-10 C18:1 and *cis*-9,cis-12 C18:2 for CH_4 yield (g/kg of DMI); *iso* C16:0, *cis*-15 C18:1 and *trans*-10+*trans*-11 C18:1 for CH_4 intensity (g/kg of milk)], have root mean square error of 58.6 g/d, 2.8 g/kg DMI and 3.7 g/kg milk, respectively, whereas complex equations that additionally used DMI, dietary NDF, EE starch contents, and BW had lower root mean square error of 42.8 g/d, 2.5 g/kg DMI and 3.3 g/kg milk, respectively). External evaluation with individual data not used for equations development, led to the better performance of the equations as compared to external evaluation with mean data. MFA are potential predictors of enteric CH_4 emissions and in order to be used on farm, prediction equations should be further developed using MFA predicted by MIRS.

Key Words: dairy cow, methane emissions, prediction models, milk fatty acids

INTRODUCTION

Enteric methane (**CH_4**) emissions have been recognized as a major source of greenhouse gases (**GHG**) in livestock farming. Dairy cows' CH_4 emissions account for 46% of the total GHG emissions in the dairy supply chain, when expressed as carbon dioxide (**CO_2**)-equivalents (Gerber et al., 2013). The global demand for livestock products is constantly increasing (FAO, 2013), and ruminants are almost the sole source of milk for humans, providing 644 million tons of fat-protein corrected milk, among which dairy cattle contribute to 80% (Gerber et al., 2013). The increasing demand for dairy products led to the expansion of dairy herds. Therefore, there is a need for strategies to reduce CH_4 emissions that would limit the negative impact of dairy cows on the environment. Several dietary strategies such as formulating diets rich in concentrate (and more particularly in starch), or supplementing diets with lipids or other chemical additives (3-NOP, nitrate, monensin; Hristov et al., 2013; Martin et al., 2010; Odongo et al., 2007) have proven their efficacy to reduce CH_4 emissions from dairy cows. Methanogenesis is the main pathway that uses H_2, an unavoidable by-product resulting from dietary carbohydrate fermentation (48 to 80%; Mills et al., 2001), while rumen biohydrogenation of unsaturated fatty acids (**FA**) uses up to 2.6% of rumen H_2 (Mills et al., 2001). Fermentation in the rumen also leads to the production of volatile fatty acids (**VFA**), which are precursors for de novo synthesis of short- and medium-chain FA in the mammary gland. In addition, certain VFA production pathways, such as acetate or butyrate, lead to production of H_2, while propionate production pathways uses hydrogen (**H_2**). Thus, direct interactions exist between rumen fermentation, CH_4 production, and milk FA (**MFA**) composition.

Predictive tools such as empirical equations or mechanistic models for estimating CH_4 emissions are useful for evaluating potential strategies for methane mitigation, especially because measurement techniques, such as open respiratory chambers or SF_6 gas tracer, are costly and may be difficult to apply on large-scale dairy farms. Although numerous models

have been developed to predict CH_4 emissions from dairy cows based only on milk FA (Van Gastelen and Dijkstra, 2016), milk FA and other variables such as milk production (Weill et al., 2008) or forage intake (Chilliard et al., 2009), the equations generally only accurately predict CH_4 emissions for specific diets and situations similar to those under which the equations were developed. For example, the prediction equations presented by Chilliard et al. (2009) were developed using data from dairy cows consuming corn-silage based diets containing linseed. Furthermore, previous studies usually involved small numbers of dairy cows [e.g. Chilliard et al. (2009) 8 cows; Mohammed et al. (2011) 16 cows; Dijkstra et al. (2011) 100 cows; Van Gastelen et al. (2017) 32 cows; Van Gastelen et al. (2018) 218 cows].

The objectives of the present study were: 1) to construct a set of empirical models based on individual data of CH_4 emissions and milk composition (milk FA) from a large number of lactating dairy cows consuming a wide range of diets; 2) to further increase level of complexity (from research to farm level) of the developed models, based on additional independent variables such as dietary chemical composition, production performance (milk yield and composition) and animal characteristics (days in milk and body weight); and (3) to evaluate the performance of these models using independent datasets.

MATERIALS AND METHODS

Databases and variable selection

Individual animal data. The database was created using measurements made on individual animals received from collaborators in order to develop prediction equation of CH_4 based on MFA. For inclusion in the database, experiments must have met the following criteria: 1) CH_4 production must have been measured on individual dairy cows by means of respiration chambers, the SF_6 gas tracer technique, or GreenFeed system, 2) MFA profiles of individual cows analyzed by gas chromatography, 3) actual measurements of daily DMI of individual cows, 4) actual measurements of dietary composition, 5) actual measurements of milk

production and composition of individual cows, and 6) characteristics of individual cows [body weight (**BW**) and days in milk (**DIM**)] must have been recorded. Details of selected experiments are given in Table S1. Briefly, the dataset contained 312 observations from published and unpublished experiments (17 experiments) by INRA-UMRH (France), 119 individual observations (5 experiments) from Aarhus University (Denmark), 218 observations (7 experiments) from Ellinbank Research Centre (Australia), and 177 observations (5 experiments) from AAFC Sherbrooke (Canada). A total of 825 observations of CH_4 emissions (g/day) from individual lactating Holstein dairy cows, and related to DMI and diet chemical composition [organic matter (**OM**); NDF; crude protein (**CP**); Starch; ether extract (**EE**)], animal characteristics (BW, DIM), milk performance (milk yield and milk composition: fat, protein, lactose) and CH_4 mitigation treatments were obtained from the 34 *in vivo* experiments (15 randomized block and 19 Latin-square designs). A wide range of dietary treatments was included in the dataset. Main dietary forages were corn silage (n = 297), grass silage (n =157) or legume hay (n =157). Main concentrate ingredients included in the diets were rapeseed (n =264), corn grain (n =198), barley (n =124), and wheat (n = 83). Database included control diets (n =198) and CH_4 mitigating treatments, as described in Martin et al. (2010), including lipid supplementation (n =198), different forage or concentrate natures (n =149; n =140), probiotics (n =58), plant extract (n =33) or nitrate (n=16). Details of experiments were summarized in Table S1. The dataset gathered studies that tested the lipid dose effects (mean EE of lipid-supplemented diets of 7 ± 3.9 % of DM), the lipid source [linseed, rapeseed, Ca-salt of palm oil, sunflower, dry distiller's grain with solubles; mean EE of 5.5 ± 0.8 % of dry matter (**DM**); n=4 experiments] or form (crushed, extruded, cake, oil; mean EE of 6.0 ± 0.4% of DM; n=1 experiment) effects, the forage source effects (mean NDF of 35 ± 6.4 % of DM; n=7), the effect of different composition of concentrate (starch-rich; sugar-rich; lipid-rich; protein-rich; mean starch of 23 ± 9.9 % of DM; n=6 experiments) on CH_4 emissions. In several

experiments, various additives were tested for the effects of nature and/or dose of additive on CH_4 emissions. These included probiotics (4 experiments), tannins (2 experiments), lipid + calcium nitrate (2 experiments), saponin (2 experiment), or other plant extracts (3 experiments). Milk FA fractions were expressed in g/100 g of total milk FA. Some studies reported co-elution of different MFA, thus when these FA were individually identified in other studies (e.g. *iso* C17:0 + *tr*-9 C16:1), they were grouped together. Furthermore, milk FA with concentration < 0.1 g/100 g of total FA were not included in the dataset.

Dataset including the individual animal observations was randomly divided into two datasets: 1) a dataset containing 70% of the data called "training dataset" and used to develop prediction models, and 2) a second dataset containing 30% of the data, called "external individual dataset", and used to evaluate the robustness of the models (Table 1).

Mean database. Another database called "external mean dataset" was built with treatment means from the literature and was used to further evaluate the robustness of the models (Table 1). A comprehensive literature search (up to January 2018) was conducted using Science Direct, CAB International, SCOPUS, and Web of Knowledge online databases, with the following search terms: "methane" or "methane emission", "dairy", "cows" or "livestock" or "cattle", and "milk fatty acid". To be included in the data set, the studies were required to meet the same criteria used for selecting individual animal data. A total of 25 studies (Table S2) were selected and used for model evaluation and a summary statistics are given in Table 1. Briefly, the external mean dataset included studies testing the effect of different dietary strategies on CH_4 emissions, as described in Martin et al. (2010): 1) lipid dose (mean EE of 5 ± 1.5 % of DM); 2) lipid nature or form; 3) forage nature or level (mean NDF of 37.68 ± 1.326 % of DM); 4) concentrate nature or level (mean Starch content of 20.7 ± 7.6 % DM; mean % of concentrate of 35.7 ± 13.4 %); 5) probiotics; 6) organic acids; 7) plant extracts; 8) feed additives such as nitrate, monensin and 3-NOP.

Statistical analyses

Variable pre-selection for model development. An exploratory data analysis was performed to evaluate the data for completeness (e.g., missing values of nutrient composition of diets), consistentancy in nomenclature of variables in question, and the presence of outliers (Pyle, 1999). When not measured, diet chemical composition, specifically NDF, starch and EE were estimated using feed composition tables in INRA (2007), NRC (2001), and Feedipedia (https://www.feedipedia.org/). Measured or calculated variables and their summary statistics are given in Table 1. We detected outliers by using boxplot function in R (version 0.98.1102, R Foundation for Statistical Computing, Vienna, Austria). The outliers' values were compared with the range of reference values. When values were outside of this range, we requested further information from the data owner in order to understand this study effect or to decide to remove the data from further analyses. The number of observations for each variable are provided in Table 1.

Correlation among variables and identification of predictors. Data including observations (n = 825) made on individual animals were used in this analysis. Firstly, Pearson's correlation coefficients were obtained for pairwise relationships among the 46 collected individual MFA (concentration >0.1 g/100 g of milk FA) and CH_4 emissions to determine and select the most correlated individual MFA among the five MFA families (saturated FA [**SFA**], odd- and branched- FA [**OBCFA**], *cis* monounsaturated FA [***cis*-MUFA**], *trans* monounsaturated FA [***trans*-MUFA**] and polyunsaturated FA [**PUFA**]). Milk FA missing for more than 50% of the observations were not considered in this analysis. Secondly, the Pearson's correlation coefficients were obtained for each selected MFA within its family, in order to determine how independent ($r \leq 0.5$) they were and thereby avoid potential collinearity in model development. Then, principal components analysis (**PCA**) was performed, using the *FactoMinR* and *MissMDA* packages (version 1.34 and 1.7.3, respectively) in R (version 0.98.1102, R

Foundation for Statistical Computing, Vienna, Austria), on the MFA significantly associated with daily CH_4 production (g/day; $r \geq |0.3|$), CH_4 yield (g/kg of DMI; $r \geq |0.3|$) and CH_4 intensity (g/kg of milk; $r \geq |0.2|$) along with other variables in order to identify additional predictors of CH_4 emissions.

Random-effects model analysis. As mentioned, data from the entire dataset (n=825) were randomly divided (*dplyr* package in R) to a training dataset (n=578 observations) for model development, and to another dataset (hereafter called external individual dataset) (n = 247) for model evaluation. A set of linear mixed-effects models was constructed to predict separately CH_4 production (g/day), yield (g/kg of DMI), or intensity (g/ kg of milk). Random-effect meta-analysis approaches (St-Pierre, 2001) were applied using *nlme* package (version 3.1-131) in R (version 0.98.1102, R Foundation for Statistical Computing, Vienna, Austria). The nlme function fits linear mixed-effects models in the framework described in Lindstrom and Bates (1990). Several models were developed with increasing level of complexity by incrementally adding different independent quantitative variables: dietary content (% DM) of CP, NDF, ADF, EE, starch, BW (kg), and DIM (d), milk yield (kg/d), milk fat, protein and lactose percentages. A first set of models began with the most representative MFA (in % of total FA) of each family selected based on pairwise correlations and PCA analysis. Then DMI was added to the simplest models based on MFA followed by milk performance, or animal characteristics, or diet composition (Figure 1).

Finally, all significant variables were included together to create highly complex models.

Furthermore, this approach enables analysis of fixed effects of independent variables such as MFA, or DMI (Figure 1), as well as the study-specific deviation of the CH_4 emission response, which was taken into account as a random effect. The general mixed-effect model for single and multiple regressions is represented as:

$$Y = \beta_0 + \beta_1 X_{1ij} + \beta_2 X_{2ij} + \ldots + \beta_n X_{nij} + e_{ij},$$

where β_0, $\beta_1 X_{1ij}$, and $\beta_2 X_{2ij}$, . . ., $\beta_n X_{nij}$ are the fixed effects of independent variables (intercept and effects), and e_{ij} are the random experiment effects (i = 1, . . ., n studies and j = 1, . . ., n_i observations).

Pairwise Pearson's correlations for variables with absolute value of $|r| \geq 0.5$ were not included simultaneously in the model development. Indeed, multicollinearity can lead to issues in developing models, such as inaccurate model parameterization, decreased statistical power and exclusion of significant predictor variables during model construction (Graham, 2003). Only variables with *P* value < 0.10 were retained in the model.

Models associated first with the lowest Bayesian information criterion (**BIC**), and then the lowest residual standard error (**RMSE**) with the highest R^2 were selected as the best models to predict each CH_4 emission response at each level of complexity. Adjusted dependent variable values were calculated based on regression parameters of the final model for each level of complexity to determine adjusted R^2 values corrected for random experiment effect (St-Pierre, 2001).

Prediction error (predicted value minus observed value) were visually inspected for any pattern. Moreover qualitative factors, CH_4 mitigation strategies classified according to Martin et al. (2010), were tested by running Anova in R using the *stats* package (version 3.6.0). These mitigation strategies were classified on the basis of forage type (alfalfa; association; barley; chicory; clover; cocksfoot grass; corn; grass; red clover; and timothy), lipid type represented by major FA from lipid supplementations (C16:0; *cis*-9 C18:1; C18:2 n-6; and C18:3 n-3), concentrate type (starch-rich, sugar-rich, lipid-rich, and protein-rich), or feed additives (nitrate, tannin, saponin, and other plant extract).

Model evaluation. The potential of each developed model to accurately predict CH_4 production was assessed on two independent datasets of individual or mean observations (Figure 1). According to Appuhamy et al. (2016), a combination of model evaluation metrics was used to

assess model performance. Briefly, square root of mean square of prediction error (**MSPE**), expressed as a % of the observed mean (**RMSPE**) was calculated. Smaller RMSPE indicates better model performance. The MSPE can be decomposed into 3 parts, error due to central tendency or mean bias (**ECT**), error due to deviation of the regression slope or slope bias (ER) and error due to the disturbance or random bias (**ED**) (Bibby and Toutenburg, 1977). The concordance correlation coefficient (**CCC**) (Lin, 1989) was calculated. The CCC is a product of the Pearson correlation coefficient of the relationship between predicted and observed values and the bias correction factor (C_b, measure of accuracy) indicating how far the best fit line deviates from the concordance or unity line of the observed values *vs.* predicted values plot. The CCC ranges from 0 to 1 with greater values better model performance. When using different data to compare the performance of models, we can use the ratio of RMSPE and standard deviation of the data (observed values), namely RMSPE-observations standard deviation ratio (**RSR**) as it takes standardized model performance relative to the variability in observations in different datasets (Moriasi et al., 2007). Smaller RSR (<1) indicates better performance given the variability of observations. Model performance were primarily ranked based on RSR, followed by RMSPE, and then the other criteria.

RESULTS

Database

The individual animal observations contained diets based on 30 to 100% (% DM) of forages that were either pasture, silage, hay, or haylage (alfalfa, barley, corn, timothy, clover, chicory, ray-grass, and cocksfoot grass). The experiments included a large variety of dietary strategies with different forage:concentrate ratio, nature of concentrate or forage, supplemented or not, with lipids (fat, oil, or fatty acids), plant extracts (essential oils, tannins, and saponins), chemical additives (nitrate), and probiotics (*Saccharomyces cerevisiae*). The dataset based on mean data

from the literature included other supplementations such as 3-NOP, monensin, or plant extracts (Table S2).

Overall, individual data show wide range of values in predictor (e.g. MFA, DMI, milk, etc…) and response (e.g. CH_4 production in g/day, CH_4 yield in g/kg of DMI and CH_4 intensity in g/kg of milk) variables, promoting the development of models capable of predicting CH_4 emissions across wide variety of production conditions in dairy cows. Individual milk FA were considerably variable with CV ranging from 20% to more than 100% (Table 1). The variables of CH_4 emissions were also very variable with an averaged CV of 28%. The average DMI and milk yield were 20.5 kg/day and 28.6 kg/day per cow, respectively. In line with individual animal observations, individual MFA in the external mean dataset had large CV. Moreover, mean DMI and milk yield were similar between individual and mean data (21.0 and 31.3 kg/day, respectively).

Pearson correlation coefficients between CH_4 emissions and individual milk FA

Among individual milk FA concentrations, C10:0 and C8:0 had positive relationships with CH_4 production (r = 0.33; r= 0.34, $P < 0.05$; Table 3). CH_4 yield and intensity were positively related to C16:0 (r= 0.26, $P < 0.05$). CH_4 production was inversely related to *iso* C17:0 (coeluted with *trans*-9 C16:1) (r=-0.32, $P < 0.05$). CH_4 yield and intensity had positive relationships with *iso* C16:0 (r=-0.27 and r=0.33, respectively; $P < 0.05$). Negative correlations between CH_4 production, yield and intensity were observed with *cis*-10 C18:1, *cis*-11 C18:1 and *cis*-15 C18:1. However, less than 50% of the data were reported for *cis*-10 C18:1 (data not shown). Methane production, yield and intensity were negatively correlated with *trans*-10 C18:1 and *trans*-10+*trans*-11 C18:1, with Pearson coefficient correlations varying from -0.34 to -0.45 ($P < 0.05$). The *trans*-11,*cis*-15 C18:2 was inversely correlated to CH_4 production (r=-0.29; $P < 0.05$), and *cis*-9,*cis*-12 C18:2 was negatively correlated to CH_4 yield (r=-0.30, $P < 0.05$).

Mixed effect models

Models for daily CH_4 production. Models to predict daily CH_4 production are given in Table 2. Daily CH_4 production had positive relationships with C10:0, DMI, NDF, milk yield, milk fat and protein percentages, and BW. There were negative relationships of several MFA, such as *iso* C17:0 (+*trans*-9 C16:1), *cis*-11 C18:1, *trans*-10 C18:1 and *trans*-11,*cis*-15 C18:2, with CH_4 production. The best simple model included 4 milk FA and had RMSE of 58.6 g/day with R^2=0.72 (14.4%; Table 2). The RSR was 0.88 and 1.22, CCC of 0.53 and 0.01, and RMSPE of 24.0 and 27.7%, with the external individual and mean datasets, respectively (equation 1; Table 2). The MFA model's error was mainly associated with error due to disturbance (ED of 96%) in the evaluation with the external individual dataset, whereas with the external mean dataset, the error was mainly due to the central tendency (56%).

When DMI was added to the simple model based on MFA, RMSE decreased from 14.4% to 12.4%, and R^2 increased from 0.72 to 0.79 (equation 2; Table 2). We also observed better prediction ability as RSR decreased from 0.88 and 1.22 to 0.67 and 0.97 in the external individual dataset and external mean dataset used for model evaluations, respectively. Moreover, CCC increased from 0.53 to 0.83 with the external individual dataset, and RMSPE (%) decreased from 24.0% and 27.7% to 17.9% and 21.9% with the external individual dataset and external mean dataset, respectively. When DMI, dietary NDF and EE contents and BW were included along with the MFA (*iso* C17:0 + *trans*-16 C16:1, *cis*-11 C18:1, and *trans*-11,*cis*-15 C18:2) in equation 9 (RMSE = 42.8 g/d; R^2=0.85), we observed the best performance. The RSR and CCC analysis showed had the lowest RSR (0.56) and the greatest CCC (0.90) for equation 9 as compared to other models, when evaluated on the external individual dataset. Consistently the equation 9 was related to the smallest RMSPE when evaluated in both external datasets (15.6 and 15.9%, respectively). Error was due to random variability of data as indicated by substantial dispersion error (99.6 and 75.8% when evaluated on external individual and mean datasets, respectively). Model including all the variables (equation 11) had RSR, RMSPE and

CCC similar to those of equation 9 (16.4%) indicating the potential to have better prediction power with simpler models.

Models for CH_4 yield. There were positive relationships between CH_4 yield and C16:0, *iso* C16:0, but negative relationships between CH_4 yield and unsaturated FA, such as *cis*-11 C18:1, *trans*-10 C18:1 and *cis*-9,*cis*-12 C18:2 (equations 12, 13, 14, 15 and 16). CH_4 yield had positive and negative relationships with dietary NDF and EE and milk yield, respectively. The MFA model (equation 12) has a RMSE of 2.8 g/kg of DMI (13.9%) with R^2=0.70 (Table 3). The evaluation resulted in RSR of 0.84 and 1.14, CCC of 0.71 and 0.27, with the external individual dataset and external mean dataset, respectively. Equation 13 (RMSE = 2.6; R^2=0.72) based on MFA (n=5), and dietary NDF and EE had the best prediction abilities (Table 3) with lower RSR (0.84 and 0.92) and higher CCC (0.76 and 0.66) in both external evaluation datasets as compared to the other models. The RMSPE values were 18.8% and 17.0% in the external individual dataset and external mean dataset, respectively. Random error accounted for the biggest part of the total prediction error (> 80%). When all variables were included (equation 17), the RSR (0.85 and 0.95) were close to those from equation 13, but RMSPE was increased when this equation was evaluated in both external evaluation datasets.

Models for CH_4 intensity. Milk *iso* C16:0 content was positively related to CH_4 intensity, whereas negative association was found with milk *cis*-15 C18:1 and *trans*-10+*trans*-11 C18:1 contents in equation 18 (Table 4), which had a RMSE of 3.7 g/kg of milk (23.9%) and R^2=0.61. The evaluation of the model in the external individual dataset and external mean dataset resulted in RSR of 0.93 and 1.42, CCC of 0.56 and 0.34, and RMSPE of 27.4 and 28.2%, respectively. Equation 22 (RMSE=3.3 and R^2=0.67; Table 4), based on milk C16:0, *iso* C16:0, *trans*-10 C18:1, dietary NDF and animal characteristics (DIM and BW), had the best prediction abilities with low RSR (0.79 and 0.63) and high CCC (0.74 and 0.79) in both external evaluation datasets as compared to the other models. The RMSPE values were 24.3% and 15.4% when equation

22 was evaluated in the external individual dataset and external mean dataset, respectively. As already observed for CH_4 yield (g/kg of DMI), there were positive relationships between CH_4 intensity and C16:0, *iso* C16:0 and NDF, whereas, there were negative relationships between CH_4 intensity and *trans*-10 C18:1.

Dietary strategies effects. Marginal effects forage type or different feed additives (nitrate, essential oil, saponin, tannin, and other plant extracts), were tested on the residuals of equations 1 and 9, but no association was observed ($P > 0.05$). The analysis of residuals indicate that the nature of the major FA in lipid-supplemented diets explained considerable proportion of the residuals of equation 12 ($P < 0.05$). In addition, several FA (C16:0, *cis*-9 C18:1, *cis*-9,*cis*-12 C18:2, C18:3n-3) tended to be associated with the residuals of equation 13 ($P < 0.10$). No effect of the other dietary mitigation strategies were observed on the residuals of CH_4 yield prediction equations. Saponin supplementation, and specifically tea saponin, tended to have an effect ($P < 0.10$) on MFA model residuals of equation 18, and essential oil (unknown nature) addition also tended to have an effect on residuals from the best models (equation 22) ($P < 0.10$). No other effect of CH_4 mitigation strategies was observed.

DISCUSSION

The compilation of experiments in the 825 individual datasets used for model development contained a larger variety of diets from experiments conducted across five countries in Europe, North America and Asian Australian (Table S1) in comparison to the data used for development of previously reported equations to predict CH_4 emissions (n=246 observations in Williams et al., 2014; n=218 observation in Van Gastelen et al., 2018).

Key MFA predictors in simple models

As expected, we observed positive relationships between *iso* C16:0 and CH_4 yield (g/kg DMI) and intensity (g/kg milk). The relationships between branched FA and CH_4 emissions have been reported in several other studies (Van Galstelen et al., 2017 and Chilliard et al., 2009 for CH_4

production in g/d; Dijkstra et al., 2011 for CH_4 yield in g/kg DMI; Van Lingen et al., 2014 for CH_4 intensity in g/kg milk). Indeed, outer membrane of fibrolytic bacteria are rich in branched-chain FA, and more specifically in *iso* FA (Vlaeminck et al., 2006), and are in great number with fiber-rich diets (Noziere et al., 1996), which are known to be linked to high CH_4 emissions. Vlaeminck et al., (2006) also reported increasing odd-*iso* FA content in milk from cows fed increasing proportion of forage. Thus, the negative relationship between *iso* C17:0 (coeluted with *trans*-9 C16:1) and CH_4 production (g/day) was unexpected. Vlaeminck et al. (2006) also observed greater *iso* C17:0 content with the inclusion of corn silage or lipid (rich in C18:3 n-3) supplementation in the diet. These dietary strategies (replacing grass silage by corn silage or adding lipids in the diet) are known to lower CH_4 emissions in dairy cows (Hristov et al., 2013). We also report negative relationships among milk *cis*-MUFA (*cis*-11 C18:1 and *cis*-15 C18:1), *trans*-MUFA (*trans*-10 C18:1; and *trans*-10+*trans*-11 C18:1) and PUFA (*trans*-11,*cis*-15 18:2; and *cis*-9,*cis*-12 C18:2) and CH_4 emissions. Negative associations between milk C18:1, C18:2 and C18:3 isomers and CH_4 emissions have also been observed by Chilliard et al. (2009), Dijkstra et al. (2011), Van Lingen et al. (2014), Rico et al. (2016) and Van Gastelen et al. (2018). Diet composition has an impact on milk *cis*-MUFA, *trans*-MUFA and some PUFA, which are RBH intermediates (Ferlay et al., 2017). For instance, diets rich in unsaturated FA often cause a shift from *trans*-11-C18:1 to more *trans*-10 C18:1 in the rumen (Bauman and Griinari, 2001 ou 2003). Furthermore, diets rich in starch (more than 35%) could lead to low pH (5.7) as observed by Zened et al. (2013), and supplementation with PUFA (more than 7.3%) could lead to low *trans*-MUFA content in milk, except for *trans*-10 C18:1 (Zened et al., 2013), depending on the completeness of the RBH. These type of diets are known to reduce CH_4 emissions (Martin et al., 2010). Consequently, negative relationships are expected between milk C18:1, C18:2 and C18:3 isomers and CH_4 emissions. Furthermore, the lowest RSR (0.84), and the greatest CCC (0.71) and RMPSE% (18.9%) values for the simple prediction equation of

CH_4 yield including only milk FA (equation 12), suggest that this equation performed better than those for CH_4 production (equation 1) and CH_4 intensity (equation 18).

Irrespective of the response of CH_4 emission (g/d, g/kg of DMI, g/kg of milk), we reported that prediction equations developed on individual data have better performance when evaluated with external individual dataset than with the external mean dataset. These discrepancies between evaluation performances could be explained by the lower range of variability observed in the external mean dataset as compared to the external individual dataset. In addition, some dietary strategies (monensin or cardanol) are only represented in the external mean dataset. This could further explain why both simple and complex prediction equations of CH_4 emissions, whatever the unit, have low performances when challenged against the external mean dataset and seem to be unsuitable for diets supplemented with such additives.

The potential relationships between CH_4 and individual MFA have been studied either in individual experiments (Mohammed et al., 2011; Williams et al., 2014; Van Gastelen et al., 2017) or in meta-analysis (Dijkstra et al., 2011; Van Lingen et al., 2014; Rico et al., 2016; Van Gastelen et al., 2018), and prediction have been developed using different individual MFA only as predictors of CH_4 emissions. Milk *cis*-11 C18:1 and *trans*-10 C18:1 were the only MFA related to CH_4 emissions that were found in this study and in multiple of the aforementioned studies (Mohammed et al. 2011; Dijkstra et al., 2011; Rico et al., 2016; Van Gastelen et al., 2018).

Moreover, the R^2 reported in this study with equation 1 (in g/d; R^2=0.85) and 12 (in g/kg of DMI; R^2=0.82) are similar to Rico et al. (2016) with R^2=0.84, but greater than Mohammed et al. (2011) with R^2=0.74, Dijkstra et al. (2011) with R^2=0.73, Van Gastelen et al., (2017) with R^2=0.63, Van Lingen et al. (2014) with R^2=0.58, Van Gastelen et al. (2018) with R^2=0.54 for CH_4 production and R^2=0.40 for CH_4 yield or Williams et al., (2014) with R^2=0.37.

On the other hand, RMSE values reported in this study with equation 1 (RMSE = 58.6 g/d) and

12 (in g/kg of DMI; RMSE = 2.8 g/kg of DMI) are greater than Rico et al. (2016) with RMSE = 26.0 g/d, Van Gastelen et al. (2018) with RMSE=35.7 g/d for CH_4 production and RMSE =1.6 g/kg of DMI for CH_4 yield, but lower than in Williams et al., (2014) with predicted standard deviation of 82.2 g/d.

Thus, few MFA are commonly found among developed prediction equations in this study and in the literature. In addition, performances of these prediction equations are not consistent, meaning that MFA on their own have a limited potential of prediction of CH_4 emissions.

Key predictors in complex models

Dry matter intake is a key factor of daily CH_4 production (Reynolds et al., 2011). A significant positive relationship between DMI and CH_4 production demonstrated that increasing DMI lead to greater CH_4 emissions because of greater availability of substrates for microbial fermentation in the rumen (Niu et al., 2018). Equation 2 further verify that DMI is a major driver of enteric CH_4 production in dairy cows, and thus is a strong predictor of CH_4 emissions (Hristov et al., 2013).

Dietary NDF, which represents the effect of forage inclusion rates, was included as positive predictor in several equations showing the best performance (equations 9, 13 and 22) for all three CH_4 emission responses. Studies focusing on the effect of types of carbohydrates have indicated that diets rich in NDF generally promote high acetate and butyrate production, and in turn high CH_4 emissions (Bougouin et al., 2018; Bannink et al., 2008; Johnson and Johnson, 1995; Moe and Tyrrell, 1979). On the other hand, non-structural carbohydrates primarily starch, favor production of propionate, resulting in less CH_4 production in the rumen. Additionally, it has been shown that substituting wheat, which is rapidly fermented in the rumen, in place of pasture, which is rich in structural carbohydrates, in the diet reduced CH_4 production and yield in dairy cows, with no negative effect on milk production, although feeding high levels (i.e., >40% of DMI) of wheat decreased milk fat content (Williams et al., 2013; Moate et al., 2014).

Regardless of the CH_4 emission response, dietary EE content was also identified as a key negative predictor variable in the best performing equations. Dietary EE is indicative of the total lipid content in the diet, and lipid mitigating effect on enteric CH_4 production is well established (Beauchemin et al., 2008; Knapp et al., 2014; Martin, et al. 2010). Increased dietary lipid content likely results in low availability of substrate for fermentation in the rumen as lipids are often supplemented at the expense of carbohydrates in the diet. Moreover, lipids can have a toxic effect on methanogens and also on protozoa known to produce great amount of H_2 that promote CH_4 production in the rumen (Guyader et al., 2014; Grainger and Beauchemin, 2011). Consistently, dietary EE in all the equations was significantly and negatively correlated with CH_4 emissions. Several prediction equations developed in the literature have also included EE as a negative predictor of CH_4 emissions, but with different effect size (regression coefficient) estimates. Indeed, Moate et al (2011) conducted a meta-analysis using 17 experiments and developed CH_4 yield prediction equation with a coefficient of -0.08 per unit increase dietary EE content (12 to 114 g/kg DM). Grainger and Beauchemin (2011) also proposed a prediction equation for CH_4 yield, developed with lactating cows fed 44 dietary treatments, with a coefficient of -0.1 per unit of dietary EE (% of DM). In the present study, coefficients for dietary EE content was -0.3 for each equations using that variable (equation 13 and 16). However, similar coefficient for dietary EE (from -0.29 to -0.45) were found in intercontinental prediction equations for CH_4 yield developed by Niu et al. (2018). Additional factors were considered in our study as compared to the studies of Moate et al. (2011), and Grainger and Beauchemin (2011), which could explain the difference of slopes observed in this study because they explain another part of the variability not taken into account with EE alone.

Body weight was positively related to CH_4 production and intensity (equations 4, 9, 10, 11, 17, 21, 22, and 23) as reported in prediction equation developed by Niu et al. (2018). As mentioned by Hristov et al. (2013), BW and DMI are positively related to each other, which lead to more

rumen feed fermentation, resulting in greater CH_4 production.

Complex equations developed in this study exhibited better performance when the above-stated variables were added to the simple equations only including MFA in predicting CH_4 production (RMSE= 42.8 vs 58.6 g/d and R^2= 0.85 vs 0.72, respectively), CH_4 yield (RMSE= 2.6 vs 2.8 g/kg DMI and R^2= 0.72 vs 0.70, respectively), or CH_4 intensity (RMSE= 3.3 vs 3.7 g/kg Milk and R^2= 0.67 vs 0.61, respectively). Moreover, we observed that accuracy of prediction of CH_4 production improved (RSR=0.56 and 0.70; -5% RMSPE with the external individual dataset; -8% RMSPE with the external mean dataset) when the independent variables (e.g. DMI, dietary NDF and EE, and BW) were added to the equation based on MFA. Our results confirm that increasing predicting equations' complexity lead to, most of the time, better goodness-of-fit (Niu et al., 2018; Moraes et al., 2014; Santiago-Juarez et al., 2016) probably because they explain additional proportion of the variability not taken into account in simple equation with MFA alone.

The R^2 reported in this study with equation 9 (R^2=0.85; RMSPE=42.8 on the internal validation dataset; model RMSE=42.8 g/d), which include MFA (iso C17:0 + tr9 C16:1, cis-11 C18:1, trans-11,cis-15 C18:2), DMI, dietary NDF and EE, and BW, is lower than in Chilliard et al. (2009) with R^2=0.95 and RMSE=28.8 g/d. Chilliard et al. (2009) used milk FA concentrations (*c*9 C14:1, C16:0, *tr*16+*c*14 C18:1, and C18:2n-6) and forage intake to developed prediction equations. However, better prediction ability was observed with equation 9 as compared to the one from IPCC (R^2=0.63) based on GEI. The RMSPE reported with the equations 9 (RMSPE=15.6 and 15.9% with the external individual dataset and external mean dataset, respectively) and 13 (RMSPE=18.8 and 17.0% with the external individual dataset and external mean dataset, respectively) are of similar magnitude as Niu et al. (2018), who reported RMSPE of 16.6% but using different predictors (DMI, EE, NDF, milk fat content and BW).

Equations' ability to predict CH_4 emissions is increased when other variables (intake, diet

composition or BW) are included along with MFA, probably because they explain another part of the variability not taken into account with MFA alone.

Effects of qualitative factors on the prediction equations' residuals

When the class of lipids, forage type and different feed additives were further tested on the prediction error, no further marginal effects were observed for CH_4 production. We only observed an effect of the major FA supplemented on the residuals for CH_4 yield equation 12. The major FA responsible for this effect was C18:3n-3 as compared to *cis*-9 C18:1 and lead to a decrease by 1.9 points of the intercept in equation 12. This result is in line with Doreau et al. (2011), who also observed an effect of the lipid class on the slope of the overall relationship between CH_4 yield and EE. On the contrary, Beauchemin and Grainger (2006) did not observed such effect in their meta-analysis. This discrepancies between results across different studies could be due to the variability in composition of the database. Indeed, not all the studies focused on the effect of lipid class on CH_4 emission were included in our dataset that was used to develop the equations. In this study, saponin or essential oil had an effect on the residuals of CH_4 intensity prediction equation. Thus, we cannot extend conclusion on the nature of all these additives because of a lack of different natures of saponin or essential oil present in the database. Furthermore, measurement of CH_4 emissions, even when done using the gold standard methods, unavoidably includes a number of associated errors because these techniques need to be correctly and appropriately used to generate reliable and accurate data (Hristov et al., 2018). In addition, even when cows are fed a fixed amount of a specific diet for a period of up to 16 weeks, there may be substantial changes over time in CH_4 emissions, probably associated with adaption of ruminal microbial populations (Moate et al., 2018). Thus, these issues continue to present challenges for the development of models that can accurately predict CH_4 production, yield and intensity.

Application of CH4 prediction equations on farm

The best CH_4 prediction equation developed in this study has a low potential of applicability on-farm. Indeed, milk samples could be routinely obtained on farm, but the GC technique, which is the gold standard method to determine MFA, is rather expensive and time-consuming. Moreover, research has been directed towards the use of near-infrared reflectance (**NIR**) or mid-infrared (**MIR**) spectrometry, which are rapid, cheaper and easier methods to determine milk FA concentrations. Furthermore, MIR is already implemented in laboratories of Milk Recording Organization, in France and Belgium for instance, to quantify major milk components used for milk payment, and can be used to estimate various milk FA such as C12:0, C14:0, C16:0, *cis*-9 C16:1, *cis*-9 C18:1 and SFA and MUFA in cow milk (Soyeurt et al., 2006). Unfortunately, the best five MFA predictors of CH_4 emissions reported in the current study are not all well quantified with MIR spectrometry, except for C8:0, C10:0, C16:0 and *cis*-11 C18:1 (Soyeur et al., 2006; Ferrand-Calmels et al., 2014). Thus, for on farm estimation of CH_4 emissions, CH_4 prediction equation should be developed using preferably MFA that can be determined accurately with MIR spectrometry.

CONCLUSIONS

In conclusion, our analysis based on a relatively large dataset including a wide range of diets from 5 different countries indicated that MFA have the ability to accurately predict enteric CH_4 production, yield and intensity of dairy cows. Equations based only on MFA performed well with RMSPE% ranging from 19% to 27%. Inclusion of DMI, dietary NDF, EE and starch contents, and BW into the equation further improved prediction performance with RMSPE% ranging from 16% to 24%. Nevertheless, DMI is difficult to be measured routinely in commercial farms. Therefore, equations to predict CH_4 emissions based on MFA only are promising for direct on farm use, but still require investigation in order to reduce the prediction error.

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TABLE 1 Summary descriptive statistics of the datasets used for the development and validation of the models

	Development					Validation									
	Training dataset (*n* = 578)					Internal validation dataset (*n* = 247)					External validation dataset (*n* = 84)				
Variables[1]	n	Mean	SD	Minimum	Maximum	n	Mean	SD	Minimum	Maximum	n	Mean	SD	Minimum	Maximum
Milk composition (fatty acid content)															
C8:0, g/100 g of total FA	578	1.23	0.32	0.0	1.98	247	1.22	0.33	0.28	2.02	74	1.28	0.62	0.54	4.20
C10:0	578	2.80	0.78	0.60	4.67	247	2.80	0.83	0.55	4.90	74	2.74	0.73	1.09	5.60
C12:0	578	3.24	0.93	0.84	6.04	247	3.28	1.02	0.78	6.41	78	3.37	0.91	1.04	6.27
C13:0	349	0.11	0.06	0.0	0.43	160	0.12	0.05	0.0	0.46	48	0.11	0.03	0.06	0.22
C14:0	578	11.17	1.89	5.35	14.76	247	11.11	2.00	5.16	15.18	78	11.27	2.81	5.88	30.6
C15:0	578	1.15	0.37	0.48	2.59	247	1.19	0.41	0.55	3.42	71	1.03	0.24	0.65	1.53
iso C15:0	449	0.28	0.20	0.10	1.50	193	0.28	0.12	0.07	1.19	39	0.21	0.05	0.11	0.32
Aiso C15:0	471	0.44	0.15	0.0	1.18	204	0.45	0.14	0.1	0.83	42	0.44	0.10	0.30	0.73
C16:0	578	29.66	6.27	15.56	44.28	247	29.77	6.58	15.34	42.8	78	29.66	4.68	15.94	36.7
iso C16:0	404	0.24	0.07	0.1	0.55	174	0.25	0.06	0.10	0.68	44	0.22	0.07	0.12	0.50
C17:0	578	0.58	0.17	0.02	1.15	247	0.58	0.17	0.19	1.17	75	0.63	0.34	0.32	1.99
iso C17:0 + *trans*-9 C16:1	405	0.44	0.18	0.0	1.05	176	0.42	0.18	0.12	1.0	35	0.38	0.11	0.15	0.67
Aiso C17:0	429	0.46	0.13	0.02	0.89	195	0.47	0.14	0.18	1.0	39	0.41	0.10	0.14	0.68
C18:0	578	9.44	3.44	2.67	20.65	247	9.23	3.33	2.26	21.7	80	9.63	2.79	1.39	14.8
C20:0	553	0.14	0.11	0.0	1.75	237	0.15	0.16	0.0	2.00	63	0.14	0.07	0.05	0.42
cis-9 C10:1	312	0.24	0.08	0.03	0.42	144	0.23	0.08	0.05	0.43	15	0.26	0.06	0.12	0.38
cis-9 C14:1	423	1.01	0.38	0.24	3.57	185	1.00	0.39	0.26	3.51	64	1.05	0.37	0.49	2.86
cis-9 C16:1	413	1.47	0.63	0.38	4.78	179	1.51	0.68	0.35	5.19	47	1.51	0.29	0.98	2.20
trans-11-C16:1	88	0.17	0.07	0.01	0.30	46	0.178	0.06	0.02	0.30	8	0.07	0.06	0.03	0.20
cis-9 C17:1	397	0.21	0.09	0.06	0.65	178	0.21	0.09	0.07	0.68	40	0.23	0.07	0.08	0.36
cis-9 C18:1	568	18.77	4.34	9.46	32.05	241	18.90	4.51	8.9	32.63	48	18.64	3.15	10.2	26.26
cis-10 C18:1	45	0.67	0.47	0.0	1.68	17	0.68	0.38	0.0	1.31	4	0.44	0.68	0.01	1.44
cis-11 C18:1	482	0.75	0.49	0.3	4.71	207	0.76	0.47	0.30	4.01	35	0.61	0.22	0.39	1.24
cis-12 C18:1	328	0.35	0.19	0.08	1.15	145	0.34	0.17	0.11	1.06	49	0.33	0.14	0.12	0.68
cis-13 C18:1	328	0.12	0.09	0.02	0.92	145	0.11	0.06	0.03	0.37	40	0.10	0.05	0.03	0.24

cis-14 C18:1	171	0.29	0.34	0.02	1.55	77	0.29	0.34	0.02	1.48					
cis-15 C18:1	279	0.22	0.23	0.01	1.53	122	0.24	0.33	0.01	1.93	16	0.41	0.84	0.08	3.50
cis-16 C18:1	81	0.08	0.05	0.0	0.22	30	0.08	0.04	0.01	0.18	8	0.07	0.03	0.03	0.13
trans-6/8 C18:1	320	0.40	0.30	0.0	1.70	146	0.41	0.30	0.0	1.50	17	0.36	0.10	0.26	0.61
trans-9 C18:1	542	0.32	0.23	0.0	2.32	229	0.31	0.23	0.0	2.48	48	0.31	0.16	0.11	0.82
trans-10 C18:1	481	0.92	1.47	0.11	11.30	207	0.95	1.50	0.1	9.52	55	0.84	1.07	0.12	5.60
trans-11 C18:1	565	1.52	1.43	0.20	12.23	239	1.55	1.40	0.29	10.14	63	1.28	0.65	0.59	4.40
sum *trans*-10+11 C18:1	477	2.40	2.12	0.46	13.40	207	2.43	2.12	0.49	10.91	59	2.03	1.57	0.84	8.80
trans-12 C18:1	316	0.48	0.31	0.09	2.12	142	0.47	0.29	0.08	1.33	30	0.50	0.20	0.19	1.11
trans-13/14 C18:1	245	0.78	0.87	0.0	4.22	107	0.78	0.90	0.0	4.21	18	1.10	0.98	0.18	4.56
trans-15 C18:1	114	0.56	0.32	0.16	1.36	48	0.55	0.36	0.13	1.48	29	0.63	0.39	0.26	2.08
trans-16+*cis*-14 C18:1	257	0.45	0.55	0.08	5.80	112	0.44	0.48	0.09	4.45	10	0.32	0.12	0.10	0.44
cis-9,*cis*-12 C18:2	568	1.82	0.69	0.05	6.79	241	1.73	0.56	0.7	4.14	78	2.20	0.76	1.28	4.35
cis-9,*trans*-12 C18:2	305	0.13	0.11	0.02	0.61	135	0.14	0.12	0.01	0.58	12	0.11	0.06	0.04	0.28
cis-9,*trans*-13 C18:2	295	0.31	0.25	0.05	2.31	130	0.32	0.27	0.06	1.54	16	0.39	0.37	0.13	1.62
trans-11,*cis*-15 C18:2	328	0.31	0.49	0.01	3.55	145	0.36	0.58	0.02	2.75	26	0.22	0.34	0.02	1.54
cis-9,*trans*-11 CLA	435	0.69	0.59	0.04	3.73	189	0.70	0.56	0.17	4.20	51	0.68	0.38	0.34	2.08
C18:3n-6	287	0.02	0.01	0.0	0.14	135	0.02	0.01	0.0	0.10	41	0.04	0.03	0.01	0.12
C18:3n-3	552	0.58	0.38	0.06	2.22	240	0.57	0.40	0.07	2.27	66	0.50	0.21	0.18	1.20
Intake															
DMI (kg/day)	578	20.5	3.4	10.8	32.2	247	20.5	3.5	11.3	30.4	84	21.0	4.2	14.2	28.6
Diet composition															
OM (% of DM)	578	92.02	2.34	84.5	95.12	247	92.12	2.25	84.5	95.01	75	92.95	1.19	89.5	95.5
CP (% of DM)	578	16.77	2.77	10.3	25.18	247	16.74	2.67	12.2	25.10	84	16.41	1.59	14.2	20.10
NDF (% of DM)	578	36.01	5.64	21.1	54.5	247	35.83	5.51	21.1	54.5	80	34.07	4.05	25.4	46.5
ADF (% of DM)	477	22.52	3.3	15.4	30.8	204	22.29	3.27	15.4	30.17	78	21.63	3.20	16.2	28.95
Ether extract (% of DM)	578	4.56	2.13	0.0	16.9	247	4.53	2.05	0.12	13.9	84	4.04	1.66	2.1	8.4
Starch (% of DM)	578	17.50	9.00	0.0	38.2	247	17.59	9.35	0.0	38.2	84	20.88	6.10	0.5	32.6
Animal Characteristics															
Days in milk	578	142	67	22	421	247	152	78	22	513	73	136	51	53	290
BW (kg/cow)	542	625	87	427	889	227	635	85	444	906	73	631	49	548	717

Dairy performance															
Milk yield (kg/day)	578	28.6	7.2	6.5	50.7	247	28.6	7.7	10.0	47.7	84	31.3	7.8	13.4	46.5
Milk Fat (%)	576	3.81	0.71	1.32	6.46	245	3.82	0.67	1.97	5.68	84	3.83	0.60	2.59	5.06
Milk Protein (%)	576	3.20	0.33	2.0	4.61	245	3.21	0.34	2.22	4.74	84	3.22	0.25	2.72	3.77
Milk Lactose (%)	479	4.84	0.31	3.49	5.62	206	4.84	0.32	3.03	5.59	63	4.72	0.22	4.1	5.2
CH_4 production (g/day)	578	414.8	106.2	84.2	707.9	247	411.3	103.3	108.6	686.5	84	380.0	85	149.2	563
CH_4 yield (g/kg DMI)	578	20.2	4.7	4.4	41.4	247	20.0	4.5	6.4	30.8	84	18.6	4.5	7.9	29.2
CH_4 intensity (g/kg of milk)	578	15.5	5.6	3.3	46.6	247	15.4	5.3	4.6	35.5	84	12.9	4.4	5.0	29.2

[1]Missing data for OM (missing value; *n* = 191), CP (*n* = 191), NDF (*n* = 191), EE (*n* = 285) and Starch (*n* = 191) have been estimated from INRA, 2007.

TABLE 2 Prediction equations of methane emissions (g/day per cow) according to different complexity levels and model performance evaluation

Model development based on Training dataset				Model performance evaluation based on Validation datasets						
Equations	Level	Prediction equation[1]	n[2]	Dataset	RMSPE, %	ECT, %	ER, %	ED, %	CCC	RSR
1	Milk composition	291.7 (±40.45) + 25.2*C10:0 (±7.45) – 176.6**iso* C17:0 + *trans*-9 C16:1 (±43.88) – 90.7**cis*-11 C18:1 (±20.97) – 46.6**trans*-11,*cis*-15 C18:2 (±10.18) **(RMSE=58.6; R^2=0.72)**	301	Internal External	24.0 27.7	0.59 55.6	3.35 0.60	96.06 43.77	0.53 0.01	0.88 1.22
2	Milk composition + intake	204.7 (±41.65) – 76.6**iso* C17:0 + *trans*-9 C16:1 (±39.90) – 75.5**cis*-11 C18:1 (±18.48) – 7.9**trans*-10 C18:1 (±3.18) – 48.9**trans*-11,*cis*-15 C18:2 (±8.22) + 15.1*DMI (±1.39) **(RMSE=50.6; R^2=0.79)**	300	Internal External	17.9 21.9	0.19 40.3	1.74 0.5	98.07 59.26	0.83 0.04	0.66 0.97
3	Milk composition + intake + diet composition	12.1 (±62.47) – 77.9**iso* C17:0 + *trans*-9 C16:1 (±38.78) – 70.3**cis*-11 C18:1 (±18.09) – 7.7**trans*-10 C18:1 (±3.10) – 49.1**trans*-11,*cis*-15 C18:2 (±8.04) + 15.7*DMI (±1.36) + 4.7*NDF (±1.17) **(RMSE=49.5; R^2=0.81)**	300	Internal External	16.9 17.5	0.01 23.5	0.15 0.05	99.84 76.47	0.88 0.11	0.62 0.77
4	Milk composition + intake + animal characteristics	272.9 (±51.42) + 13.0*C10:0 (±6.74) – 195.5**iso* C17:0 + *trans*-9 C16:1 (±41.61) – 116.4**cis*-11 C18:1 (±18.72) – 39.3**trans*-11,*cis*-15 C18:2 (±9.07) + 0.4*BW (±0.06) **(RMSE=50.3; R^2=0.79)**	265	Internal External	19.7 26.3	0 37.5	0.06 0.11	99.95 50.70	0.83 0.02	0.71 1.16
5	Milk composition + intake + dairy performance	122.1 (±43.57) – 69.8**iso* C17:0 + *trans*-9 C16:1 (±19.29) – 9.2**cis*-11 C18:1 (±3.07) – 52.5**trans*-11,*cis*-15 C18:2 (±8.03) + 15.4*DMI (±1.29) + 11.9*Milk Fat (±5.76) **(RMSE=51.3; R^2=0.81)**	324	Internal External	18.4 19.0	0 49.3	0.05 0.06	97.63 43.31	0.86 0.05	0.65 0.83
6	Milk composition + dairy performance	412.3 (±42.99) + 21.2*C10:0 (±7.30) – 163.4**iso* C17:0 + *trans*-9 C16:1 (±42.58) – 94.4**cis*-11 C18:1 (±20.39) – 50.2**trans*-11,*cis*-15 C18:2 (±9.94) + 3.1*Milk (±0.72) **(RMSE=58.6; R^2=0.72)**	301	Internal External	22.0 22.1	0.14 52.0	2.90 0.22	96.96 47.78	0.73 0.02	0.80 1.16
7	Milk composition + dairy performance	327.5 (±55.70) + 20.3*C10:0 (±7.28) – 142.3**iso* C17:0 + *trans*-9 C16:1 (±43.18) – 77.0**cis*-11 C18:1 (±21.60) – 49.8**trans*-11,*cis*-15 C18:2 (±9.87) + 3.4*Milk (±0.73) + 15.6*Milk Fat (±6.92) **(RMSE=56.5; R^2=0.74)**	299	Internal External	21.6 24.5	0.24 56.4	2.13 0.3	99.94 62.44	0.71 0.02	0.79 1.08

8	Milk composition + intake + dairy performance + diet composition	-6.2 (±97.98) + 16.5*C10:0 (±6.95) – 68.9**iso* C17:0 + *trans*-9 C16:1 (±38.35) – 57.8**cis*-11 C18:1 (±18.89) – 44.7**trans*-11,*cis*-15 C18:2 (±8.60) + 14.8*DMI (±1.40) + 13.3*Milk Fat (±5.90) + 3.4*NDF (±1.80) - 1.7*Starch (±1.20) **(RMSE=48.7; R^2=0.81)**	299	Internal External	17.1 17.0	0.08 23.9	0.37 0.31	99.55 75.83	0.86 0.12	0.63 0.75
9	Milk composition + intake + diet composition + animal characteristics	-42.0 (±60.57) – 112.1**iso* C17:0 + *trans*-9 C16:1 (±36.09) – 104.3**cis*-11 C18:1 (±15.55) – 31.7**trans*-11,*cis*-15 C18:2 (±8.57) + 12.5*DMI (±1.32) + 4.8*NDF (±1.04) - 4.7*EE (±2.34) + 0.2*BW (±0.05) **(RMSE=42.8; R^2=0.85)**	265	Internal External	15.6 15.9	0.03 16.2	0.21 7.3	99.75 76.52	0.90 0.17	0.56 0.70
10	Milk composition + intake + dairy performance + animal characteristics	164.9 (±56.85) – 166.1**iso* C17:0 + *trans*-9 C16:1 (±40.07) – 114.6**cis*-11 C18:1 (±18.70) – 46.5**trans*-11,*cis*-15 C18:2 (±8.26) + 3.2*Milk (±0.06) + 14.8*Milk Fat (±6.25) + 0.4*BW (±0.66) **(RMSE=48.3; R^2=0.81)**	263	Internal External	17.7 24.1	0.56 35.1	0.26 0	99.18 64.94	0.77 0.03	0.63 1.06
11	Milk composition + intake + dairy performance + diet composition + animal characteristics	-99.2 (±83.10) + 14.3*C10:0 (±6.50) – 164.6**iso* C17:0 + *trans*-9 C16:1 (±39.10) – 107.1**cis*-11 C18:1 (±18.30) – 39.2* *trans*-11,*cis*-15 C18:2 (±8.60) + 3.4*Milk (±0.60) + 33.4*Milk Protein (±13.1) + 4.8*NDF (±1.1) + 0.4*BW (±0.1) **(RMSE=47.3; R^2=0.81)**	263	Internal External	16.4 19.5	0.21 30.0	0.24 7.03	99.54 62.93	0.85 0.06	0.59 0.86

DMI: dry matter intake; EE: ether extract; NDF: neutral detergent fiber; BW: body weight; DIM: days in milk; RMSPE: root mean square prediction error; RSR: root mean square deviation; ECT: error from mean bias; ER: error from regression linear bias; ED: error from disturbance; CCC: concordance correlation coefficient.

[1]RMSE express in g/day

[2]n, number of observations used to construct equations.

TABLE 3 Prediction equations of methane yield (g/kg of DMI) according to different complexity levels and model performance evaluation

Equation	Level	Model development based on Training dataset: Prediction equation[1]	n[2]	Model performance evaluation based on Validation datasets: Dataset	RMSPE, %	ECT, %	ER, %	ED, %	CCC	RSR
12	Milk composition	21.8 (±1.07) + 10.7*_iso_ C16:0 (±2.45) – 2.8*_cis_-11 C18:1 (±0.56) – 0.8*_trans_-10 C18:1 (±0.14) – 0.8*_cis_-9,_cis_-12 C18:2 (±0.28) **(RMSE=2.8; R^2=0.70)**	393	Internal External	18.9 20.9	0.83 31.53	1.20 1.68	97.97 66.78	0.71 0.27	0.84 1.14
13	Milk composition + diet composition	7.6 (±3.02) + 0.1*C16:0 (±0.04) + 9.1*_iso_ C16:0 (±2.37) – 2.8* _cis_-11 C18:1 (±0.54) – 0.6* _trans_-10 C18:1 (±0.14) – 0.7* _cis_-9,_cis_-12 C18:2 (±0.28) + 0.3*NDF (±0.06) – 0.3*EE (±0.12) **(RMSE=2.6; R^2=0.72)**	398	Internal External	18.8 17.0	0 6.81	6.80 13.11	93.20 80.08	0.76 0.66	0.84 0.92
14	Milk composition + dairy performance	23.5 (±1.37) + 10.3*_iso_ C16:0 (±2.45) – 2.7* _cis_-11 C18:1 (±0.56) – 0.8* _trans_-10 C18:1 (±0.14) – 0.8* _cis_-9,_cis_-12 C18:2 (±0.28) - 0.1*Milk (±0.01) **(RMSE=2.8; R^2=0.69)**	390	Internal External	18.7 21.1	0.49 29.17	0.85 3.54	98.67 67.29	0.72 0.28	0.83 1.14
15	Milk composition + dairy performance	20.2 (±1.77) + 0.1*C16:0 (±0.04) + 9.6*_iso_ C16:0 (±2.44) – 2.7* _cis_-11 C18:1 (±0.56) – 0.7* _trans_-10 C18:1 (±0.14) – 0.6* _cis_-9,_cis_-12 C18:2 (±0.28) - 0.1*Milk (±0.03) **(RMSE=2.7; R^2=0.70)**	392	Internal External	19.0 19.0	0.37 1.08	1.44 1.86	98.19 70.07	0.71 0.35	0.85 1.08
16	Milk composition + diet composition + dairy performance	9.3 (±3.17) + 0.1*C16:0 (±0.04) + 8.7*_iso_ C16:0 (±2.37) – 2.8* _cis_-11 C18:1 (±0.54) – 0.6* _trans_-10 C18:1 (±0.14) – 0.7* _cis_-9,_cis_-12 C18:2 (±0.28) + 0.3*NDF (±0.06) – 0.3*EE (±0.12) - 0.1*Milk (±0.03) **(RMSE=2.6; R^2=0.72)**	392	Internal External	19.0 17.5	0.06 5.80	7.61 16.09	92.33 78.11	0.76 0.66	0.85 0.95
17	Milk composition + dairy performance + animal characteristics	18.2 (±2.40) + 0.1*C16:0 (±0.04) + 8.0*_iso_ C16:0 (±2.39) – 3.0* _cis_-11 C18:1 (±0.53) – 0.6* _trans_-10 C18:1 (±0.14) – 0.7* _cis_-9,_cis_-12 C18:2 (±0.27) - 0.1*Milk (±0.03) + 0.005*BW (±0.003) **(RMSE=2.5; R^2=0.73)**	356	Internal External	19.0 17.5	0.06 5.80	7.61 16.09	98.11 76.10	0.76 0.66	0.85 0.95

DMI: dry matter intake; EE: ether extract; NDF: neutral detergent fiber; BW: body weight; DIM: days in milk; RMSPE: root mean square prediction error; RSR: root mean square deviation; ECT: error from mean bias; ER: error from regression linear bias; ED: error from disturbance; CCC: concordance correlation coefficient.

[1]RMSE express in g/kg of DMI

[2]n, number of observations used to construct equations.

TABLE 4 Prediction equations of methane intensity (g/kg of milk) according to different complexity levels and model performance evaluation

Model development based on Training dataset				Model performance evaluation based on Validation datasets						
Equation	Level	Prediction equation[1]	n[2]	Dataset	RMSPE, %	ECT, %	ER, %	ED, %	CCC	RSR
18	Milk composition	13.8 (±1.43) + 16.2*_iso_ C16:0 (±3.75) – 3.1*_cis_-15 C18:1 (±1.57) – 0.5*_trans_-10+trans-11 C18:1 (±0.15) **(RMSE=3.7; R^2=0.61)**	277	Internal External	27.4 28.2	5.09 46.08	1.76 24.44	93.15 29.49	0.56 0.34	0.93 1.42
19	Milk composition + diet composition	16.5 (±1.77) + 13.3*_iso_ C16:0 (±3.28) – 1.0*_trans_-10 C18:1 (±0.15) – 0.3*EE (±0.15) – 0.1*Starch (±0.05) **(RMSE=3.7; R^2=0.77)**	393	Internal External	28.3 22.7	2.50 20.87	1.78 0.52	95.72 78.61	0.61 0.44	0.91 0.99
20	Milk composition + dairy performance	11.9 (±5.55) + 8.3*_iso_ C16:0 (±2.86) – 0.4*_trans_-10+trans-11 C18:1 (±0.11) + 1.9*Milk fat (±0.35) + 3.00*Milk protein (±0.76) - 3.1*Milk lactose (±0.97) **(RMSE=3.0; R^2=0.67)**	293	Internal External	25.0 18.1	4.77 63.19	5.36 4.41	89.87 32.40	0.65 0.40	0.90 1.22
21	Milk composition + animal characteristics	4.3 (±2.34) + 7.86*_iso_ C16:0 (±3.01) – 0.7*_trans_-10+trans-11 C18:1 (±0.11) + 0.02*BW (±0.01) + 0.01*DIM (±0.003) **(RMSE=3.3; R^2=0.66)**	353	Internal External	40.0 38.2	69.79 90.19	4.13 0.18	26.08 9.63	0.07 0.07	1.88 2.30
22	Milk composition + diet composition + animal characteristics	- 11.9 (±3.87) + 0.2*C16:0 (±0.04) + 11.5*_iso_ C16:0 (±3.08) – 0.8*_trans_-10 C18:1 (±0.15) + 0.3*NDF (±0.07) + 0.02*BW (±0.01) + 0.01*DIM (±0.003) **(RMSE=3.3; R^2=0.67)**	357	Internal External	24.3 15.4	3.41 19.70	1.40 1.38	95.20 78.92	0.74 0.79	0.79 0.63
23	Milk composition + diet composition + dairy performance + animal characteristics	-10.8 (±6.08) + 6.6*_iso_ C16:0 (±2.66) – 0.4* _trans_-10+trans-11 C18:1 (±0.10) + 0.3*NDF (±0.06) + 1.8*Milk fat (±0.32) + 2.1*Milk protein (±0.76) - 1.7*Milk lactose (±0.91) + 0.02*BW (±0.01) + 0.01*DIM (±0.003) **(RMSE=2.7; R^2=0.72)**	284	Internal External	49.5 15.4	35.38 19.07	0.61 40.28	64.01 40.65	0.16 0.75	1.17 0.96

DMI: dry matter intake; EE: ether extract; NDF: neutral detergent fiber; BW: body weight; DIM: days in milk; RMSPE: root mean square prediction error; RSR: root mean square deviation; ECT: error from mean bias; ER: error from regression linear bias; ED: error from disturbance; CCC: concordance correlation coefficient.

[1]RMSE express in g/kg of milk

[2]n, number of observations used to construct equations.

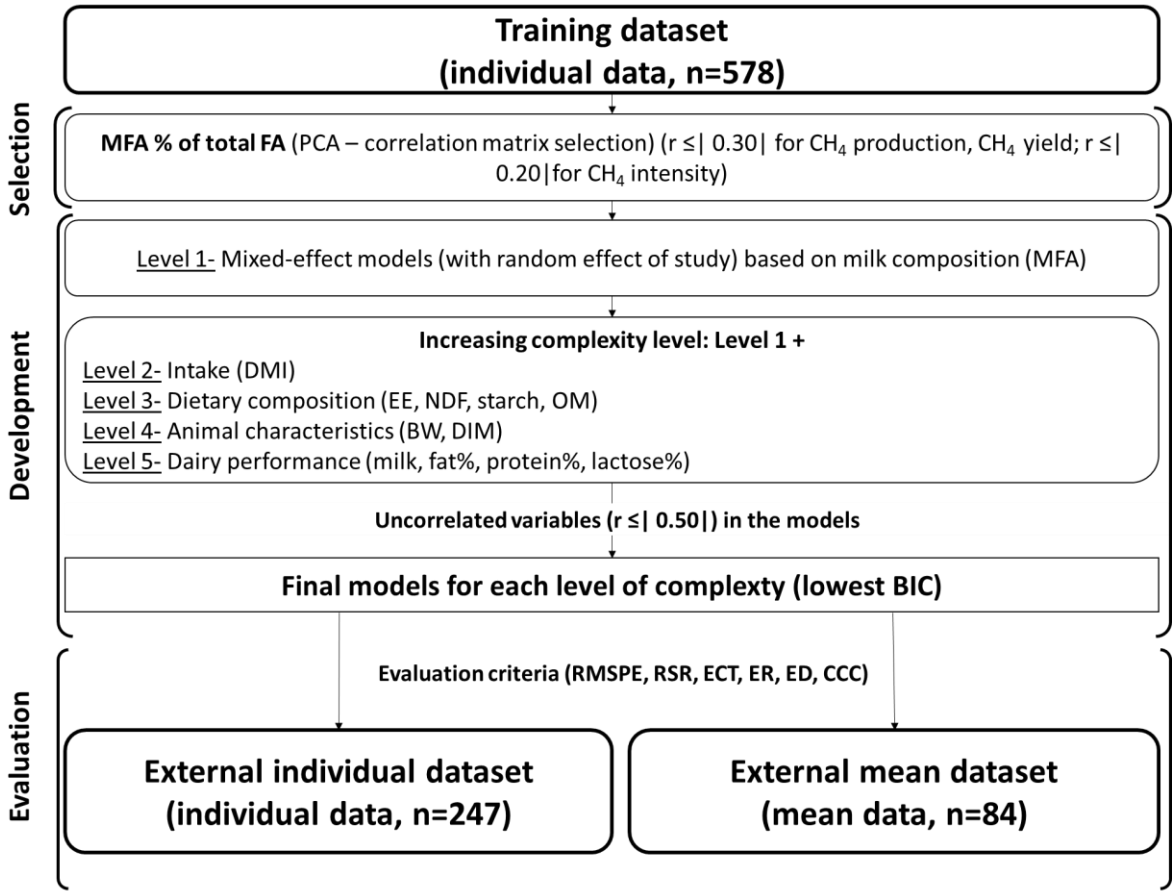

Figure 1. Diagram illustrating variable selection and model development and validation (MFA: milk fatty acid; PCA: principal component analysis; CH_4: methane; DMI: dry matter intake; EE: ether extract; NDF: neutral detergent fiber; OM: organic matter; BW: body weight; DIM: days in milk; BIC: bayesian information criteria; RMSPE: root mean square prediction error; RSR: root mean square deviation; ECT: error from mean bias; ER: error from regression linear bias ; ED: error from disturbance; CCC: concordance correlation coefficient).

SUPPLEMENTARY TABLE 1 Data sources and characteristics of included studies in the individual database (*n* = 825)

Related published article	n	No. Trt	CH_4 techniques[1]	Diet composition of basal diet/control	Treatments
LIPIDS					
Ferlay et al., 2013	16	4	SF6	50% of grass hay + 50% of concentrate	5, 10 or 15 % of extruded linseed
Ferlay et al., 2013	16	4	SF6	60% of corn silage and hay + 40% of concentrate	5, 10 or 15 % of extruded linseed
Martin et al., 2011 Lerch et al., 2012	20	3	SF6	60% of grass silage and cockfoot grass + 40% of concentrate	extruded linseed or rapeseed
Martin et al., 2011 Lerch et al., 2012	20	3	SF6	80% of cockfoot grass + 20% of concentrate	extruded linseed or rapeseed
Bougouin et al., (in progress)	16	4	OC	60% of corn silage and hay + 40% of concentrate	Ca salt of Palm oil, or extruded rapeseed, or extruded sunflower
Martin et al., (in progress)	32	4	OC	60% of grass silage and hay + 40% of concentrate	1.5, 3.0 or 4.0 % of extruded rapeseed
Brask et al., 2013	15	4	OC	50% of corn and grass silage + 50% of concentrate	rapeseed form: crushed, oil, cake
Alstrup et al., 2015	48	4	OC	48% of corn and clover silage + 52% of concentrate	rapeseed, rumen protected fat, +/- HMBi
Benchaar et al., 2013	48	4	OC	60% of corn silage, or alfalfa silage and timothy hay, + 40% of concentrate	DDGS at different level 10, 20, or 30
Brask et al., 2013	24	4	OC	Forage [grass silage or corn silage (65% of the diet DM), with two stages of maturity], +/- lipid (rapeseed)	
FORAGE & CONCENTRATE					
Hassanat et al., 2013	27	3	OC	Forage [(60% of timothy hay and different forages (100% of corn silage, or 100% of alfalfa silage, or 50:50 of corn silage and alfalfa silage)]	
Benchaar et al., 2014	27	3	OC	Forage [(60% of timothy hay and different forages (100% of corn silage, or 100% of barley silage, or 50:50 of corn silage and barley silage)]	
Hassanat et al., 2014	27	3	OC	Forage [(60% of timothy hay and different forages (100% of timothy silage, or 100% of alfalfa silage, or 50:50 of timothy silage and alfalfa silage)]	
Benchaar et al., 2015	48	4	OC	Forages (60% of either corn silage or red clover silage) + 40% of concentrate including linseed	
Williams et al., 2016	32	3	SF6	30 to 75% of either harvested chicory or brassica or alfalfa hay + concentrates	
Eugène et al., 2015	18	3	SF6	Forage (100% raygrass, or 70% raygrass+30% chicory, or 70% raygrass+30% clover)	
Moate et al., 2018*	27	2	OC	45% of alfalfa cubes + 55% of concentrate	Concentrate (corn or wheat)
Bougouin et al., 2018	15	4	OC	50% of grass silage and hay + 50% of concentrate	Concentrates (rich in fiber or Starch) +/- bicarbonate
Hellwing et al., 2012	15	4	OC	50% of clover silage + 50% of concentrate	Concentrate (beet molasses or wheat)
Moate et al., (in progress)	32	4	SF6	43 to 75% of harvested pasture + concentrate	Concentrate (15%; 30% or 45% of wheat)
Moate et al., 2017	32	4	SF6	47% of alfalfa hay, and 53% of concentrate	Concentrate (corn or wheat or barley)
Moate et al., unpublished	31	2	SF6	45% of alfalfa cubes + 55% of concentrate	Concentrate (rich in corn or wheat)
CHEMICAL FEED ADDITIVE: Nitrate (NO3)					
Guyader et al., 2016	16	2	OC	60% of corn silage and hay + 40% of concentrate	Lipid (extruded linseed) + calcium NO3

Guyader et al., 2016	16	2	OC	60% of grass silage and hay +40% of concentrate	Lipid (extruded linseed) + calcium NO3
PLANT EXTRACTS					
Guyader et al., 2016	14	2	OC	60% of corn silage and hay + 40% of concentrate	Saponin (Tea)
Unpublished data (France)	33	3	SF6	70% forage-mixture (corn silage, alfalfa hay, beetpulp silage, alfalfa silage and grass hay) + 30% of concentrate	Essential oil (2 different unknown types)
Lejonklev et al., 2016	16	4	OC	65% of corn and clover silage + 35% of concentrate	Plant extract (low or high concentration of oregano or caraway)
Williams et al., 2018	32	3	SF6	45 to 63% of alfalfa hay + 50% of concentrate	Plant extract (almond or citrus pulp)
Moate et al., (in progress)	32	3	SF6	50% to 70% of harvested pasture + 50% of concentrate	Plant extract (white or red grape marc)
PROBIOTICS					
Bayat et al., 2015	16	4	SF6	50% of grass silage + 50% of concentrate	*Saccharomyces cerevisiae* lipid or lipid (Camelina oil)
Philippeau et al., 2017	16	4	SF6	60% of corn silage and hay + 40% of concentrate	*Saccharomyces cerevisiae*
Philippeau et al., 2017	16	4	SF6	60% of grass silage and hay + 40% of concentrate	*Saccharomyces cerevisiae*
Jeyanathan et al., (in progress)	16	4	OC	55% of corn silage and hay + 45% of concentrate	*Saccharomyces cerevisiae*
Jeyanathan et al., (in progress)	16	4	OC	55% of grass silage and hay + 45% of concentrate	*Saccharomyces cerevisiae*

[1]OC: Open-circuit respiratory chamber; SF6: SF6 tracer technique

[2]CS= corn silage, CTR = control treatment, GS= grass silage, AS= alfalfa silage, BS= barley silage, H= grass hay, AH= alfalfa hay, AHL= alfalfa haylage, HL= grass haylage, S=straw, TH= timothy hay

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SUPPLEMENTARY TABLE 2 Data sources and characteristics of studies included in the external validation database (*n* = 84)

Authors	n	No. Trt	CH_4 techniques[1]	Diet composition of basal diet/control (on DM basis)	Treatments
LIPIDS					
Chilliard et al., 2009	4	3*	SF6	65% of CS + 35% of concentrate	Linseed in three different forms: crude, extruded, oil
Mohammed et al., 2011	4	3*	OC	45% of barley silage + 55% of concentrate	Steam-rolled barley + lipids at 3.3% added fat (DM basis): calcium salts of long-chain FA (palm oil; control) or crushed oilseeds from sunflower, flax, or canola
Hollmann et al., 2012	4	3*	OC	50% of alfalfa hay + 50% of concentrate	Coconut oil (1.3, 2.7, or 3.3% DM)
Moate et al., 2013	4	3*	OC	75% of alfalfa hay + 25% of concentrate	Algal meal at 125, 250, or 375 g/day
Johnson et al., 2002	3	2*	SF6	51% of alfalfa hay and silage + 49% of concentrate.	Whole cottonseeds and ground canola oilseeds at 4.0 or 5.6% of dietary fat
Sauer et al., 1998	4	2*	OC	65% of CS, alfalfa haylage and hay + 35% of concentrate	Soybean oil or feed additive (monensin)
Lopes et al., 2017	3	2*	GF	61% of a mixture of corn silage, alfalfa haylage and grass hay + 39% concentrate	Conventional variety of extruded soybean, or commercial extruded soybean (Plenish variety), or commercial heated soybeans (Plenish variety)
Pirondini et al., 2015	4	4*	OC	52% of alfalfa hay + 48% of concentrate.	Concentrate rich in dietary starch (low vs. high: 23.7 and 27.7% on a DM basis) +/- Fish oil
FORAGE & CONCENTRATE					
Livingstone et al., 2015	4	2*	OC	Forage 50% with CS or GS (75:25 or 25:75) + 50% concentrate	Forage +/- Extruded linseed
Van Knegsel et al., 2007	2	2*	OC	60% of corn silage + 40% of concentrate	Lipogenic nutrients or in glucogenic nutrients
Van Gastelen et al., 2015	4	3*	OC	80% of silage + 20% of concentrate	Forage: 100% GS, 67% GS and 33% CS, 33% GS and 67% CS, or 100% CS (all DM basis).
Hatew et al., 2016	4	4	OC	80% of CS + 20% of concentrate	Forage (four different maturity of corn silage: 25, 28, 32, and 40 to reflect the DM contents at harvest).
Van Gastelen et al., 2017	2	1*	OC	80% of silage + 20% of concentrate	Forage (100% GS, or 100% CS, or a mixture of both silages (66.7% GS and 33.3 % CS; 33.3 GS and 67.7% CS).
Doreau et al., 2014	4	4	SF6	45% of CS + 55% of concentrate	Concentrate: rich in starch or in fiber, +/- organic acid (malate acid)
Hart et al., 2015	4	4*	SF6	Corn silage to grass silage ratio of 70:30 or 30:70 offered *ad libitum*, + concentrate (6.1 kg DM).	Concentrate either rich in starch or fiber
CHEMICAL FEED ADDITIVE					
Hamilton et al., 2010	4	2*	OC	36% of GS + 64% of concentrate	Monensin

Reynolds et al., 2014	3	2*	OC	51% of GS + 49% of concentrate	3NOP: 500 or 2,500 mg/day; delivered directly into the rumen
Klop et al., 2016	4	3*	OC	70% of GS + 30% of concentrate, control contained urea as alternative non-protein N source to nitrate	NO3 (nitrate), or Lipid (DHA), or NO3 + DHA.
PLANT EXTRACT					
Benchaar et al.,2015	4	3*	SF6	54% of CS + 46% of concentrate	Eugenol: 25, 50, 75 mg/kg DM
Hristov et al., 2013	4	3*	SF6	59% of alfalfa hay + 41% of concentrate	Origanum Vulgare leaves: 250, 500, and 750 g/day
Branco et al., 2015	2	1*	GF	63% of CS + 37% of concentrate	Cashew nut shell
PROBIOTICS					
Hristov et al., 2010	2	1*	SF6	51% of CS + 49% of concentrate	*Saccharomyces cerevisiae*
ORGANIC ACIDS					
Hristov et al., 2011	3	2*	SF6	58% of CS + 42% of concentrate (containing stearic acid)	Lauric or myristic acids
Odongo et al., 2007	2	1*	OC	60% of CS, haylage and hay + 40% of concentrate	Myristic acid
Van Zijderveld et al., 2011	2	1*	OC	53% of CS + 47% of concentrate	Lauric acid, myristic acid, linseed oil, or calcium fumarate: included at 0.4, 1.2, 1.5, and 0.7% DM

*Dietary treatments including a control diet

[1]OC: Open-circuit respiratory chamber; SF6: gas tracer SF6; GF: GreenFeed

[2]CS= Corn silage, GS= grass silage.

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SUPPLEMENTARY TABLE 3 Pearson correlation coefficient between individual milk fatty acid (g/100 g of total FA) and CH_4 production, yield and intensity

	CH_4 production (g/day)		CH_4 yield (g/kg DMI)		CH_4 intensity (g/kg milk)	
	r	P-value	r	P-value	r	P-value
Saturated FA						
C4:0	0,16	0,00	0,13	0,00	-0,03	0,35
C5:0	-0,03	0,54	-0,20	0,00	-0,15	0,01
C6:0	0,30	0,00	0,24	0,00	0,11	0,00
C7:0	0,03	0,60	-0,18	0,00	-0,12	0,04
C8:0	0,35	0,00	0,25	0,00	0,18	0,00
C9:0	0,05	0,42	-0,13	0,02	-0,05	0,38
C10:0	0,33	0,00	0,21	0,00	0,18	0,00
C11:0	0,15	0,00	-0,14	0,00	-0,04	0,38
C12:0	0,29	0,00	0,16	0,00	0,18	0,00
C13:0	0,06	0,22	-0,13	0,00	-0,05	0,27
C14.0	0,28	0,00	0,10	0,00	0,10	0,01
C15.0	-0,04	0,26	-0,09	0,01	0,02	0,51
C16.0	0,27	0,00	0,24	0,00	0,26	0,00
C17.0	-0,19	0,00	0,03	0,43	0,17	0,00
C18.0	-0,02	0,51	0,06	0,10	-0,06	0,08
Branched-FA						
iso C13:0	0,26	0,00	0,31	0,00	0,25	0,00
anteiso C13:0	-0,30	0,00	0,12	0,02	0,34	0,00
iso C15:0	0,16	0,00	0,09	0,02	0,05	0,24
anteiso C15:0	-0,01	0,88	0,15	0,00	0,09	0,02
iso C16:0	0,22	0,00	0,27	0,00	0,33	0,00
iso C17:0	-0,32	0,00	-0,12	0,00	-0,03	0,37
anteiso C17:0	-0,17	0,00	0,05	0,18	0,03	0,48
***cis*-MUFA**						
cis-9 C10:1	0,34	0,00	0,21	0,00	0,18	0,00
cis-9 C14:1	-0,03	0,49	-0,08	0,05	0,00	0,00
cis-9 C16:1	-0,28	0,00	-0,17	0,00	-0,11	0,01
cis-9 C17:1	-0,34	0,00	-0,02	0,72	0,09	0,04
cis-9 C18:1	-0,19	0,00	-0,10	0,00	-0,09	0,01
cis-10 C18:1	-0,58	0,00	-0,35	0,01	-0,42	0,00
cis-11 C18:1	-0,34	0,00	-0,33	0,00	-0,19	0,00
cis-12 C18:1	-0,01	0,85	-0,19	0,00	-0,22	0,00
cis-15 C18:1	-0,43	0,00	-0,36	0,00	-0,24	0,00
cis-9 C20:1	-0,22	0,00	0,06	0,22	0,09	0,07
***trans*-MUFA**						
tr-4 C18:1	-0,19	0,00	-0,16	0,00	-0,17	0,00
tr-5 C18:1	-0,14	0,00	-0,12	0,01	-0,14	0,00
tr-6.8 C18:1	-0,34	0,00	-0,35	0,00	-0,33	0,00
tr-9 C18:1	-0,18	0,00	-0,24	0,00	-0,24	0,00
tr-10 C18:1	-0,45	0,00	-0,42	0,00	-0,34	0,00
tr-11 C18:1	-0,27	0,00	-0,18	0,00	-0,19	0,00
sum *tr*-10*tr*-11 C18:1	-0,42	0,00	-0,35	0,00	-0,34	0,00
tr-12 C18:1	-0,26	0,00	-0,28	0,00	-0,25	0,00
tr-13.14 C18:1	-0,31	0,00	-0,28	0,00	-0,22	0,00
tr-15 C18:1	-0,35	0,00	-0,34	0,00	-0,33	0,00
tr-16 C18:1	-0,03	0,52	-0,17	0,00	-0,15	0,01
PUFA						
cis-9,*cis*-12 C18:2	-0,24	0,00	-0,30	0,00	-0,19	0,00
cis-9,*tr*-12 C18:2	-0,39	0,00	-0,17	0,00	-0,14	0,00
cis-9,*tr*-13 C18:2	-0,29	0,00	-0,28	0,00	-0,25	0,00
tr-11,*cis*-15 C18:2	-0,29	0,00	-0,25	0,00	-0,23	0,00
cis-9,*tr*-11 CLA	-0,24	0,00	-0,18	0,00	-0,12	0,00
C18:3*n-6*	0,19	0,00	0,10	0,04	0,04	0,41

V. CHAPTER V

General discussion

Predictive tools for estimating large-scale enteric CH_4 emissions from individual ruminants are needed for evaluating potential strategies of CH_4 mitigation and consequently to reduce carbon footprint of livestock systems. Individual measurements are valuable for genetic selection as well as for governmental inventories in order to improve their accuracy. Direct *in vivo* measurement techniques, such as respiration chambers, SF_6 tracer gas, or Greenfeed, for the most used in research institutes so far, present different degrees of accuracy (Hammond et al., 2016). These techniques present several advantages and limits in research, but all are expensive, labor intensive and difficult to apply on a routine-basis and on large-scale dairy farms for individual animal measurements (Negussie et al., 2017; *for more details see Chapter I*). Therefore, prediction equations using proxies represent non-invasive measurements in ruminants and are a good opportunity to quantify CH_4 production and to assess the effect of different CH_4 mitigation strategies (diets, farming practices and genetic selection).

Current researches are directed toward finding proxies, or predictors, specifically in dairy cattle because of the great contribution of dairy products *versus* meat products to human diets (Food and Agriculture Organization of the United Nations, 2017). Finding proxies in milk can be easy because it is convenient to sample milk in dairy cows on a routine basis, either individually (robotic milking or milk parlor) or from bulk milk in farm conditions. Moreover, research focusing on proxies for prediction equations would enhance inventories accuracy (Hristov et al., 2018). Inventories are nowadays mostly calculated according to the IPCC Tier II prediction equations, which have limits such as the need to determine gross energy intake, a parameter difficult to measure on farm. Indeed, the amount and the quality of the feed consumed by the herd on a farm are difficult to record routinely and accurately, contrary to milk production and composition. Thus, the empirical prediction equations based on proxies, as proposed in this PhD thesis, would allow studying the effects of mitigation strategies on a large scale on farm compared to what is currently done in controlled experimental conditions. Milk FA composition reflects rumen digestion and mammary metabolism (Ferlay et al., 2017) and has been first used as proxies that represent CH_4 emissions by Chilliard et al., (2009). These authors have been the pioneers in this research area, with the development of the first prediction equations relating milk FA concentrations and CH_4 emissions in dairy cows. Other prediction equations based on milk FA concentrations have been published since then and reviewed by van Gastelen and Dijkstra (2016), but, up to now, no "generic standard" prediction equations have been found to be used in the dairy sector. Indeed, several other prediction equations have been published (Mohammed et al., 2011; Dijkstra et al., 2011; Williams et al., 2014; van Lingen et al., 2014;

Rico et al., 2016; van Gastelen et al., 2017) but have been developed on either a narrow range of diets, with few animals, or with few experiments, limiting their applicability domain.

The objective of this PhD thesis was to confirm the potential of milk FA as proxies to predict enteric CH_4 emissions in dairy cows under different diets used worldwide and by using two complementary approaches. The *in silico* approach aimed at creating two databases (based on individual observations thanks to an international collaboration or on mean observations of the literature) in order to develop prediction equations of CH_4 emissions from milk FA concentrations in dairy cows receiving a wide range of diets. The created databases pointed out the lack of data with some diets, which were studied in dairy cows using an in vivo approach.

The general discussion of the PhD thesis is divided in three parts. In the first section, we will give a quick feedback on the effects of the dietary treatments, tested *in vivo,* on enteric CH4 emissions and milk FA composition by highlighting the more original results and comparing them to the literature. The second part of this discussion concerns the *in silico* approach with the development of the prediction equations of CH_4 using milk FA as predictor variables and their validation. In addition, the performance of prediction equations developed in this PhD will be compared to prediction equations from the literature. The perspectives of this PhD work for the short- and long- terms will be proposed in the last section.

1. *In vivo* approach: links among enteric CH_4 emissions and milk FA according to diet

In this section, we will consider the original results from the two *in vivo* experiments together. The objectives of these experiments were to compare within-experiment the carbohydrates type with grass silage based-diets (named: Exp. 1 - **CARBO**) and different lipid sources with corn silage-based diets (named: Exp. 2 – **MFD** for diets susceptible to induce milk fat depression) on methanogenesis and dairy milk FA composition. In Exp. 1, dairy cows received 4 dietary treatments with forage-to-concentrate ratio of 50:50. Diets consisted of 42% of grass silage and 8% of hay, and 40% of fiber-rich concentrate or starch-rich concentrate with bicarbonate addition or not (1% of the DMI). In Exp. 2, diets consisted of 56% corn silage, 4% hay, and 40% concentrates rich in: (1) SFA from Ca-salts of palm oil; (2) starch from corn and wheat; (3) MUFA (*cis*9-C18:1) from extruded rapeseed; and (4) PUFA (C18:2n-6) from extruded sunflower. These dietary treatments were chosen after screening the diets from the 2 databases.

1.1. Variation of CH_4 emissions according to diets

Results from Exp. 1 concerning CH_4 emissions, rumen environment, and dairy performance were consistent with results reported in the literature.

In summary, we observed:

- A reduced DMI for the starch-rich diet as compared to the fiber-rich diet.
- No effect on milk production and composition, BW and energy balance according to diets.
- Lower CH_4 emissions (on average, -18% in g/d; -15% in g/kg DMI; and -19% in g/kg milk) in cows fed the starch-rich diets *vs*. fiber-rich diets. Mitigation effect of increasing dietary starch content (e.g. increasing concentrate level in the diet, instead of forage level) has been widely documented (Martin et al., 2010; Hristov et al., 2013) and our results also confirm that the carbohydrates type directly influences methanogenesis with diets based on grass silage.
- Greater rumen propionate proportion and lower butyrate proportion in total VFA, which can be linked to the lower rumen protozoa number (-36%) with the starch-rich diets compared to the fiber-rich diets.
- Carbohydrates type did not affect digestion of nutrients, except starch digestibility, which increased with cows fed starch-rich diets.
- Bicarbonate addition did not influence CH_4 emissions or nutrients digestibility regardless of the carbohydrates type in the diets. Rumen pH increased with bicarbonate addition, as expected, but the other rumen parameters were comparable with and without bicarbonate addition.

In conclusion, we show that feeding dairy cows with high-starch diets, even based on grass silage, is an effective dietary approach for reducing CH_4 emissions without altering dairy performance.

To the best of our knowledge, no direct comparison between different energy nature effect (starch *vs* lipids) on CH_4 emissions has been carried out in the past and it is known that these feeding strategies (increasing dietary starch or lipid content) decrease CH_4 emissions in dairy cows (Martin et al., 2010). However, feeding dairy cows with diets rich in starch (due to a high level of concentrate and/or corn silage-based diets), supplemented or not with unsaturated FA, can induce MFD, also called low milk fat syndrome (Bauman and Griinari, 2003).

Table 6 Summary of the treatments effects on CH_4 emissions in the 2 *in vivo* experiments from this PhD

	Associated experiment	Animal type (*n*) Experimental design Based-forage diet	Dietary treatments	Methane emissions			
				g/d	g/kg of DMI	g/kg of milk	% GE intake
Bougouin et al., 2018	Exp. 1-CARBO	Lactating Holstein dairy cows (*n* = 4) Latin square 2x2 factorial design Grass silage	1/ Starch-rich diet	408	18.5	14.0	5.5
			2/ Starch-rich diet + bicarbonate	381	17.3	13.2	5.2
			3/ Fiber-rich diet	470	20.9	16.9	6.2
			4/ Fiber-rich diet + bicarbonate	495	21.3	17.0	6.3
Bougouin et al., 2018	Exp. 2 - MFD	Lactating Holstein dairy cows (*n* = 4) Latin square design 4x4 Corn silage	1/ Starch-rich diet	346	18.7	12.7	5.6
			2/ Ca-salt of palm oil	374	20.2	15.2	5.8
			3/ Rapeseed	353	19.0	12.9	5.5
			4/ Sunflower	349	18.8	13.2	5.5

Therefore, the main objective of Exp. 2 was to study the effects of diets either rich in starch or supplemented with different lipid sources rich in unsaturated FA, susceptible to induce MFD, on milk yield, fat yield and FA profile in dairy cows fed corn silage-based diets in comparison to a diet inducing no MFD. At the same time, the effects on methanogenesis and on other digestive processes (rumen fermentation parameters, total tract digestibility), as well as the links among individual milk FA and CH_4 emissions, were also investigated.

In summary, we observed:

- Similar CH_4 emissions among diets (Table 6) whatever the unit considered (on average 356 (± 36.0) g/d, 19.2 (±1.61) kg/DMI, 5.6 (±0.47) % GEI, and 13.5 (±1.45) g/kg milk).
- No effect of the lipid type (saturated *vs* unsaturated FA) with corn silage-based diets on methanogenesis, even if greater effect of PUFA on CH_4 emissions was expected, as reported by Doreau et al. (2011).
- Typical milk FA profiles representative of MFD syndrome (Bauman and Griinari, 2003) with all diets, even with cows receiving the diet supplemented with Ca-salt of palm oil. This situation led to low CH_4 emissions. Consequently, we suggest that the mitigation effects of dietary lipids on methanogenesis were hidden because of the MFD observed whatever the diet.
- No change in the rumen fermentation parameters (VFA, and protozoa number) according to the diet. This lack of effects observed counteracts the previous point.

In conclusion, we did not observe CH_4 mitigation effect of starch-rich or lipid-supplemented diets based on corn silage. The expected CH_4 mitigation effect of these dietary strategies was not demonstrated but all these diets induced a MFD.

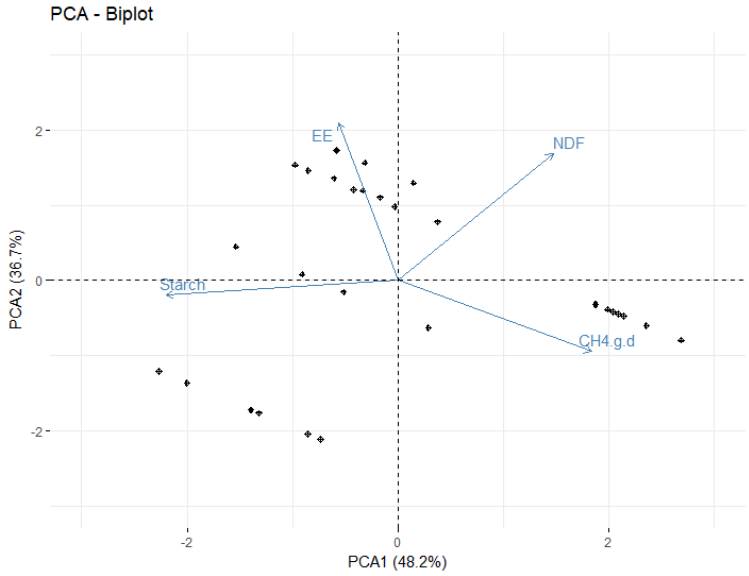


Figure 11 PCA – Biplot for CH_4 production (g/d) and diet composition (NDF, EE, and Starch contents in % of DM) for the 2 *in vivo* experiments of this PhD (n = 31)

Overall, CH_4 production appears to be negatively related to the dietary starch content but positively related to dietary NDF content as illustrated in the PCA biplot (Figure 11). Indeed, dietary starch and CH_4 production are on the opposite side on PCA axis 1, which represents 48% of the variance, whereas dietary NDF is on the same side, showing potential positive links. The dietary EE is on the opposite side from CH_4 production on PCA axis 2, which represents 37% of the variance, showing that these two variables are negatively linked. **Thus, dietary starch and EE contents explain a large part of CH_4 production variations with corn- or grass-silage based diets. This is in agreement with the recent publication of Niu et al. (2018) on enteric CH_4 emissions prediction using an intercontinental database.**

1.2. Variations of milk FA composition according to diets

In Exp. 1 (CARBO), as compared to fiber-rich diets, milk from cows fed starch-rich diets had:

- Lower concentrations of *trans*-11 C18:1, sum *cis*-C18:1, *cis*-9, *trans*-11 CLA and sum of CLA
- Greater concentrations of some minor isomers of CLA (*trans*-10,*trans*-12, *trans*-7,*cis*-9 + *trans*-8,*cis*-10, and *cis*-11,*trans*-13) and SFA (from C5:0 to C17:0).

Bicarbonate addition did not change milk FA composition whatever the carbohydrates type in the diets. *Further information are available in Chapter II*.

In Exp. 2 (MFD), when cows were fed the diet supplemented with Ca-salt of palm oil or the MFD-Starch diet, we observed:

- Higher milk SFA concentration in comparison to MFD-Rapeseed and MFD-Sunflower diets
- Lower milk MUFA concentrations in comparison to MFD-Rapeseed and MFD-Sunflower diets
- Lower milk total *trans* FA concentration in comparison to MFD-Sunflower, with the value for MFD-RS being intermediate
- Milk *trans*-11 C18:1 did not change among diets, but lower *trans*-10 C18:1 contents were observed for PALM and MFD-Starch diets in comparison to the others diets.

The MFD seems more severe with MFD-Sunflower and MFD-Rapeseed than diet supplemented with Ca-salt of palm oil and MFD-Starch diet, because of a decrease in milk SFA concentration and a stronger shift from *trans*-11 C18:1 to *trans*-10 C18:1 in milk with these diets. The MFD-Sunflower diet increased more milk *trans* FA (+60%), *trans*-10 C18:1 (+31%), *trans*-10,*cis*-12 CLA (+27%) and PUFA (+36%) concentrations than MFD-Rapeseed diet, which explains the numerically lowest milk fat yield with MFD-Sunflower diet. These modifications suggest that rumen biohydrogenation pathways of unsaturated FA differ between these two diets.

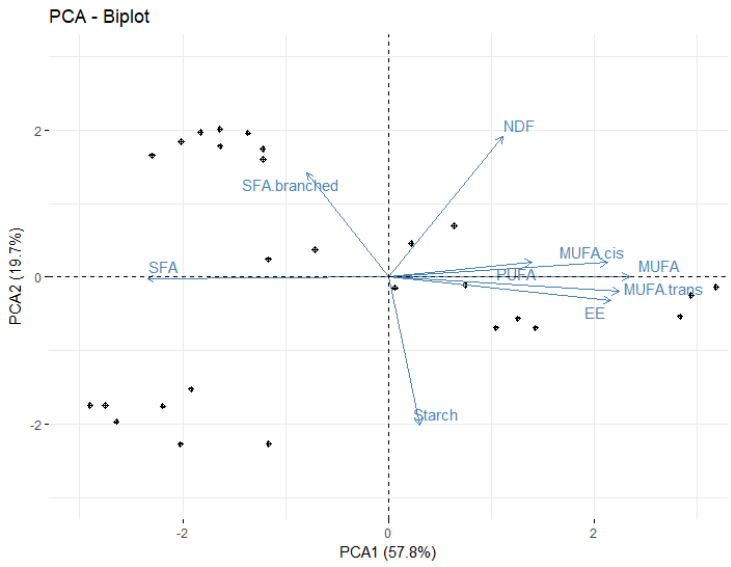


Figure 12 PCA – Biplot for diet composition (NDF, EE, and Starch contents in % of DM) and milk FA sums representing the different milk FA families, from the 2 *in vivo* experiments of this PhD (n = 31)

When Exp. 1 and 2 are studied together in the PCA analysis, we observed that the two first axes explained together 78% of the total variance (Figure 12). On PCA 1 axis, dietary EE is positively related to milk MUFA family (either *cis*- or *trans*-), as well as with PUFA. However, all of these variables are on the opposite side of SFA, meaning that they are negatively linked to SFA. Dietary NDF and milk branched SFA are on the opposite side from starch on PCA 2 axis, and are not related to dietary EE or milk SFA, MUFA and PUFA.

Overall, milk FA from the different families (SFA, MUFA, and PUFA) are related to each other and with dietary EE, but are not related to dietary NDF or starch, except for milk branched FA. Thus, the dietary conditions fed to the cows influence milk FA from the different families. Results on the effects of high-starch diets (Exp. 1) or diets supplemented with Ca salts of palm oil (Exp. 2) on higher *de novo* synthesized FA are in agreement with the literature. In contrast, the corn silage-based diets supplemented with unsaturated FA (rapeseed or sunflower) produced milk fat with low *de novo* synthesized FA and a MFD. In addition, the corn silage-based diets rich in starch or supplemented with Ca salts of palm oil or rapeseed or sunflower seeds induced low milk fat contents and a shift of *trans*-11 C18:1 to *trans*-10 C18:1 in milk fat, leading to a MFD in dairy cows with a numerically decreased milk fat yield.

1.3. Links among milk FA and CH_4 emissions according to diets

Several authors have shown relationships among CH_4 emissions and individual milk FA concentrations (Table 7) using various dietary strategies in either individual experiments (Chilliard et al., 2009; Mohammed et al., 2011; Rico et al., 2016) or using a meta-analysis approach (Dijkstra et al., 2011; van Lingen et al., 2014; van Gastelen et al., 2017). The Figure 13 summarizes the various links found among milk FA and CH_4 production (g/d) in the 2 *in vivo* experiments according to the Pearson correlation coefficients (Table 8).

Table 7 Principal published relationships between individual milk FA and CH_4 emissions

References (CH_4 unit)	SFA	branched FA	*cis*-MUFA	*trans*-MUFA	PUFA
Chilliard et al., (2009) (g/d)	C8:0 (+) C10:0 (+) C15:0 (+) C16:0 (+)		*cis*-13 C18:1 (-)	*trans*-10 C18:1 (-)	C18:2 *cis*-9 + *trans*-13 (-)
Mohammed et al., 2011 (g/d)	C8:0 (+) C15:0 (-)		*cis*-11 C18:1 (-) *cis*-13 C18:1 (-)	*trans*-10 C18:1 (-)	
Dijkstra et al., (2011) (g/kg of DMI)	C8:0 (+) C10:0 (+) C16:0 (+)	*anteiso* C17:0 (+) *iso* C15:0 (+) *iso* C16:0 (+)	*cis*-11 C18:1 (-) *cis*-13 C18:1 (+)		*cis*-9,*trans*-12 C18:2 (-) *cis*-9,*cis*-12 C18:2 (-)
Van Lingen et al., 2014 (g/kg of DMI)	C16:0 (+)	*iso* C16:0 (+)	*cis*-11 C18:1 (-)		*cis*-9,*cis*-12 C18:2 (-)
Rico et al., 2015 (g/d)	C10:0 (+) C15:0 (-)		*cis*-13 C18:1 (-)	*trans*-10 C18:1 (-)	
Van Gastelen et al., 2017 (g/d or kg of DMI)		*iso* C15:0 (+)		*trans*-10 C18:1 (-)	*cis*-9,*cis*-12 C18:2 (-)

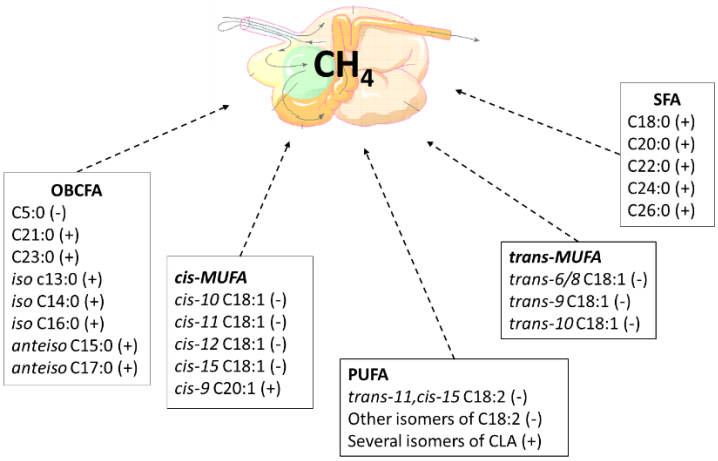


Figure 13 Links among milk FA (SFA, OBCFA, *cis*-MUFA, *trans*-MUFA, PUFA families) and CH_4 emissions (g/d) from the 2 *in vivo* experiments of this PhD. RBH: Rumen biohydrogenation; FA: fatty acids; OBCFA: odd- and branched-chain fatty acids; MUFA: monounsaturated fatty acids; PUFA: polyunsaturated fatty acids

To the best of our knowledge, no experiments have linked CH_4 emissions and milk FA in dairy cows in MFD status.

- Several individual **SFA** had significant positive relationships to CH_4 production, yield or intensity (Table 8), with C18:0, C20:0, C24:0 and C26:0 being the most positively correlated ($r > 0.70$; $P < 0.05$ for production and $r > 0.60$; $P < 0.05$ for intensity, respectively), while only C5:0 had negative correlation ($r = -0.66$; $P < 0.05$ for production).
- Several positive relationships among **branched FA** and CH_4 production, yield and intensity have been observed. Overall, the greatest positive correlations were obtained among *iso* C15:0, *anteiso* C15:0 and CH_4 emissions (production, yield, and intensity).
- Overall, several individual ***cis* MUFA** were negatively correlated to CH_4 production with the strongest negative correlation observed for *cis*-11 C18:1 (Table 8; $P < 0.05$). Milk *cis*-15 C18:1 was the most negatively correlated to CH_4 yield.
- Milk *trans*-10 C18:1 and other *trans* isomers of C18:1 (5, 6/7/8, and 9) and ***trans* MUFA** were negatively correlated to CH_4 production and yield (Table 3; $P < 0.05$).
- Several **PUFA** were negative correlated to CH_4 production, yield and intensity (*cis*-9,*trans*-13 C18:2, *cis*-9,*trans*-14 C18:2, *trans*-11,*cis*-15 C18:2, and *trans*-7,*cis*-9 CLA + *trans*-8,*cis*-10 CLA), while *trans*-12,*trans*-14 CLA and *trans*-7,*trans*-9 CLA had positive relationships. We observed negative relationships among *trans*-9,*trans*-12

C18:2, *trans*-9,*cis*-12 C18:2 and CH_4 production and yield. Milk C22:5n-3 was negatively correlated to CH_4 intensity.

The strongest positive correlations among milk FA and CH_4 production (g/d) were found for C24:0 (SFA family), *iso* C14:0 (branched FA family), and *trans*-12, *trans*-14 CLA (PUFA family), while the strongest negative relationships were with *cis*-11 C18:1, *trans*-10 C18:1 and *trans*-9,*trans*-12 C18:2 (belonging to the *cis* MUFA, *trans* MUFA, and PUFA families, respectively).

In the PCA analysis (Figure 14), milk SFA and branched SFA are linked to CH_4 production (g/d), but are on the opposite side of milk MUFA (*cis* and *trans*) and PUFA, and dietary EE content on PCA axis 1 (54.9% of the total variance). Dietary NDF and CH_4 production are opposite to starch content on PCA axis 2 (20.3% of total variance). Thus, milk FA, dietary composition and CH_4 production are related to each other.

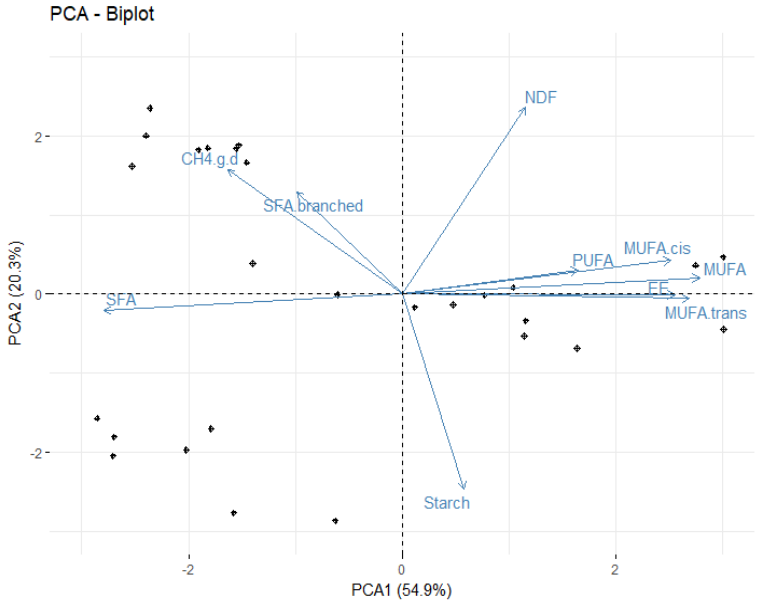


Figure 14 PCA – Biplot for diet composition (NDF, EE, and Starch contents in % of DM), milk FA sums, which represent the different milk FA families, and CH_4 production (g/d) from the 2 *in vivo* experiments of this PhD (n = 31)

In conclusion, relationships among milk FA concentrations and CH_4 emissions observed in our work are not always consistent with the literature. We show that these relationships are strongly influenced by the dietary EE content suggesting that these relationships are diet dependent.

Table 8 Significant Pearson correlation coefficients among individual milk FA concentrations and CH_4 emissions for the 2 *in vivo* experiments of this PhD

	CH_4 production (g/d)		CH_4 yield (g/kg of DMI)		CH_4 intensity (g/kg of milk)	
Milk FA (% of total FA)	r	P value	r	P value	r	P value
C5:0	-0.66	0.008	-0.77	0.001	-0.54	0.037
C18:0	0.73	0.002	0.76	0.001	0.68	0.005
C20:0	0.74	0.002	0.76	0.001	0.68	0.005
C21:0	0.45	0.093	0.45	0.09	0.59	0.022
C22:0	0.69	0.005	0.77	0.001	0.70	0.004
C23:0	0.64	0.01	0.78	0.001	0.60	0.018
C24:0	0.77	0.001	0.70	0.004	0.54	0.036
C26:0	0.71	0.005	0.66	0.01	0.63	0.017
iso C13:0	0.50	0.056	0.62	0.014	0.67	0.006
iso C14:0	0.76	0.001	0.71	0.003	0.48	0.073
iso C15:0	0.71	0.003	0.87	0	0.71	0.003
anteiso C15:0	0.75	0.001	0.73	0.002	0.65	0.009
iso C16:0	0.69	0.004	0.60	0.018	0.36	0.183
anteiso C17:0	0.49	0.065	0.56	0.03	0.61	0.015
cis-6/7/8 C16:1	-0.71	0.003	-0.34	0.221	-0.15	0.6
cis-9 C17:1	-0.75	0.001	-0.28	0.304	-0.18	0.514
cis-10 C18:1	-0.61	0.015	-0.57	0.028	-0.42	0.123
cis-11 C18:1	-0.77	0.001	-0.46	0.085	-0.41	0.133
cis-12 C18:1	0.51	0.051	0.04	0.876	-0.12	0.66
cis-13 C18:1	-0.63	0.012	-0.58	0.024	-0.42	0.118
cis-15 C18:1	-0.51	0.054	-0.63	0.012	-0.45	0.089
cis-9 C20:1	0.70	0.004	0.65	0.009	0.45	0.096
cis-11 C20:1	-0.71	0.003	-0.57	0.027	-0.36	0.192
trans-5 C18:1	-0.48	0.07	-0.59	0.021	-0.47	0.077
trans-6/7/8 C18:1	-0.52	0.047	-0.53	0.04	-0.38	0.166
trans-9 C18:1	-0.58	0.022	-0.49	0.064	-0.32	0.246
trans-10 C18:1	-0.74	0.002	-0.74	0.002	-0.59	0.022
trans-9,*trans*-12 C18:2	-0.77	0.001	-0.75	0.001	-0.50	0.059
cis-9,*trans*-13 C18:2	-0.61	0.016	-0.73	0.002	-0.61	0.015
cis-9,*trans*-12 C18:2 + *cis*-9,*trans*-14 C18:2	-0.55	0.032	-0.72	0.003	-0.62	0.014
trans-11,*cis*-15 C18:2	-0.72	0.002	-0.64	0.011	-0.58	0.024
trans-9,*cis*-12 C18:2 + *trans*-10 C19:1	-0.71	0.005	-0.57	0.035	-0.43	0.129
trans-12,*trans*-14 CLA	0.79	0	0.65	0.009	0.52	0.047
trans-11,*trans*-13 CLA	0.46	0.087	0.48	0.072	0.40	0.136
trans-9,*trans*-11 CLA	0.67	0.006	0.52	0.048	0.46	0.083
trans-7,*trans*-9 CLA	0.76	0.001	0.62	0.014	0.63	0.011
trans-12,*cis*-14 CLA	0.50	0.06	0.32	0.247	0.19	0.49
C22:5n-3	-0.58	0.023	-0.48	0.073	-0.56	0.031
iso SFA	0.73	0.002	0.79	0	0.61	0.015
anteiso SFA	0.70	0.011	0.71	0.003	0.68	0.006
trans MUFA	-0.63	0.011	-0.60	0.017	-0.42	0.12

2. *In silico* approach: Potential of milk fatty acids to predict CH_4 emissions in different units

Potential of milk FA to predict CH_4 emissions has been described several times in the literature, and the 2 *in vivo* experiments conducted in this PhD work confirm that there are links among individual milk FA and CH_4. However, it has also been stated that relationships should be investigated using larger datasets on dairy cows fed a wide range of diets (Hristov et al., 2018). Thus, a large dataset based on individual observations was created. The research hypothesis is that milk FA have the potential to accurately predict CH_4 emissions whatever the diet considered.

2.1. Description of the datasets and the modelling approach

In this section, we will briefly introduce the material and methods of the *in silico* approach with the important details about the description of the created dataset and the modeling approach. *For further details, please refer to the Chapter IV.*

Description of the databases

Individual dataset. A dataset was created thanks to an international scientific collaboration. For inclusion in the database, the *in vivo* experiments must have met different criteria:

- The CH_4 production was measured on individual dairy cows by respiration chambers, SF_6 tracer gas or GreenFeed measurement techniques.
- Milk FA profile of individual cows was determined by gas chromatography.
- Data on daily DMI, dietary composition, milk production and composition, and characteristics (BW and DIM) of individual cows have been recorded.

Among INRA experiments, we included the 2 *in vivo* experiments described in *Chapter II (Exp.1 - CARBO) and III (Exp.2 - MFD),* because of the originality of the dietary treatments used. We observed that 1) data are comprised within the variability of the individual dataset and are suitable for inclusion in this database, and 2) a positive relationship between DMI and CH_4 production (Figure 15), comparable to the global relationship developed with the other studies from the individual database. Several authors have already reported this positive relationship, indicating that DMI is one of the main driver of the daily CH_4 production (Reynolds et al., 2011; Niu et al., 2018).

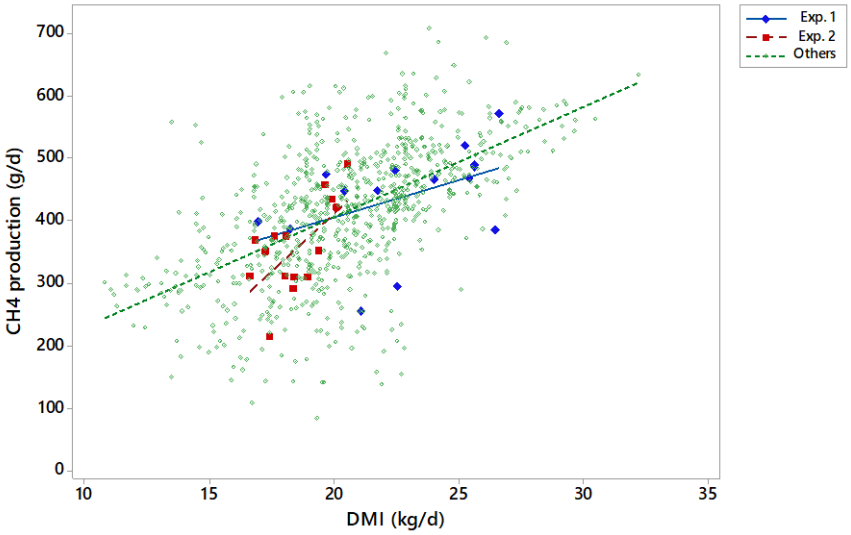


Figure 15 Relationships between dry matter intake (DMI) and CH_4 production (g/d) within the individual database (*n* = 825). Blue and red points indicate the position of our data obtained in the 2 *in vivo* experiments of this PhD (*n* = 31)

External mean dataset. Another dataset based on published studies was also created (mean data). The same filters as used to create the individual dataset were applied in the search of published studies. The objective was to obtain the same variables in both datasets. Data from the literature presented a similar range of CV for milk FA than the individual dataset (from 20% to more than 100%). The CH_4 response variables in the individual dataset strongly varied with an averaged CV of 28%. The average DMI and milk yield were 20.5 (± 3.4) and 28.6 (± 7.4) kg per day per cow, respectively. The DMI and milk yield were greater in the literature dataset with 21.0 (±4.2) and 31.3 (± 7.8) kg per day per cow, respectively. In addition, minimum and maximum values for DMI, milk yield and composition, and CH_4 emissions (g/d, g/kg of DMI, and g/ kg of milk) fell within the range of values from the individual dataset.

Database quality assessment. Pre-processing of data was performed on both datasets because some records were either incomplete (missing values, missing certain variables of interest), inconsistent (containing heterogeneity in codes or names) or noisy (containing errors or outliers).

Modeling approach

The modeling approach comprised 3 steps (Figure 16): 1) **Selection** of the milk FA from the complete individual database; 2) **Development** of the CH_4 prediction equations based on the training dataset (constituted by a random selection of data from the whole individual database); and 3) **Validation** of the developed CH_4 prediction equations on the internal validation dataset (individual data) and external validation dataset (mean data).

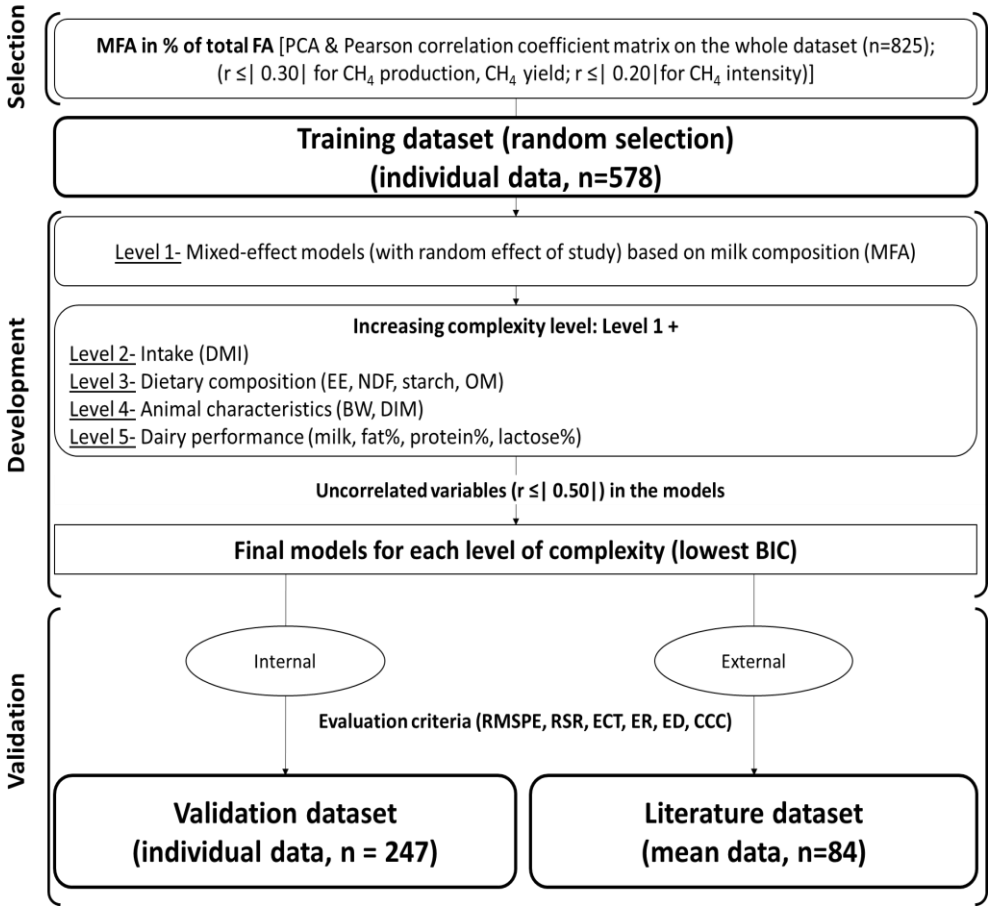


Figure 16 Diagram illustrating variable selection, equation development and validation

MFA: milk fatty acids; PCA: principal component analysis; CH_4: methane; DMI: dry matter intake; EE: ether extract; NDF: neutral detergent fiber; OM: organic matter; BW: body weight; DIM: days in milk; BIC: Bayesian information criteria; RMSPE: root mean square prediction error; RSR: root mean square deviation; ECT: error from mean bias; ER: error from regression linear bias; ED: error from disturbance; CCC: concordance correlation coefficient.

Selection. **A)** Pearson's pairwise correlation coefficients were obtained among the 46 individual milk FA initially selected (having a concentration > 0.1 % of total milk FA) with CH_4 emissions (expressed as g/d; g/kg of DMI; or g/kg of milk) to determine the most correlated individual milk FA per family (SFA, branched FA, *cis*-MUFA, *trans*-MUFA and PUFA; *details are available in Chapter IV with Supplementary Table 3*).

B) Several Principal Components Analyses (**PCA**) were also performed, using the FactoMinR package in R, on 1) the milk FA significantly correlated to CH_4 production ($r \geq |0.3|$), CH_4 yield ($r \geq |0.3|$), and CH_4 intensity ($r \geq |0.2|$), and 2) the selected milk FA and other variables (diet composition, animal characteristics and milk performance) in order to identify potential predictors of CH_4 emissions. Missing values for individual milk FA were estimated and

replaced with plausible values using the MissMDA package in R.

PCA for CH_4 production. The two first axes of the PCA explained together 75.2% of the total variance (Figure 17). Variable (C10:0) on the red circle was positively related to CH_4 production (g/d). On the other side of PCA1 axis, the two green circles included *cis*-11 C18:1 + *trans*-10 C18:1, and *trans*-11,*cis*-15 C18:2 + *iso* C17:0 coeluted with *trans*-9 C16:1 (% of total FA), respectively. These variables are negatively related to C10:0 and CH_4 production, but positively related together.

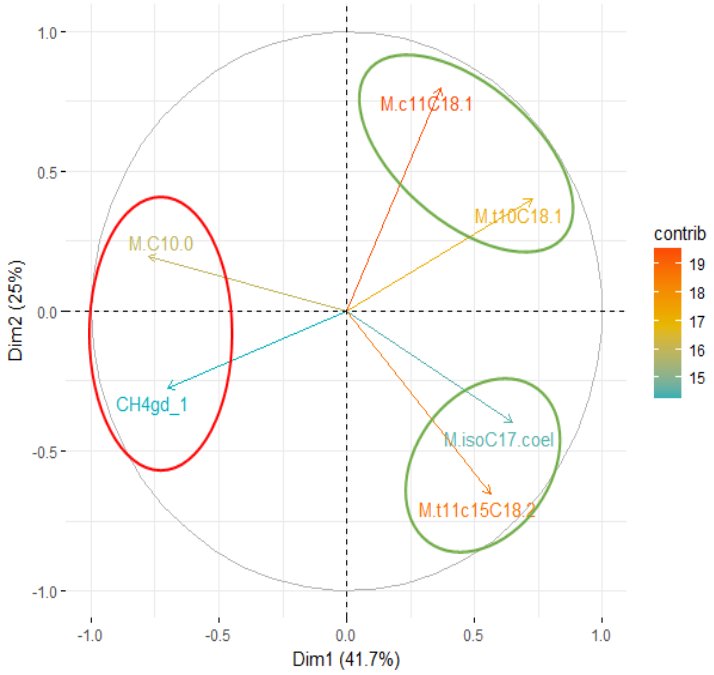


Figure 17 Results of a principal components analysis (PCA) based on the 5 selected milk FA (% of total FA) and CH_4 production (g/d) with the first (PCA1) and second (PCA2) dimensions

When milk FA and other variables are included in the PCA, C10:0, milk fat content and CH_4 production are positively linked together (red circle; Figure 18), but negatively related to the variables included in the red green circle [*trans*-10 C18:1, *trans*-11,*cis*-15 C18:2, *iso* C17:0+*trans*-9 C16:1 (% of total FA), and dietary NDF (%)].

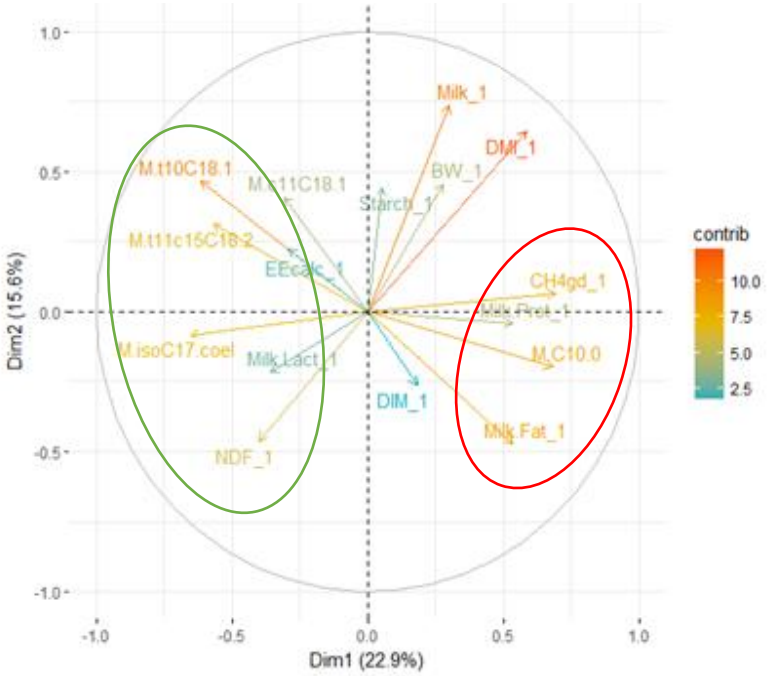


Figure 18 Results of a principal components analysis (PCA) based on selected milk FA (C10:0, *iso* C17:0+*trans*-9 C16:1, *cis*-11 C18:1, *trans*-10 C18:1, *trans*-11,*cis*-15 C18:2, % of total FA), diet composition (NDF, EE, and Starch contents; % of DM), milk performance [milk yield (kg/d), milk fat (%), milk protein (%), milk lactose (%)], animal characteristics [DIM (d), BW (kg)] and CH_4 production (g/d) with the first (PCA1) and second (PCA2) dimensions

PCA for CH_4 yield. The two first axes of the PCA explained together 65.2% of the total variance (Appendix 4). Milk C16:0 and *iso* C16:0 were positively related to CH_4 yield. On the other side of PCA 1 axis, the two green circles included *cis*-11 C18:1 + *trans*-10 C18:1 + *cis*-9,*cis*-12 C18:2. This green circle is negatively related to C16:0 and *iso* C16:0, but positively related together (Appendix 4, Figure 1). Milk C16:0, milk fat content and CH_4 yield were positively linked together (red circle; Figure 2, Appendix 4), but negatively related to the variables included in the red green circle [*trans*-10 C18:1, *cis*-9 C18:1, *cis*-9,*cis*-12 C18:2 (% of total FA), and dietary Starch content (%)].

PCA for CH_4 intensity. The two first axes of the PCA explained together 71.8% of the total variance (Figure 1; Appendix 5). Milk C16:0 and *iso* C16:0 are positively related to CH_4 intensity (g/kg of milk) similarly to CH_4 yield. Milk *cis*-15 C18:1, *trans*-10 C18:1 and *trans*-10+*trans*-11 C18:1 are negatively related to C16:0 and *iso* C16:0, but positively related together each other. As observed for CH_4 yield, milk C16:0, milk fat content and CH_4 intensity were positively linked together, as well as with *iso* C16:0 (red circle; Figure 2, Appendix 5), but negatively related to *trans*-10 C18:1, *trans*-10+*trans*-11 C18:1, *cis*-15 C18:1 (% of total FA), and dietary EE content (%)].

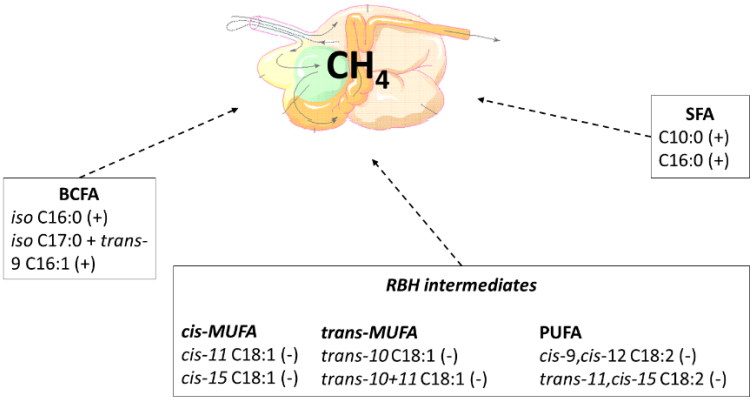


Figure 19 Selected milk FA for inclusion in the CH_4 prediction equations developed in this PhD

To conclude, the milk FA selected as potential predictors of CH_4 emissions (Figure 19) are different for each CH_4 units used, such as:

- **For CH_4 production (g/d): C10:0, *iso* C17:0 (+*trans*-9 C16:1), *cis*-11 C18:1, *trans*-11,*cis*-15 C18:2**
- **For CH_4 yield (g/kg DMI): C16:0, *iso* C16:0, *cis*-11 C18:1, *cis*-9,*cis*-12 C18:2**
- **For CH_4 intensity (g/kg milk): C16:0, *iso* C16:0, *cis*-15 C18:1, *trans*-10 C18:1 or *trans*-10+*trans*-11 C18:1 (these 2 latter FA are not used together in prediction equation development), *cis*-9,*cis*-12 C18:2**

Furthermore, all the variables (milk FA selected, diet composition, animal characteristics and dairy performance) included in the aforementioned PCA had $|r| \leq 0.5$ with CH_4 emissions (Appendix 6), thus they are suitable for development of complex prediction equations.

Model development. A first set of models began with a primary pool including the most representative milk FA (% of total FA) of each family selected based on pairwise correlations and PCA analysis. Then, DMI was added to the simplest models based on milk FA followed by milk performance, plus animal characteristics, plus diet composition (Figure 16). Finally, all significant variables were included to create the most complex models.

Table 9 Pearson correlation coefficients reported in the literature between individual milk FA and CH_4 emissions

References	Chilliard et al. 2009 (g/d)	Dijkstra et al. 2011 (g/kg DMI)	Mohammed et al. 2011 (g/d)	van Lingen et al. 2016 (g/kg DMI)	Rico et al. 2015 (g/d)	van Gastelen et al. 2017 (g/d)
C6:0	0.88	0.19		0.19		0.20
C11:0	0.78	0.42				
C16:0	0.91	0.34		0.34		0.31
iso C15:0		0.42				0.26
iso C17:0		-0.37	-0.38[1]			-0.13
cis-9 C10:1						
cis-11 C18:1		-0.61	-0.64	- 0.52		
cis-11 C20:1					-0.44	
trans-10 C18:1	-0.66		-0.34		-0.41	-0.48
trans-10+*trans*-11 C18:1		-0.46		-0.56		
cis-9,*cis*-12 C18:2		-0.32	-0.39	-0.25		-0.48
cis-9,*trans*-12 C18:2		-0.61				
C18:3 n-3			-0.36			0.09

[1]*iso* C17:0 coeluted with *trans*- 6/7/8 C16:1

Correlated variables ($|r| \geq 0.5$) were not regressed together to minimize multicollinearity and avoid inaccurate parameters, a decreased statistical power, and a risk for excluding variables having significant effects during equations construction (Graham, 2003).

Model validation. The potential of each developed equations to accurately predict CH_4 production was assessed on two independent datasets of observations as detailed previously (Figure 16). Equations were evaluated on both individual data from the internal validation dataset, and on the external validation dataset based on the mean data from the literature. According to Appuhamy et al. (2016), a combination of model evaluation metrics was used to assess equation performance including RMSPE, ETR, ER, ED, CCC, and RSR. The attention was first drawn to RSR and RMSPE to choose the best models, and then all the criteria were taken into account. The calculation details for each of the metrics are available on Appendix 7.

2.2. Methane prediction equations based on milk FA

2.2.1. Methane prediction equations based on milk FA only or with other proxies

For the past decades, several authors have investigated the relationships between individual or sums of milk FA and CH_4 emissions (production, yield, and intensity), because of their common metabolic pathways in the rumen (*See Chapter I, section 3*). These relationships have been studied in individual experiments (Mohammed et al., 2011; van Gastelen et al., 2017) or in meta-analysis (Dijkstra et al., 2011; van Lingen et al., 2014; Williams et al., 2014; Rico et al., 2016; van Gastelen et al., 2018). Authors have highlighted different significant correlations (Table 9), and developed prediction equations using different individual milk FA (Table 10). Relationships between *cis*-11 C18:1 and CH_4 production (g/d) was found in our prediction models, which was also reported by Mohammed et al. (2011), Rico et al. (2016) and van Gastelen et al. (2017). In addition, the *trans*-10 C18:1 is the only common milk FA related to CH_4 yield in this study and in the literature (Dijkstra et al., 2011; van Gastelen et al., 2018).

The identified milk FA to predict CH_4 emissions in this PhD (Table 11) are of multiple origins (Figure 20). The positive relationships between SFA and CH_4 could be explained by the fact that milk SFA are *de novo* synthesized in the mammary gland from blood acetate and *β*-hydroxybutyrate (Bernard et al., 2008), precursors coming from the substrates fermentation in the rumen and which are positively associated with enteric CH_4 emissions (Czerkawski, 1986; Ellis et al., 2008). Milk C10:0 is *de novo* synthesized, whereas C16:0 has double origin: directly coming from the feedstuffs and taken up from the blood stream, or from *de novo* mammary

Table 10 Methane prediction equations from the literature

Authors	n_{animal}	$n_{treatment}$	CH_4	Units	Prediction equations	R^2	RMSE
IPCC, 2007	NA	NA	NA	kg/yr	CH_4= [Intake (MJ/d) × Ym × (365 days/yr)] / [55.65 MJ/kg of CH_4]	0.63	NA
Chilliard et al., 2009	32	4	SF6	g/d	CH_4 = 9.46 × C16:0 – 97.6 × *trans*-16+*cis*-14 C18:1 + 13.3 × forage intake (kg of DM/d) – 78.3 × *cis*-9 C14:1 + 77.4 × 18:2 n-6 – 21.2	0.95	28.8 g/d
Mohammed et al., 2011	32	4	RC	g/d	CH_4 = 272.4 – 486.2 × *cis*-9 C17:1 – 122.7 × *cis*-11 C18:1 + 2.22 × *trans*-CLA – 11.76 × ∑*trans*-C18:1 + 260.1 × *anteiso* C15:0	0.74	NA
Dijkstra et al., 2011	50	10	RC	g/kg DMI	CH_4 = 24.6 + 8.74 × *anteiso*-C17:0 − 1.97 × *trans*-10+11 C18:1 − 9.09 × *cis*-11 C18:1 + 5.07 × *cis*-13 C18:1	0.73	NA
van Lingen et al., 2014	146	30	RC	g/kg DMI	CH_4 = 23.39 + 9.74 × *iso*-C16:0 − 1.06 × *trans*-10+11 C18:1 − 1.75 × C18:2 n-6	0.58	NA
Williams et al., 2014	246		RC, SF6	g/d	CH_4 = 539 + 50.8 × C8:0 – 5.26 × ∑C18	0.37	82.2 g/d
Rico et al., 2016	81		RC	g/d	CH_4 = 669.1 + 838.7 × *cis*-11 C14:1 – 493.2 × *cis*-9 C17:1 – 44.2 × *cis*-11 C18:1 - 963.7 × *trans*-8, *cis*-13 C18:2	0.84	23.5 g/d
van Gastelen et al., 2017	32	4	RC	g/d	CH_4 = 211,2 + 50,4 × C4:0 + 77,7 × *cis*-9 C14:1 – 82,0 × *trans*-11 C18:1	0.63	NA
			RC	g/kg DMI	CH_4 = 27.2 – 7.0 × cis-9,trans-11 C18:2	0.54	NA
van Gastelen et al., 2018	218	30	RC	g/d	CH_4 = 507.0 + 62.9 × C15:0 – 240.6 ×*cis*-9 C17:1 – 202.8 ×*trans*-10 C18:1 – 59.3 ×*trans*-11 c18:1 + 48.1 ×C18:2n-6 – 187.1 ×C18:3n-3 + 326.4 ×C20:4n-3 – 816.8 ×C24 :0	0.54	35.7 g/d
			RC	g/kg DMI	CH_4 = 22.9 + 20.9 ×*iso* C15:0 – 9.6 ×*anteiso* C15:0 + 7.6 ×C17:0 – 2.4 ×*trans*-11 C18:1 – 2.8 ×trans-15+cis-11 C18:1 – 4.4 × C18:3n-3	0.40	1.6 g/kg DMI
Niu et al., 2018	2566	NA	RC, SF_6, GF	g/d	CH_4 = -60.5 + 12.4 × DMI - 8.78 × EE + 2.10 × NDF + 16.1 × MF + 0.148× BW	NA	16.8%
		NA		g/kg DMI	CH_4 = 15.4 - 0.291 × EE + 0.144 × NDF - 0.104 × ECM + 1.34 × MF - 1.12 × MP + 0.00330 × BW	NA	16.1%

GF: GreenFeed; RC: respiration chamber; SF6: SF_6 gas tracer method; DMI: Dry matter intake; NA: not available.

synthesis. Our results confirm the hypothesis reported by several authors about the common biological pathway and the positive relationships between milk SFA and CH_4 emissions.

Milk branched FA are of microbial origin and milk *iso* C16:0 and *iso* C17:0 are both synthesized in the rumen from branched amino-acid precursors (valine and *iso* leucine, respectively), and minor VFA (*iso* butyrate and valerate) (Vlaeminck et al., 2006). In the equation, we observed that *iso* C16:0 and *iso* C17:0 are positively and negatively related to CH_4, respectively. Butyrate is the precursor of *iso* C16:0, as well as valine. It has been proven that butyrate production by rumen microorganisms is releasing H_2 (Mills et al., 2001), thus explaining the positive link with CH_4 production. Similarly, valerate, which is involved in the production of *iso* C17:0 along with leucine, is considered as an H_2 sink (Mills et al., 2001). Thus, more *iso* C17:0 implies more valerate produced in the rumen and, in turn, less H_2 available for methanogenesis. However, Vlaeminck et al. (2006) reported that outer membrane of fibrolytic bacteria is generally enriched in *iso* FA, whereas amylolytic bacteria contain high amounts of linear odd-chain FA and *anteiso* FA. Therefore, it might be expected that an increased cellulolytic bacteria number leads to higher milk *iso* FA content, whereas an increased number of amylolytic bacteria could increase milk *anteiso* and linear odd-chain FA at the duodenal level and then in the milk. Consequently, the negative relationship between milk *iso* C17:0 is surprising but could be due to the coelution with *trans*-9 C16:1 that might hide the true relationship.

The C18:1, C18:2 and C18:3 isomers are coming from the diet or from the RBH which is either total or partial. The RBH is influenced by the rumen conditions, such as pH, which modulate bacteria number and activity. This latter parameter is directly linked to the carbohydrate nature, and thus to CH_4 emissions (Figure 20). Indeed, certain dietary strategies rich in readily fermentable carbohydrates (high starch- and low-fiber diets) are well known to decrease ruminal pH, which impairs methanogens activity resulting in low CH_4 emissions, as well as modifies RBH pathways of unsaturated FA inducing a strong production of RBH intermediates.

The RBH of dietary unsaturated FA can also serve as a H_2 sink even if a very limited amount of H_2 may be used for this purpose (1 to 2% of the rumen metabolic H_2; Czerkawski and Clapperton, 1984; Jenkins et al., 2008). In addition, dietary unsaturated FA and certain unsaturated RBH intermediates have some anti-bacterial and methanogens proprieties (Goel et al., 2009) decreasing in turn methanogenesis. Dietary unsaturated FA are known to be negatively associated with CH_4 production and yield as reported by (Patra, 2012), which support our results.

Table 11 Methane prediction equations [simple (1, 12, and 18); and best complex (9, 13, and 22)] developed from the individual datatset

Eq.	n_{animal}	$n_{treatment}$	Units	Prediction equation	R^2	RMSE[1]
1	301	20	g/d	291.7 (±40.45) + 25.2*C10:0 (±7.45) – 176.6**iso* C17:0 + *trans*-9 C16:1 (±43.88) – 90.7**cis*-11 C18:1 (±20.97) – 46.6**trans*-11,*cis*-15 C18:2 (±10.18)	0.72	58.6
9	265	18	g/d	-42.0 (±60.57) – 112.1**iso* C17:0 + *trans*-9 C16:1 (±36.09) – 104.3**cis*-11 C18:1 (±15.55) – 31.7**trans*-11,*cis*-15 C18:2 (±8.57) + 12.5*DMI (±1.32) + 4.8*NDF (±1.04) - 4.7*EE (±2.34) + 0.2*BW (±0.05)	0.85	42.8
12	393	20	g/kg DMI	21.8 (±1.07) + 10.7**iso* C16:0 (±2.45) – 2.8**cis*-11 C18:1 (±0.56) – 0.8**trans*-10 C18:1 (±0.14) – 0.8**cis*-9,*cis*-12 C18:2 (±0.28)	0.70	2.8
13	398	24	g/kg DMI	7.6 (±3.02) + 0.1*C16:0 (±0.04) + 9.1**iso* C16:0 (±2.37) – 2.8* *cis*-11 C18:1 (±0.54) – 0.6* *trans*-10 C18:1 (±0.14) – 0.7* *cis*-9,*cis*-12 C18:2 (±0.28) + 0.3*NDF (±0.06) – 0.3*EE (±0.12)	0.72	2.6
18	277	17	g/kg milk	13.8 (±1.43) + 16.2**iso* C16:0 (±3.75) – 3.1**cis*-15 C18:1 (±1.57) – 0.5**trans*-10+*trans*-11 C18:1 (±0.15)	0.61	3.7
22	357	22	g/kg milk	- 11.9 (±3.87) + 0.2*C16:0 (±0.04) + 11.5**iso* C16:0 (±3.08) – 0.8**trans*-10 C18:1 (±0.15) + 0.3*NDF (±0.07) + 0.02*BW (±0.01) + 0.01*DIM (±0.003)	0.67	3.3

[1]RMSE expressed in the CH_4 units of the prediction equation

In fact, negative correlations were found among individual *cis* MUFA, especially with *cis*-11 C18:1 and CH_4 emissions in our 2 *in vivo* experiments and in the study of Dijkstra et al., 2011). However, we highlighted for the first time negative relationships among PUFA (*trans*-9,*cis*-12 C18:2) and CH_4 production and yield. Dijkstra et al. (2011) noted a negative correlation between *cis*-9,*trans*-12 C18:2 and CH_4 yield, which was not observed in our results. Similarly, Dijkstra et al. (2011), van Lingen et al. (2014) and van Gastelen et al. (2017) reported a negative correlation between *cis*-9,*cis*-12 C18:2 and CH_4 yield, which we did not reproduce.

The best CH_4 prediction equations developed in this work included different milk FA depending on the CH_4 units, as well as different other variables, (Table 11; *for further details, see Chapter IV*). Vanlierde et al. (2015) and Vanrobays et al. (2016) highlighted the importance of using lactation stage in order to predict CH_4 emissions because variations exist among CH_4 emissions and milk FA concentrations according to the lactation stage. Based on these findings, we used DIM for the development of more complex prediction equations. However, this proxy was only retained in the best prediction equations of CH_4 intensity. Other proxies (dietary NDF, EE or starch content, DMI, and BW) included in CH_4 prediction equations will not be discussed in details, because their influence has already been discussed in *Chapter IV*. Considering the milk composition, milk protein content was included as a positive predictor in prediction equations for CH_4 yield and intensity, milk fat content was included in CH_4 production and intensity prediction equations, and milk lactose content was only included on CH_4 intensity prediction equations. Niu et al. (2018) also observed a positive relationship between milk protein content and CH_4 yield, and between milk fat content and CH_4 production and yield.

In conclusion, individual milk FA selected in published CH_4 prediction equations based on milk FA are different from one paper to another, which could be due to the different experimental conditions (e.g. diet, parity or lactation stage). Therefore, milk FA included in the prediction equations seem to be dependent on the dietary strategy. The dataset used in this PhD presents probably the widest domain used so far to develop prediction equations regarding the variety of diets used in France, Denmark, Finland, USA, Canada or Australia. Therefore, the common milk FA found between this work and published studies reinforce the idea that they are good predictors of CH_4 emissions from dairy cows fed different diets.

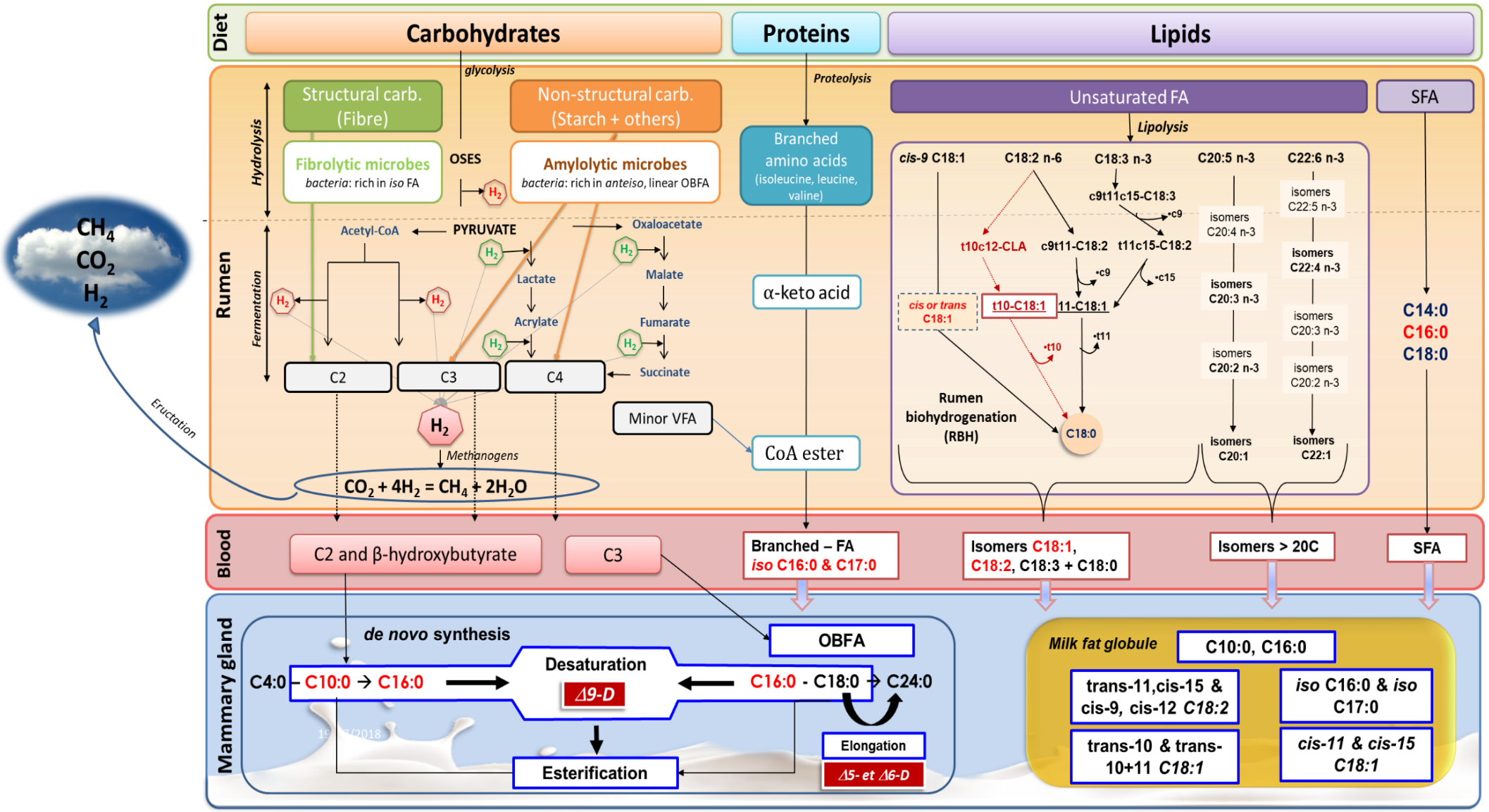


Figure 20 Potential common metabolic pathways between CH_4 emissions and milk FA: focus on the selected milk FA (in red) to predict CH_4

2.2.2. Validation of the CH_4 prediction equations developed in this PhD

Simple versus complex prediction equations. Simple CH_4 prediction equations based on milk FA (See Table 11, and *Chapter IV*) developed in this thesis showed good performance (RMSPE from 19% to 27% depending on the CH_4 unit used) and have proven the ability of milk FA as single proxy to predict CH_4. However, complex equations including milk FA and other proxies (DMI, dietary NDF, EE and starch contents, DIM and BW) had better performance (RMSPE from 16% to 24% depending on the CH_4 unit). In their review, Negussie et al. (2017) have argued the advantage of combining proxies to predict CH_4 emissions because: 1) proxies may explain different sources of CH_4 variations (carrying different information); 2) one proxy allows correction for shortcomings in the way the other proxy describes CH_4 emissions. Previously, several authors had used a combination of proxies such as:

- milk FA and forage intake (Chilliard et al. (2009).
- milk FA, rumen indicators (VFA, pH, protozoa counts), feed intake (DMI, forage intake, or FA intake), and milk yield and composition (Mohammed et al., 2011)

However, van Gastelen et al. (2018), who used milk FA and three types of milk metabolites (e.g. volatile and non-volatile metabolites), did not report any improvement of inclusion of other proxies in CH_4 prediction performance of their models.

Validation on individual versus mean data. Irrespective of the CH_4 emissions unit (g/d, g/kg of DMI, or g/kg of milk), we reported that prediction equations developed on individual data have better performance when evaluated with individual data (internal validation dataset) than with mean data (external validation dataset). Variability observed for the mean data from published studies (external validation dataset) was narrower and comprised within the variability of the individual dataset (min and max values). In addition, some dietary strategies (monensin, 3-NOP or cardanol) were only represented in the external validation dataset. Thus, the developed CH_4 prediction equations, whatever the unit, seem to be unsuitable for diets containing such additives. Moreover, all of the above-mentioned differences (narrower variability, different dietary strategies) between the 2 independent validation datasets may explain the lower performance of the prediction equations on the external validation dataset.

In conclusion, CH_4 prediction equations developed in this PhD showed good prediction performance on individual dataset but not on mean dataset. This suggests that their applicability is limited to predict CH_4 emissions in dairy cows fed diets with certain additives (monensin, 3-NOP or cardanol). In addition, the CH_4 prediction equations

Table 12 Comparison of CH_4 prediction equations based on milk FA developed in this PhD with those of the literature

		Model performance evaluation					
Authors	Dataset	RMSPE, %	ECT, %	ER, %	ED, %	CCC	RSR
This work, prediction equation 1 (g/d)	Internal	24.0	0.59	3.35	96.06	0.53	0.88
	External	27.7	55.6	0.60	43.77	0.01	1.22
Williams et al., 2014 (g/d)	Internal	44.2	8.51	53.4	38.1	0.05	1.56
	External	27.0	45.7	14.9	39.4	0.01	1.48
van Gastelen et al., 2017 (g/d)	Internal	54.9	16.8	53.7	29.5	0.02	1.78
	External	45.4	16.6	49.7	33.6	0.03	1.65
Mohammed et al., 2011 (g/d)	Internal	102.6	61.2	19.5	19.3	0.00	2.27
	External	187.8	70.4	19.3	10.3	0.00	3.05
van Gastelen et al., 2018 (g/d)	Internal	128.7	20.5	72.2	7.3	0.01	3.24
	External	47.7	35.1	53.0	11.9	0.00	2.79
This work, prediction equation 12 (g/kg of DMI)	Internal	18.9	0.83	1.20	97.97	0.71	0.84
	External	20.9	31.53	1.68	66.78	0.27	1.14
van Lingen et al., 2014 (g/kg of DMI)	Internal	22.0	0.05	8.73	91.20	0.57	0.98
	External	27.1	53.1	0.24	46.7	0.12	1.38
Dijkstra et al., 2011 (g/kg of DMI)	Internal	34.7	7.28	52.1	40.6	0.50	1.42
	External	28.5	50.8	7.01	42.2	0.13	1.47
van Gastelen et al., 2017 (g/kg of DMI)	Internal	27.8	35.5	4.07	60.4	0.16	1.46
	External	27.4	51.4	1.16	47.4	0.11	1.40
van Gastelen et al., 2018 (g/kg of DMI)	Internal	34.0	0.0	59.1	40.9	0.44	1.53
	External	29.5	33.9	21.2	44.9	0.20	1.42

would need a better prediction ability in order to highlight small changes in CH_4 emissions (< 16%) due to their variability in prediction errors.

2.3. Comparison of the performance of the CH_4 prediction equations developed in this PhD with those of the literature

To predict enteric CH_4 emissions, several prediction equations are available in the literature (Table 10). Among them, we selected 8 prediction equations of CH_4 based on milk FA (Table 12) and 2 others global prediction equations (IPCC, 2007 and Niu et al., 2018) including predictors not based on milk FA (Table 13) for comparison with prediction equations from this PhD. Prediction equations were selected for the following reasons:

- Mohammed et al. (2011) and van Gastelen et al. (2017) developed CH_4 emissions prediction equations based only on milk FA and on individual data from dairy cows.
- Chilliard et al. (2009), Dijkstra et al. (2011), van Lingen et al. (2014), and Niu et al. (2018) included the level of forage intake and/or dietary NDF, starch or EE content, which are all known to be good predictors of CH_4 emissions.
- Several authors (Dijkstra et al., 2011; van Lingen et al., 2014; Williams et al., 2014; Niu et al., 2018; van Gastelen et al., 2018) developed prediction equations using a meta-analysis approach that included several studies (individual *in vivo* data from dairy cows fed different diets).

The IPCC equation (IPCC, 2007) was also selected because it is the most widely used equation up to know to predict CH_4 emissions (*for more information, refer to Chapter I, section 1*).

The R^2 and RMSE reported in the literature are ranging from 0.37 to 0.84, and 23.5 and 82.2 g/d (Table 10), respectively. The R^2 and RMSE reported in this PhD with Equations 1 (R^2 = 0.85; RMSE = 58.6 g/d) are comprised in this range and show good performances. However, prediction equations from Rico et al. (2016) and van Gastelen et al. (2018) had the lowest RMSE with 23.5 and 35.7 g/d, respectively. In addition, the coefficient of variation (**CV**) reported in Rico et al. (2016) and van Gastelen et al. (2018) for CH_4 production prediction equation were smaller (4,9 and 9.7%, respectively) as compared to the CV of Equation 1 (14.7%). Similarly, van Gastelen et al. (2018) reported a CV of 7.1% for CH_4 yield prediction equation, which is lower than CV from equation 12 (14.2%).

Table 13 Comparison of the performance of the CH_4 prediction equations developed in this PhD with those of the literature

		Model performance evaluation					
Authors	Dataset	RMSPE, %	ECT, %	ER, %	ED, %	CCC	RSR
This work, Prediction equation 9 (g/d)	Internal	15.6	0.03	0.21	99.75	0.9	0.56
	External	15.9	16.2	7.3	76.52	0.17	0.70
This work, Prediction equation 13 (g/kg of DMI)	Internal	18.8	0	6.80	93.20	0.76	0.84
	External	17.0	6.81	13.11	80.08	0.66	0.92
Niu et al., 2018 (g/d)	Internal	20.7	8.47	5.09	86.4	0.15	0.77
	External	26.2	0.21	30.2	69.6	0.43	1.16
IPCC, 2007 (g/d)	Internal	20.8	12.8	5.33	81.8	0.12	0.85
	External	18.9	11.0	4.75	84.3	0.07	0.93
Chilliard et al., 2009 (g/d)	Internal	27.9	16.0	28.2	54.9	0.04	1.25
	External	33.6	41.7	35.7	22.7	0.01	1.92

The milk FA-based prediction equations from the literature (Mohammed et al., 2011; Dijkstra et al., 2011; van Gastelen et al., 2017; van Lingen et al., 2014; Williams et al., 2014; van Gastelen et al., 2018) were evaluated on our two validation datasets[1], and had moderate prediction potential (Table 12; Appendix 8). This is probably due to the wider range of diets used in this work, and thus confirming that literature prediction equations have a specific domain of applicability for accurate CH_4 estimations. The CH_4 yield prediction equations developed in this PhD (equation 12, Tableau 12) had better performance than that from van Lingen et al. (2014), when evaluated on both independent validation datasets (Tableau 12). Indeed, when evaluated on internal validation dataset, RSR (0.84) was greater and RMSPE (18.9%) was lower from our equations than those of van Lingen et al. (2014), who used only milk FA to predict CH_4 yield (RSR = 0.98 and RMSPE = 22.0%).

Prediction potentials from the other equations described in the literature (Mohammed et al., 2011; Dijkstra et al., 2011; Williams et al., 2014; van Gastelen et al., 2017, 2018) were rather low, with RSR values > 1.0, indicating that the variability linked to the CH_4 prediction was greater than that of the CH_4 observed in the datasets.

We observed better performance of the equations based on milk FA developed in this study (equations 1 and 12) when evaluated on the internal validation dataset as compared to the external validation dataset. The same pattern was also observed for the equations from the literature, except for van Gastelen et al. (2017, 2018). Thus, prediction equations seem to better perform when applied on individual data when compared to mean data.

The R^2 reported in this work with equation 9 (R^2=0.85; model RMSE=42.8 g/d), was lower than R^2 reported previously by Chilliard et al. (2009), with R^2=0.95 and model RMSE=28.8 g/d), using milk FA concentrations (*cis*-9 C14:1, C16:0, *trans*-16+*cis*-14 C18:1, and C18:2n-6) and forage intake in their prediction equation. Thus, equation 9 had higher RMSE than Chilliard et al. (2009) but similar CV (10.8% *versus* 9.6%). In addition, Niu et al. (2018) reported a similar RMSPE of 16.6% for CH_4 production prediction equations in comparison to RMSPE from equation 9 (15.6 and 15.9% on the internal and external validation datasets, respectively). However, better prediction ability was observed with Equation 9 as compared to the one from IPCC (R^2 = 0.63) based on GEI.

In addition, the complex equations developed in this study performed better than those from Niu et al. (2018), or IPCC (2007), or Chilliard et al. (2009) when challenged against the internal

[1] Except for the prediction equation developed by Rico et al. (2016) because our datasets did not include all the predictive variables used in this equation.

validation datasets (Table 13) from this PhD thesis. Indeed, we observed lower RSR (0.56 to 0.92) for the Equations 9 and 13 as compared to higher RSR (0.77 to 1.92) for the published equations. The IPCC equation presented good performance on both validation datasets but still having greater RSR (> 0.85) than our Equation 9 (RSR < 0.70). When challenged against the external validation datasets, equations from Chilliard et al. (2009) and Niu et al. (2018) had low performance with RSR < 1. However, Chilliard et al. (2009) developed their prediction equation on few data (16 observations) and specific diets such as corn silage-based diets supplemented with linseed, which might explain the low performance.

The CH_4 prediction equations (Appendix 9) developed in this PhD showed better performance than published prediction equations when evaluated on the external validation datasets (individual and mean).

To conclude, simple prediction equations based on milk FA from this PhD did not lead to better performance than the one developed by Rico et al. (2016) and van Gastelen et al. (2018). However, the milk FA included in the aforementioned published equation are present in very low concentration and the relationships with methanogenesis is yet to be demonstrated. Performances of complex prediction equations developed in this PhD thesis were similar as compared to Chilliard et al. (2008) or Niu et al. (2018). Additionally, prediction equations developed in this PhD thesis seemed to better perform when evaluated on a similar dataset than that used for their development, i.e. with the individual dataset. Overall, evaluating published prediction equations on similar subsets than those used for their development would lead to better performance because they are obviously related to their domain of validity.

2.4. Practical use "on farm" of the CH_4 prediction equations developed in this PhD: CG- vs MIR-analyzed FA

Up to now, GC techniques have been the most accurate and precise methods to quantify a large number of individual milk FA, even when present at low concentrations in milk fat of dairy ruminants. However, this method requires high expertise, is expensive and time-consuming, and, therefore, it is not feasible for the analyses of a large number of samples on a routine basis. Other measurement methods have been developed and NIR and MIR spectroscopies appear to be a good alternative to quantify certain milk FA (Coppa et al., 2010) because of their low costs, rapid utilization and good prediction of certain milk components. In addition, the MIR spectrometry is already implemented in laboratories of Milk Recording Organisation to quantify major milk components used for milk payment (e.g. milk fat, protein,

Table 14 Performance of prediction equations based on MIR spectrometry-analyzed milk FA

		Models performance evaluation					
Equations	Dataset	RMSPE, %	ECT, %	ER, %	ED, %	CCC	RSR
Simple CH_4 (g/d) equation (24) including	Internal	24.9	1.3	2.8	95.9	0.29	0.96
C10:0	External	28.6	3.1	2.8	94.1	0.23	1.38
Complex CH_4 (g/d) equation (25) including	Internal	23.7	1.2	1.0	97.8	0.41	0.88
C10:0, *cis*-11 C18.1, *cis*-9,*cis*-12 C18.2	External	22.8	22.6	0.4	77.0	0.04	0.98
Simple CH_4 (g/kg of milk) equation (26)	Internal	32.2	0.14	0.2	99.6	0.50	0.94
including C16:0	External	29.4	23.9	1.6	74.5	0.27	1.05
Complex CH_4 (g/kg of milk) equation (27)	Internal	32.3	0.4	0.6	99.0	0.53	0.94
including C16:0, *cis*-11 C18.1	External	36.1	33.2	13.4	53.4	0.00	1.32

and lactose contents) and can be used also to estimate various milk FA (Soyeurt et al., 2006; Ferrand-Calmels et al., 2014). Because there is an increasing social demand for healthy products and composition labeling of dairy products, some European countries (e.g. France and The Netherlands) have introduced milk FA composition as a parameter to be considered to determine milk price (Coppa et al., 2014). Furthermore, milk FA could be used in predicting enteric CH_4 emissions from dairy cattle (*Chapter IV*) that would help developing and studying large-scale strategies effect on CH_4 emissions, as well as identifying environmentally friendly animals and farming systems (lowering their carbon footprint due to decreasing enteric CH_4 emissions). Therefore, it appears necessary to develop routine, low cost methods for the evaluation of the milk FA composition. This PhD work has highlighted the potential of several milk FA (C10:0, C16:0, *iso* C16:0, *iso* C17:0 +*trans*-9 C16:1, *cis*-11 C18:1, *cis*-15 C18:1, *trans*-11,*cis*-15 C18:2, *cis*-9,*cis*-12 C18:2) to predict CH_4 emissions according to the unit used, among which some FA are well measured by MIR spectrometry. Indeed, milk C8:0, C10:0, C16:0, the sum of SFA, *cis*-9 C18:1, *cis*-11 C18:1 or *cis*-9,*cis*-12 C18:2 could be well estimated with MIR spectrometry (Soyeurt et al., 2006; Ferrand-Calmels et al., 2014). Thus, to investigate the potential of use of milk FA well estimated by MIR spectrometry, CH_4 prediction equations (in g/d and g/kg of milk) were developed on a restricted selection of milk FA based on the results of Soyeurt et al. (2006) and Ferrand-Calmels et al. (2014). The best developed prediction equations were the equation 24 (Figure 21 & 22) and 26 (Figure 22 & 23):

- CH_4 (g/d) = 385.9 (±26.60) + 42.9*C10:0 (±5.59) – 89.5**cis*-11 C18:1 (±11.34) – 17.8**cis*-9,*cis*-12 C18:2 (±5.82)

(RMSE=61.8 g/d; R^2=0.70; *n* = 482)

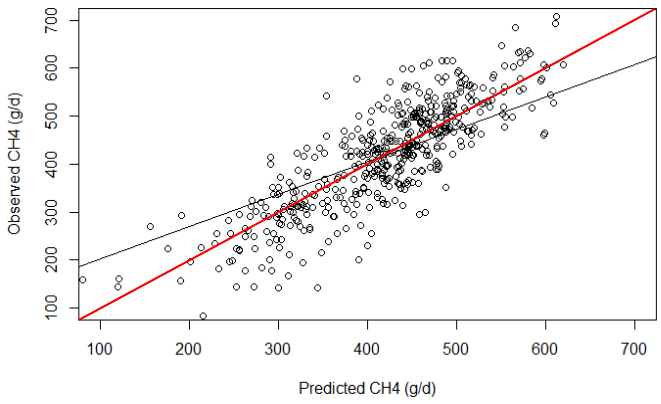


Figure 21 Observed and predicted CH_4 production (g/d) using milk FA potentially analyzed by MIR spectrometry. The black and red solid lines represent the fitted regression line for the relationship between predicted and observed values and the identity line (y = x), respectively

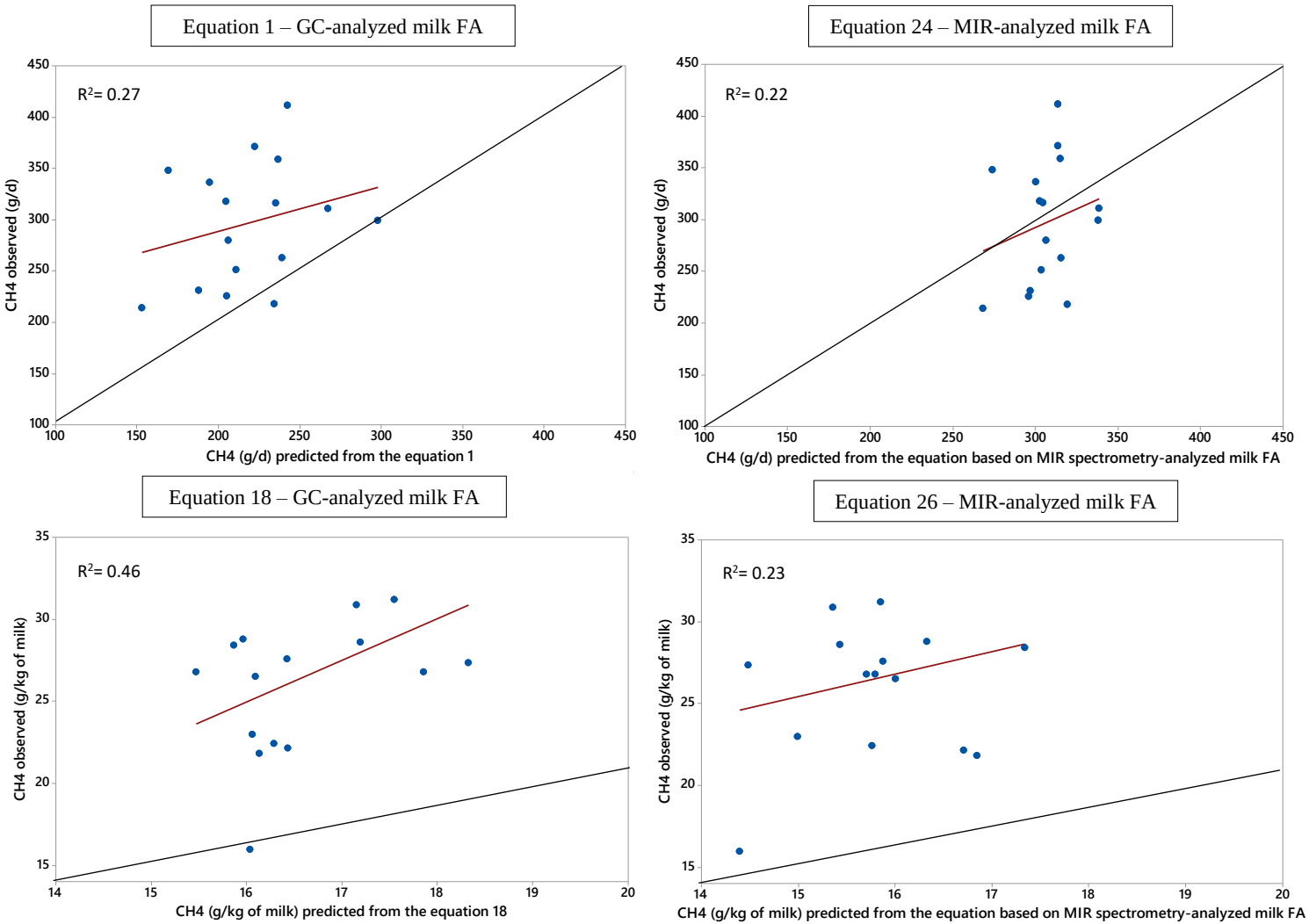


Figure 22 Observed and predicted CH_4 production (g/d) and intensity (g/kg of milk) using GC- and MIR -analyzed milk FA. The red and black solid lines represent the fitted regression line for the relationship between predicted and observed values and the identity line (y = x), respectively

- CH_4 (g/kg of milk) = 8.2 (±1.29) + 0.25*C16:0 (±0.04)

(RMSE=3.8 g/kg of milk; R^2=0.55; *n* = 578)

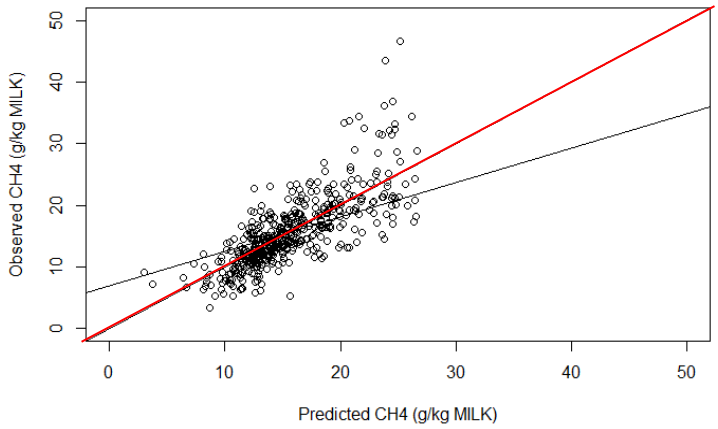


Figure 23 Observed and predicted CH_4 intensity (g/kg of milk) using milk FA potentially analyzed by MIR spectrometry. The black and red solid lines represent the fitted regression line for the relationship between predicted and observed values and the identity line (y = x), respectively

Similar modeling approach (Figure 16) was used to assess performance of the equations using the two independent validation datasets. Performance (Table 14) of CH_4 prediction equations (g/d) based on MIR-analyzed milk FA (equation 24) led to slightly lower predictive potential than predictions obtained from equation 1 based on GC-analyzed milk FA (*Chapter IV).* For instance, using CH_4 production prediction equations based on either GC- or MIR spectrometry-analyzed milk FA present an error of on average 108 and 96 g/d, respectively. As compared to the simple CH_4 production prediction equations based on C10:0 analyzed by MIR spectrometry, the performance was slightly enhanced with a more complex equation including *cis*-11 C18:1 and *cis*-9,*cis*-12 C18:2 (equation 25 with lower RSR and RMSPE on both validation datasets). With CH_4 intensity prediction equation, performance were similar for simple and complex equations (equations 26 and 27), respectively, when evaluated on the internal validation dataset, but simple prediction equation performed better than the complex one when challenged on the external validation dataset (1.05 versus 1.32 for RSR value, respectively)

In conclusion, CH_4 prediction equations based on MIR-analyzed milk FA (equations 24 and 26) led to slightly lower performance (Figure 22) than those from this PhD based on GC-analyzed milk FA. However, these equations (equations 24 and 26) would be ready to be used on field. Nevertheless, the error due to the MIR spectrometry analysis of the selected milk FA needs to be reduced. Indeed, a reduction greater than 16% previously

mentioned for GC-based prediction equation, should be observed to assess significant effect of mitigation strategies on CH_4 emissions.

Ability of the prediction equations based on MIR spectrometry-analyzed milk FA to highlight CH_4 emissions reduction. In the WP3 of the consortium project (*See Text Box 1*), an *in vivo* experiment was carried out on 16 multiparous lactating Holstein dairy cows allocated to one of the two groups based on their calving dates (control vs treated groups). Both groups were fed a basal diet (on DM basis) of 35% of corn silage, 30% of grass hay and 35% of concentrate, which included a placebo (control group) or a feed additive (treated group) known to mitigate CH_4 emissions. As expected, dairy cows fed the additive emitted 23% less CH_4 (g/d, g/kg DMI, g/kg milk). Simple CH_4 prediction equations developed in this PhD either based on GC- or on MIR spectrometry-analyzed milk FA, were used to predict CH_4 emissions and prediction equations performance were assessed on this *in vivo* experiment (Table 15).

Table 15 Performance of prediction equations based on MIR spectrometry- and GC- analyzed milk FA for CH_4 production and intensity on an *in vivo* experiment on dairy cows (n = 16)

Equation n° (CH_4 unit)	Milk FA measurement technique	RMSPE, %	CCC	RSR
1 (in g/d)	GC	44.5	0.00	1.63
25 (in g/d)	MIR spectrometry	18.7	0.18	0.96
18 (g/kg of milk)	GC	65.0	0.02	2.27
26 (g/kg of milk)	MIR spectrometry	73.7	0.01	2.45

Performance were rather poor (RSR ≥ 1.0; CCC very low) with all the prediction equations whatever the unit of CH_4 emissions. By running simple Anova analysis, the mitigating effect of the feed additive was only detected with the prediction equation 18 based on GC-analyzed milk FA ($P < 0.05$).

To conclude, the developed equation from this work poorly predicted CH_4 emissions from dairy cows fed the feed additive tested, probably because this feed additive was not represented in the individual dataset used to develop the prediction equations. Thus, this study is not in the domain of applicability of these prediction equations, which support the fact that applicability of the prediction equations depend on the dataset used to build them.

3. Perspectives

This PhD thesis deepened the knowledge about the links among milk FA and CH_4 emissions, in order to propose new prediction equations able to be used on farm, and to evaluate new strategies to mitigate CH_4 emissions in dairy cows. To complete this work, several perspectives can be drawn to improve prediction equation applicability on farm.

3.1. Complex CH_4 prediction equations based on milk FA analyzed by MIR spectrometry and other proxies

Prediction equations of CH_4 production and intensity based on milk FA analyzed by MIR spectrometry can lead to slightly lower performance as the prediction equations based on GC-analyzed milk FA. Hence, MIR equations could be "preferred " as a promising option for "on farm" applicability. It has been shown both in this PhD and in the literature (Niu et al., 2018) that more complex prediction equations present better prediction. Dietary NDF, EE and Starch contents are nowadays estimated by NIR spectrometry, but present limits in their estimation accuracy for TMR. However, they represent an easy and low cost tool ready to be used on farm. Therefore, the CH_4 prediction equations based on MIR spectrometry-analyzed milk FA and on diet composition determined by NIR spectrometry could be developed. In order to assess the potential of such prediction equations based on such proxies, a dataset including these proxies could be created in order to develop CH_4 prediction equations and to compare their performance with prediction equations developed in this work.

We have developed prediction equations with milk FA that are well estimated by MIR spectrometry as well as with diet composition that could be estimated by NIR spectrometry. Prediction equations present good performance with RSR < 1.0 and RMSPE of 21 to 25% (Table 16), which are slightly lower to prediction equations based on GC-analyzed milk FA. Thus, it seems good opportunity for using these equations on farm. Further researches should be done in order to explore and reduce the error linked to the predictions. These equations could also be assessed on independent data collected on dairy cows on farm. In that sense, as part of the Consortium project, results from a large experiment (n = 45 dairy cows during the first 6 months of lactation, CH_4 measured daily with the GreenFeed system, milk FA analyzed monthly by GC or MIR) could be used to assess the performance of the developed CH_4 prediction equations throughout an entire lactation period.

Table 16 Methane production prediction equations based on MIR spectrometry-analyzed milk FA and diet composition

Equations	Dataset	RMSPE %	CCC	RSR
CH_4 (g/d) = 226.7 + 38.4*C10:0 (±6.27) - 89.1**cis*-11 C18.1 (±11.33) – 15.7**cis*-9,*cis*-12 C18.2 (±5.64) + 5.4*NDF (±1.05) – 7.8*EE (±2.57)	Internal	25.7	0.28	0.95
	External	21.7	0.16	0.89
CH_4 (g/d) = 208.8 + 40.8*C10:0 (±6.38) + 3.0*C18:0 (±1.61) - 81.4**cis*-11 C18.1 (±12.02) – 16.9**cis*-9,*cis*-12 C18.2 (±5.66) + 5.1*NDF (±1.06) – 9.5*EE (±2.72)	Internal	25.5	0.28	0.94
	External	21.3	0.12	0.88

3.2. Specific CH_4 prediction equations depending of diets or on farm variability: creating sub-databases

Specific prediction equations based on milk FA according to diets could be developed in order to increase the prediction ability. Indeed, some diets could induce modification of CH_4 emissions and great production of specific milk FA, such as diets rich in PUFA or in starch. Thus, the individual database could be divided according to the dietary strategies in order to develop and validate prediction equations with a similar *in silico* approach as described in this PhD, as well as comparison with published prediction equations. This would help understanding the relationships among milk FA and CH_4 in specific dietary conditions.

In addition, in order to assess applicability of developed CH_4 prediction equations, the individual database could be restrained according to the range of variations in milk FA and CH_4 emissions represented commonly on farm.

3.3. Prediction equations of CH4 based on other proxies from milk

According to Negussie et al. (2016), developing prediction equations based on milk Fourier-transform infrared spectroscopy (**FTIR**) and other proxies such as the lactation stage, is a promising project. In that sense, several authors have already attempted to develop prediction equations based on milk FTIR spectra and showed good prediction potential (Dehareng et al., 2012; Vanlierde et al., 2016) (Dehareng et al, 2012, 2015, 2017; Vanlierde et al, 2018). In these studies, different methods (SF_6 and respiration chambers) to measure CH_4 were used as well as a large variety of diets. For instance, Dehareng et al. (2012) have developed the first CH_4 prediction equations based on FTIR analysis of milk in dairy cows fed either pasture grass or corn silage based diets. The CH_4 prediction obtained showed better results based on R^2_c (calibration coefficient of determination), by considering the CH_4 in g/kg of milk instead of g/d

(R^2_c of 0.80 to 0.93 vs. 0.77 to 0.84, respectively). These equations had better R^2 than in our prediction equations, but the dataset used only gathered two experiments with a limited number of cows (*n* = 60). Vanlierde et al. (2015b) developed prediction equations using 446 observations from dairy cows, but prediction performance were lower than in Dehareng et al. (2012). However, these authors reported a RMSE of calibration of 10% of the overall daily CH_4 production, which is lower than the RMSPE reported in our prediction equations (< 16% for equation 9). van Gastelen et al. (2018) compared the prediction potential of milk FTIR (using milk spectra) for CH_4 emissions with that of milk FA (using GC technique) on data from 9 experiments (218 observations and 30 dietary treatments that included either grass- or corn-silage based diets, and 15 to 30% of concentrate). The prediction models of CH_4 production and yield based on GC-analyzed milk FA presented RMSE of 35.7 g/d and 1.6 g/kg of DMI, respectively, as well as CCC of 0.72 and 0.59, respectively. The prediction models based on FTIR-milk spectra estimated CH_4 production and yield with RMSE of 43.2 g/d and 1.9 g/kg of DMI, respectively, and with CCC of 0.52 and 0.40, respectively. The cross-validation results indicated that all CH_4 prediction models (both GC-analyzed milk FA and FTIR-based models) are robust within their validity domain. Thus, it appears that FTIR-based models have the ability, even though lower than with GC analyzed milk FA-based models, to predict CH_4 emissions. However, both techniques do not seem able to predict CH_4 emission of dairy cows in practice without controlled diets. Additional CH_4 measurements (whatever the technique used) coupled with simultaneous FA measurements by GC or spectra determination by MIR are needed to improve the accuracy and robustness of these prediction models.

3.4. Ability of the prediction equations to highlight CH_4 emissions reduction

Several authors have tested the effect of CH_4 mitigation strategies, such as linseed supplementation, increasing concentrate proportion, or using chemical additive (e.g. 3-NOP, nitrate), and reported a CH_4 emissions reduction, whatever the unit of expression, varying from 8 to 47 % (Martin et al., 2010; Hristov et al., 2013; Guyader et al., 2016). Based on the RMSPE, our prediction equations tools would be able to discriminate variation in CH_4 emissions higher than 16%, which represents a limit of the ability to highlight the potential of mitigation strategies.

3.5. Use of prediction equations for small ruminants

As observed with animal-to-animal variations in CH_4 emissions, differences can also exist between ruminant species fed the same diets. Indeed, CH_4 emissions are function of the

digestive morphology and/or rumen microbial composition (Mills et al., 2001). Sheep tend to have lower CH_4 emissions when expressed as a percentage of GEI (Ulyatt et al., 2002), potentially due to sheep ability to select more digestible feedstuffs in the diet (Baumont, 1996). However, IPCC equation used to predict CH_4 emissions (% of GEI) includes different correcting factors only for beef cattle, while sheep and dairy cows have the same (*More details in Chapter I, Section 1*). Milk FA-based prediction equations have not been developed to be used for every ruminant species. Indeed, differences in mammary gland lipid metabolism exist between goats, ewes and cows as well as in subsequent milk FA secretions (Bernard et al., 2008, 2013). Fougère et al. (2018) reported also that milk FA profile from dairy cows and goats were different when animals received similar diets. For example, diet rich in starch from wheat induced an increase in milk *trans*-10,*cis*-12 CLA concentration by 18 fold in cows and only 7 fold in goats and in milk *trans*-10 18:1 concentration by 13 fold in cows and 3 fold in goats. Thus, specific prediction equations of CH_4 emissions from milk FA should probably be develop for small ruminants.

Our work highlights that some milk FA have the potential to predict enteric CH_4 emissions, but combining proxies from milk composition, diet chemical composition, or animal characteristic allow increasing the performance of prediction of the models. In addition, the domain of applicability of the prediction equations seem to be linked to the dietary strategies of the dataset used to build them. Thus, prediction of CH_4 is still a challenge that will probably keep researchers looking for answers for years to come. The results of this PhD have certainly contributed to increase the knowledge on the potential of milk FA as predictors of CH_4 emissions in dairy cows. Even though their prediction potential could be enhanced, they deliver important insights on rumen digestion and methanogenesis. Hence, it would be of great benefit gathering observations from recent experiments in order to build a larger dataset. In that sense, international collaborations should be continued. Regarding practical aspects, gas chromatography to determine milk FA composition is a complex technique that is not applicable on large scale. In addition, spectrometry techniques are promising and milk FA-based prediction equations would be a very useful "on farm" tool for CH_4 emissions estimation in dairy ruminants. However, further researches are needed in order to increase accuracy and precision of milk FA analysis determined by MIR spectrometry before to be considered as a valuable proxy.

Appendix

Appendix 1 Standard CH_4 measurement methods

Respiration Chambers. Respiration chambers have been used for more than 120 years as indirect calorimeters for the measurement of respiratory exchange and CH_4 energy losses of ruminants (e.g. Armsby and Armsby, 1903; Kellner, 1913). The principle of this technique is to continuously measure the total quantity of all gases coming from the animal's exhaled breath. The CH_4 output is then estimated from the airflow rate and CH_4 concentration inside the chamber corrected by the CH_4 concentration in the ambient air: CH_4 (g/d) = airflow rate x [CH_4]

Among several type of respiration chambers, there are two main types where the air composition is measured: 1) the closed circuit; 2) the open-circuit respiration chambers, such as the ones used at INRA facilities during this PhD (See illustrating picture; source: INRA). Respiratory chambers allow animals to see each other in order to avoid animal isolation and thus limit negative impact on welfare.

See guideline: Technical manual on respiration chambers design.
http://www.globalresearchalliance.org

A pump is pumping air from the chamber through a flow meter and different gas sensors (CH_4, CO_2, H_2, O_2…). Fresh air for the animal is drawn from outside. In some systems, fresh air is drawn through an air conditioning system to control humidity, temperature and mixing of air in the chamber. The CH_4 emissions are calculated from flow and gas concentration in inlet and outlet air from the chamber, but more complex calculations have been developed that also take into account the small differences in inflow and outflow and changes in chamber concentration of gases (Brown et al., 1984). The difference between the outgoing and incoming amount of CH_4 corresponds to the CH_4 emissions from the animal. Many different chambers have been constructed based on this principle including insulated chambers with controlled temperature and humidity (Derno et al., 2009), more simple types with no insulation of chambers and fresh air inlet from the room (Waghorn and Pinares, 2012).

SF_6 tracer gas. The racer gas technique has been used with tracers such as [3H–] methane or [l4C–] methane to quantify CH_4 emissions (Storm et al., 2012). But the most commonly gas used is the Sulphur hexafluoride (SF_6) and the first study estimating CH_4 emissions from ruminant using the SF_6 tracer gas method was implemented by Johnson et al. (1994).

The SF_6 tracer technique is based on the dilution principle of the tracer gas in the gas produced from microbial fermentation in the rumen. This technique relies on the basis that the excretions of two gases (SF_6 and CH_4) have identical dispersion into the animal's environment, and thus have the same probability of being exhaled by the animal's breath and then collected by a canister device sampler located near the nasal cavity (See illustrative picture; source : INRA).

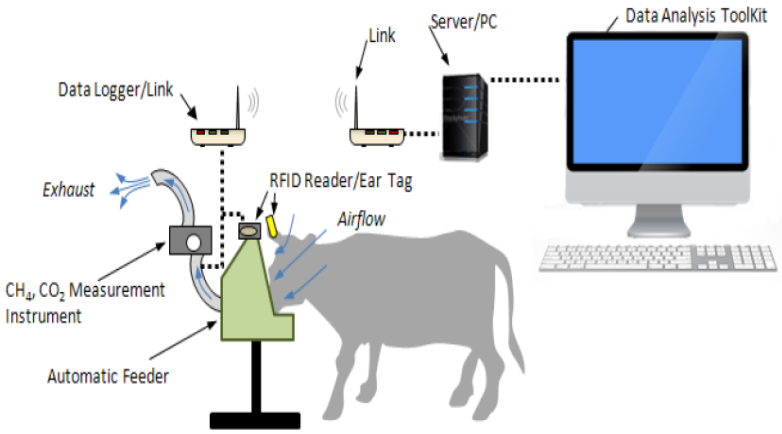
Animal equipped with a gas collection device composed of a capillary tube with in-line flow restrictors (to regulate gas sampling rate) that are placed near the nose and mouth and are connected to a pre-evacuated gas collection canister.

See guideline for use of the SF_6 tracer technique.
http://www.globalresearchalliance.org

A permeation tube (bolus) is loaded with SF_6 and calibrated by regular weighing to know the release rate of the SF_6. Then, the bolus is introduced in the rumen of the animal. Capillary tubing is placed at the nose of the animal and is connected with an evacuated collection canister, which samples the exhaled gas. By varying the length and diameter of the capillary tube the duration of sampling may be regulated. After measurement period (most of the time 24h), the canister is pressurized with nitrogen and the concentration of SF_6 and CH_4 in the sample is collected and then analyzed by gas chromatography (GC-FID and GC-ECD for CH_4 and SF6, respectively). Methane emission rate is calculated as: CH_4 (g/d) = SF_6 bolus x $[CH_4]/[SF_6]$; whereSF_6 bolus is the known SF6 release rate from permeation tubes, $[CH_4]$ $[SF_6]$ are the measured gas concentrations in the canister corrected by the gas concentrations in the ambient air (Johnson and Johnson, 1995).

GreenFeed system. The GreenFeed system (C-Lock Inc., Rapid City, South Dakota, USA) is a more recent equipment developed to measure CH_4 emissions in cattle. It is a static short-term measurement device that measures gases emissions including CH_4, CO_2, H_2 and O_2 consumption from individual cattle by integrating measurements of airflow, gas concentration, and detection of head position during each animal's visit (Huhtanen et al., 2015). Animals can visit the system at any time during which feed rewards are provided in order to encourage the

animals' visits (See illustrative picture; Source: Société C-Lock, inc; *http://c-lock.com)*. The CH_4 emissions are then measured over a short (3–7 min) periods and for several times within a day, over several days/weeks/months; time laps between visits are determined by the investigator.



Animal visiting the GreenFeed system

The CH_4 emissions are determined by using an extractor fan to draw air over the animals head and past the nose and mouth into an exhaust pipe. The collected air is mixed, filtered and airflow rate measured using a hot-film anemometer. The concentration of the different gases (CH_4, CO_2) in the sample is measured using non-dispersive infrared analysis. Daily CH_4 emissions is calculated from the air flow rate and the CH_4 concentration of the gas expired (corrected by CH_4 concentration in the ambient air). This calculation is done at each visit and cumulated for the day (Huhtanen et al., 2015).

Appendix 2 Milk fatty acids nomenclature

Fatty acids present a carbon chain with repeated –CH2- or =CH- and carboxylic acid molecule –COOH at the delta end and a methyl molecule –CH3 at the omega end. The FA carbon bones length varies from 4 to 26 atoms, with more frequently even number of carbons. Short-chain FA have up to 12 carbons, medium-chain FA have from 12 to 16 carbon atoms and long-chain FA have more than 16 carbon atoms.

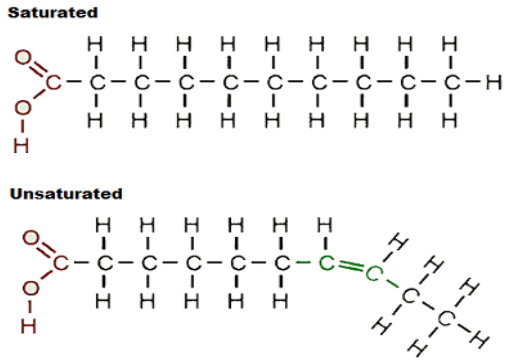


Figure 1 Molecular structure of saturated and unsaturated fatty acids

Saturated FA (**SFA**) do not have double bonds in their carbon chain, and have linear shape (Figure 1), except for branched FA (Figure 2).

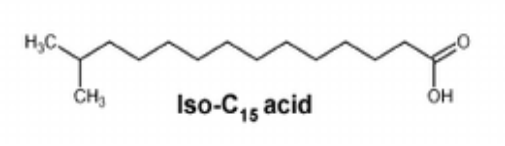


Figure 2 Molecular structure of branched fatty acids

Unsaturated FA (**UFA**) have one double bond (Figure 3; monounsaturated FA, **MUFA**) or 2 and more double bonds (polyunsaturated FA, **PUFA**). Each double bond could be found with two different geometric shapes: *cis*- or *trans*-. The *cis*- shape, also called Z-shape from the german Zusammen (=together), is the most commonly found double bond. In this case, the two H atoms are located on the same side of the double bound (Figure 3) and lead to a 30 degrees curve in the carbon chain.

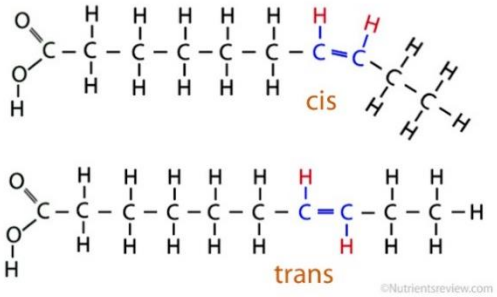


Figure 3 *Cis* and *trans* shapes of unsaturated fatty acids

The *trans*- shape, also called E-shape from the german Entgegen (=opposite), has two H atoms located on each side of the double bound and lead to a linear structure, close to the SFA structure. Most of the time, double bound in PUFA are separated by 3 carbons, but it is also possible to find double bonds separated by 4 carbons or by 2 carbons, also called conjugated FA. Isomerization process is responsible of the double bound addition to the carbon chain in the rumen of ruminants or by technological treatments of oil. Among the UFA, differences are made between omega 3 (n-3, for instance α-linolenic acid: C18:3n-3 or *cis*-9,*cis*-12,*cis*-15 C18:3), omega 6 (n-6, for instance linoleic acid: C18:2n-6 or *cis*-9,*cis*-12 C18:2), omega 9 (n-9, for instance oleic acid: C18:1n-9 or *cis*-9 C18:1), and conjugated linoleic acid (**CLA**, rumenic acid: *cis*-9,*trans*-11 CLA). In the SFA family, differences are made between iso-FA, which have iso-methyl or anteiso methyl (*iso* C15:0 or *anteiso* C15:0), odd-FA (C15:0) and even-FA (C16:0). Thus, there is a great variability among FA in terms of length, number and type of double bonds, shapes, physical proprieties (fusion temperature point), chemical and physiological proprieties.

Appendix 3 Milk fatty acid measurement method

Gas chromatography method. Fatty acid methyl ester (**FAME**) preparation is necessary for gas chromatography (**GC**) analysis, and FAME are used because of a greater volatility and great separation in GC as compared to ethylic, propylic, isopropylic or butyric esters. However, some corrections coefficients have to be applied to short-chain FAME in order to take into account their volatility (Christie, 1993). Two esterification methods are used either with an acid [hydrochloric acid (HCL), or sulfuric acid (H_2SO_4), or bore trifluoride (BF_3)] or with a base [sodium (NaOMe) or potassium (KOMe) methoxide]. The acid-esterification method has the advantage of being used for all lipid types but can lead to isomerization of conjugated FA *cis,trans* to *trans,trans* leading to measurement errors. However, this phenomenon only occurs when temperature is greater than 60°C and a reaction time lesser than 30 min (Yeonhwa Park et al., 2001). The other esterification method using a base does not lead to isomerization of conjugated FA but does not allow esterify free FA or *trans* esterified-sphingolipids (Christie, 1993).

HPLC method. HPLC technique relies on pumps to pass a pressurized liquid solvent containing the milk sample through a column filled with a solid adsorbent material (Figure 1; HPLC Column), such as silica gel impregnated with silver nitrate. Each component in the sample interacts slightly differently with the adsorbent material, causing different flow rates for the different components and leading to the separation of the components as they flow out of the column.

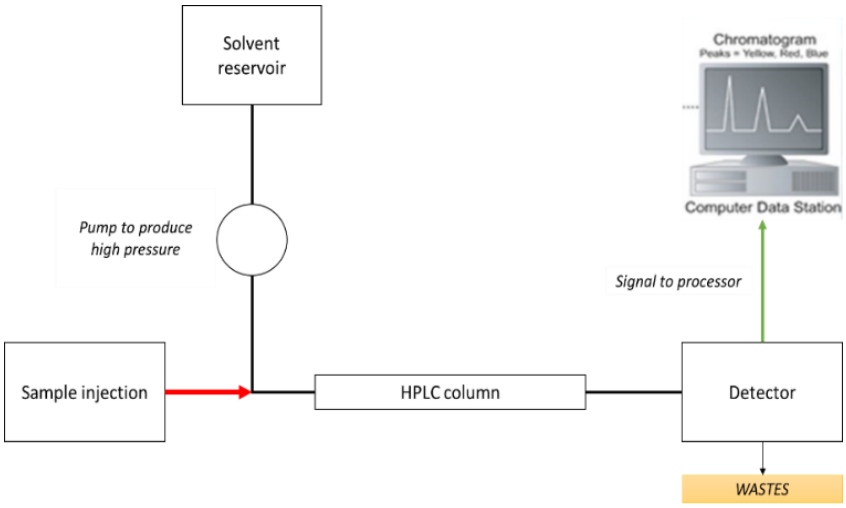


Figure 1 General scheme for HPLC process

Appendix 4 PCA analysis for CH_4 yield

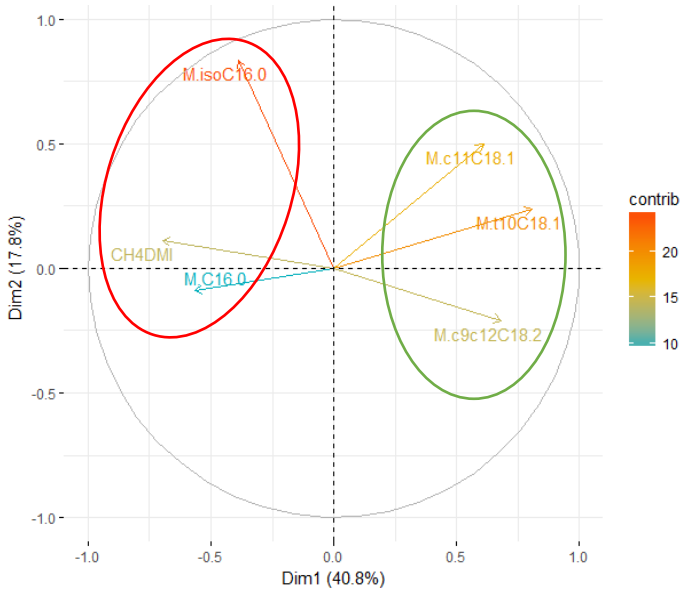


Figure 1 PCA score plot for the contribution of the selected milk FA (% of total FA) and CH_4 yield (g/kg of DMI) with the first (PCA1) and second (PCA2) dimension (right side). Scree plot for the contribution of the different dimensions of the PCA (left side)

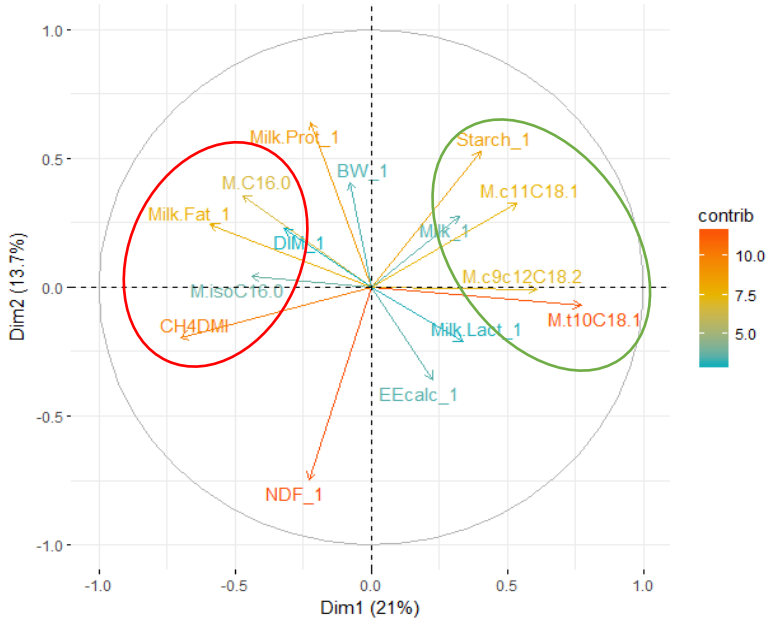


Figure 2 PCA score plot for the contribution of the selected milk FA (% of total FA), diet composition (NDF, EE, Starch; % of DM), performance [milk yield (kg/d), milk fat (%), milk protein (%), milk lactose (%)], animal characteristics (DIM, BW) and CH_4 yield (g/kg of DMI) with the first (PCA1) and second (PCA2) dimension (right side)

Appendix 5 PCA analysis for CH_4 intensity

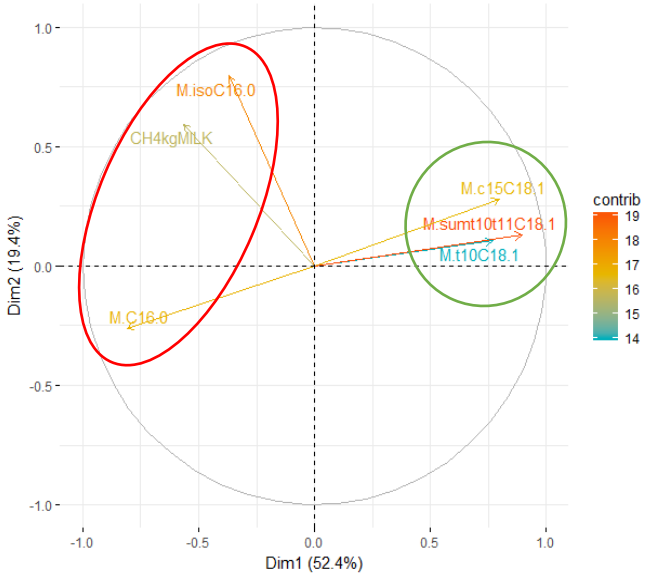


Figure 1 PCA score plot for the contribution of the selected milk FA (% of total FA) and CH_4 intensity (g/kg of milk) with the first (PCA1) and second (PCA2) dimension (right side). Scree plot for the contribution of the different dimensions of the PCA (left side)

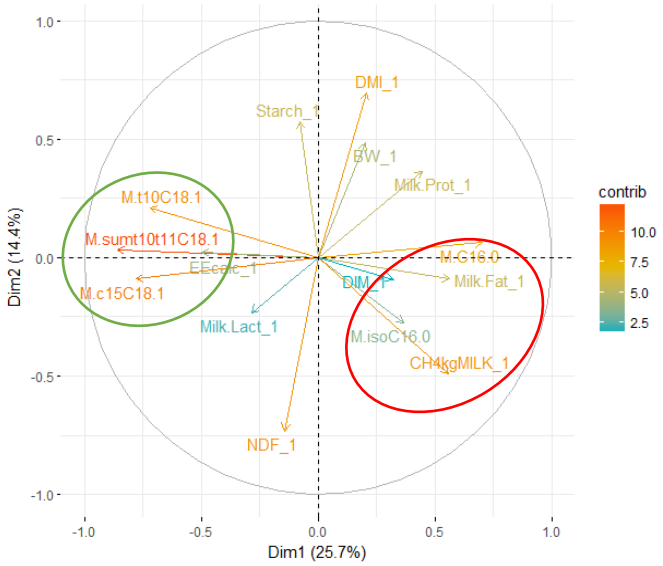


Figure 2 PCA score plot for the contribution of the selected milk FA (% of total FA), diet composition (NDF, EE, Starch; % of DM), performance [milk yield (kg/d), milk fat (%), milk protein (%), milk lactose (%)], animal characteristics (DIM, BW) and CH_4 yield (g/kg of DMI) with the first (PCA1) and second (PCA2) dimension (right side). Scree plot for the contribution of the different dimensions of the PCA (left side)

Appendix 6 Pearson correlation matrix for all variables included in the modeling approach

	CH4 (g/d)	CH4 (g/kg DMI)	CH4 (g/kg mi Milk	Fat %	Protein %	Lactose %	NDF	EE	Starch	BW (kg)	DIM (d)	C16:0	C10:0	iso C17:0*	cis-11 C18:1	cis-15 C18:1	tr-10 C18:1	tr-10+11 C18:1	tr-11,cis-15 C	c9c12C18:2
CH4 (g/kg DMI)	0.726																			
	0																			
CH4 (g/kg milk)	0.496	0.741																		
	0	*0*																		
Milk	0.272	-0.226	-0.616																	
	0	*0*	*0*																	
Fat	0.241	0.264	0.252	-0.164																
	0	*0*	*0*	*0*																
Prot	0.17	0.019	0.146	-0.086	0.399															
	0	*0.587*	*0*	*0.014*	*0*															
Lact	-0.299	-0.207	-0.183	-0.087	-0.046	-0.167														
	0	*0*	*0*	*0.023*	*0.226*	*0*														
NDF	0.017	0.324	0.309	-0.372	-0.032	-0.289	0.083													
	0.627	*0*	*0*	*0*	*0.352*	*0*	*0.031*													
EEcalc	-0.072	-0.11	-0.173	0.09	-0.131	-0.071	-0.201	0.179												
	0.038	*0.002*	*0*	*0.01*	*0*	*0.042*	*0*	*0*												
Starch	-0.085	-0.248	-0.207	0.154	-0.107	0.169	0.113	-0.354	-0.057											
	0.015	*0*	*0*	*0*	*0.002*	*0*	*0.003*	*0*	*0.103*											
BW (kg)	0.331	0.025	0.041	0.257	-0.041	0.132	-0.341	-0.117	0.064	0.079										
	0	*0.495*	*0.259*	*0*	*0.26*	*0*	*0*	*0.001*	*0.078*	*0.028*										
DIM (d)	0.129	0.191	0.436	-0.355	0.152	0.39	-0.146	0.003	-0.046	-0.075	0.215									
	0	*0*	*0*	*0*	*0*	*0*	*0*	*0.936*	*0.184*	*0.031*	*0*									
C16:0	0.27	0.236	0.262	-0.068	0.261	0.16	-0.095	-0.13	-0.38	0.079	-0.006	-0.096								
	0	*0*	*0*	*0.05*	*0*	*0*	*0.013*	*0*	*0*	*0.023*	*0.86*	*0.006*								
C10:0	0.332	0.205	0.182	0.086	0.372	0.295	-0.014	-0.252	-0.338	0.118	-0.11	-0.026	**0.533**							
	0	*0*	*0*	*0.014*	*0*	*0*	*0.707*	*0*	*0*	*0.001*	*0.002*	*0.46*	*0*							
iso C17:0*	-0.316	-0.117	-0.031	-0.261	-0.287	-0.369	0.153	0.367	0.104	-0.079	0.097	0.001	-0.315	-0.414						
	0	*0.001*	*0.37*	*0*	*0*	*0*	*0*	*0*	*0.003*	*0.024*	*0.007*	*0.983*	*0*	*0*						
cis-11 C18:1	-0.338	-0.33	-0.188	-0.021	-0.272	0.156	0.142	-0.225	-0.205	0.297	0.015	0.109	-0.148	-0.167	-0.018					
	0	*0*	*0*	*0.579*	*0*	*0*	*0.001*	*0*	*0*	*0*	*0.715*	*0.004*	*0*	*0*	*0.639*					
cis-15 C18:1	-0.432	-0.36	-0.237	-0.1	-0.301	-0.172	0.17	0.16	**0.515**	-0.045	-0.103	-0.006	**-0.618**	**-0.521**	0.323	-0.012				
	0	*0*	*0*	*0.045*	*0*	*0.001*	*0.001*	*0.001*	*0*	*0.37*	*0.043*	*0.903*	*0*	*0*	*0*	*0.808*				
tr-10 C18:1	-0.449	-0.424	-0.341	0.003	-0.413	0.052	0.169	-0.064	0.198	0.214	-0.032	-0.057	-0.407	-0.428	0.258	0.446	0.417			
	0	*0*	*0*	*0.944*	*0*	*0.177*	*0*	*0.095*	*0*	*0*	*0.426*	*0.135*	*0*	*0*	*0*	*0*	*0*			
tr-10+11 C18:1	-0.423	-0.348	-0.342	0.006	-0.362	-0.119	0.135	0.087	0.404	-0.015	-0.005	0.003	-0.714	**-0.565**	0.484	0.214	**0.545**	**0.667**		
	0	*0*	*0*	*0.883*	*0*	*0.002*	*0.001*	*0.022*	*0*	*0.699*	*0.895*	*0.937*	*0*	*0*	*0*	*0*	*0*	*0*		
tr-11,cis-15 C18:2	-0.289	-0.245	-0.23	-0.005	-0.192	-0.156	-0.008	0.11	**0.518**	-0.06	0.01	-0.012	**-0.574**	-0.439	0.313	-0.101	**0.799**	0.199	**0.621**	
	0	*0*	*0*	*0.917*	*0*	*0.001*	*0.878*	*0.017*	*0*	*0.191*	*0.842*	*0.789*	*0*	*0*	*0*	*0.029*	*0*	*0*	*0*	
c9c12C18:2	-0.238	-0.299	-0.193	0.026	-0.247	0	0.186	-0.054	0.092	0.186	-0.059	-0.068	-0.238	-0.174	0.207	0.267	-0.022	0.441	0.336	-0.087
	0	*0*	*0*	*0.452*	*0*	*0.992*	*0*	*0.127*	*0.009*	*0*	*0.104*	*0.052*	*0*	*0*	*0*	*0*	*0.659*	*0*	*0*	*0.058*
DMI	0.572	-0.115	-0.17	**0.688**	0.013	0.217	-0.216	-0.364	0.014	0.15	0.476	-0.044	0.121	0.222	-0.313	-0.032	-0.219	-0.13	-0.197	-0.15	0.041
	0	*0.001*	*0*	*0*	*0.716*	*0*	*0*	*0*	*0.697*	*0*	*0*	*0.202*	*0.001*	*0*	*0*	*0.401*	*0*	*0.001*	*0*	*0.001*	*0.243*

Appendix 7 Calculation details of metrics used to assess prediction equations' performance

Mean Square Prediction Error (MSPE) was calculated according to Bibby and Toutenburg (1977) as:

$$MSPE = \frac{1}{n}\sum_{i=1}^{n}(O_i - P_i)^2$$

where n is the number of observations, $\boldsymbol{O_i}$ is the i[th] observed value and $\boldsymbol{P_i}$ is the i[th] predicted value. The square root of the MSPE (RMSPE) is a most convenient criteria used to evaluate model prediction because it has the same unit as observed values:

$$RMSPE = \sqrt{\frac{1}{n}\sum_{i=1}^{n}(O_i - P_i)^2}$$

Smaller RMSPE indicates better performance of models. The MSPE can be decomposed into 3 parts: error due to central tendency (overall bias) (ECT), error due to deviation of the regression slope (ER) and error due to the disturbance (random error) (ED) (Bibby and Toutenburg, 1977). The ECT, ER and ED are respectively calculated as:

$$ECT = (\bar{P} - \bar{O})^2$$

$$ER = (S_p - R \times S_o)^2$$

$$ED = (1 - R^2) \times S_o^2$$

where $\bar{P}$ and $\bar{O}$ are the predicted and observed mean values, respectively, S_p is the standard deviation of predicted values, S_o is the standard deviation of observed values, and R is the Pearson correlation coefficient.

RMSPE-Observations Standard Deviation Ratio. When using different data to compare the equations' performance, we can use the ratio of RMSPE and S_o, namely RMSPE-observations standard deviation ratio (RSR) to take into account the data variability (Moriasi et al., 2007).

$$RSR = \frac{RMSPE}{S_o}$$

Smaller RSR (<1) indicates better performance given the variability of observations, otherwise the model gives higher variability of the predicted values than the observed ones.

Concordance Correlation Coefficient (CCC) (Lin, 1989) was calculated as a product of Pearson correlation coefficient (R, measure of precision) and the bias correction factor (C_b, measure of accuracy):

$$CCC = R \times C_b$$

where C_b indicates how far the best fit line deviates from the concordance or unity line of the observed values *versus* predicted values plot. The C_b ranges from 0 to 1 with greater values indicating less deviation from the concordance line. C_b is calculated as:

$$C_b = \frac{2}{v + \frac{1}{v} + u^2}$$

where v provides a measure of scale shift:

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

u provides a measure of location shift:

$$u = \frac{\bar{O} - \bar{P}}{\sqrt{S_o \times S_p}}$$

The v-value indicates the change in standard deviation between predicted and observed values. A positive u-value indicates under prediction, whereas a negative u indicates over prediction. CCC varies from optimum of 1 to lower positive values. The greater CCC the better the model performance.

Appendix 8 Observed versus predicted CH_4 production (g/d) and yield (g/kg of DMI) by the different published equations based on milk FA only and challenged on the two validation datasets

- Mohammed et al., 2011

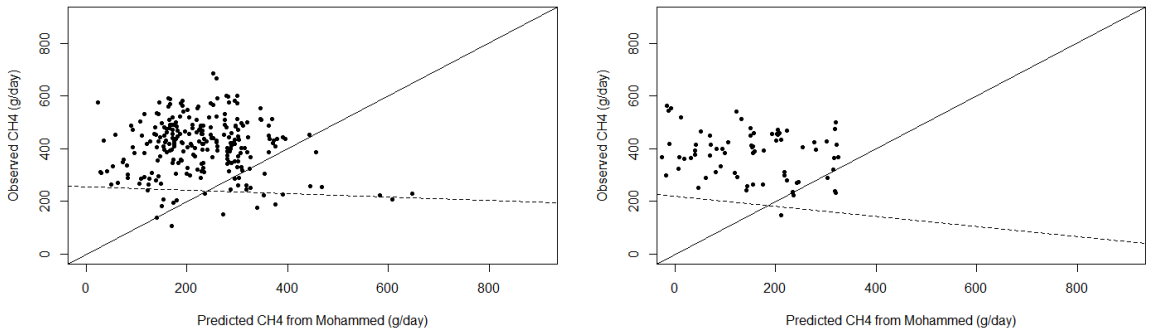


Figure 1 Observed and predicted CH_4 production (g/d) using published equation from Mohammed et al. (2011) on the internal (left) and external (right) validation datasets. The black and red solid lines represent the fitted regression line for the relationship between predicted and observed values and the identity line (y = x), respectively

- Dijkstra et al., 2011

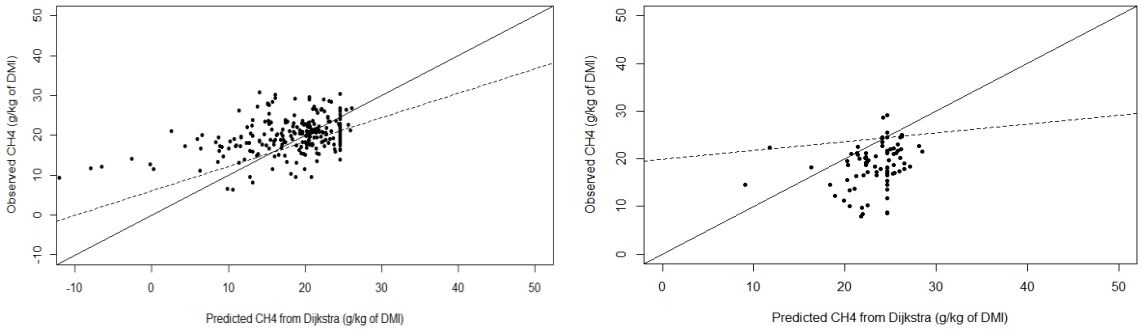


Figure 2 Observed and predicted CH_4 yield (g/kg of DMI) using published equation from Dijkstra et al. (2011) on the internal (left) and external (right) validation datasets. The black and red solid lines represent the fitted regression line for the relationship between predicted and observed values and the identity line (y = x), respectively

- Williams et al., 2014

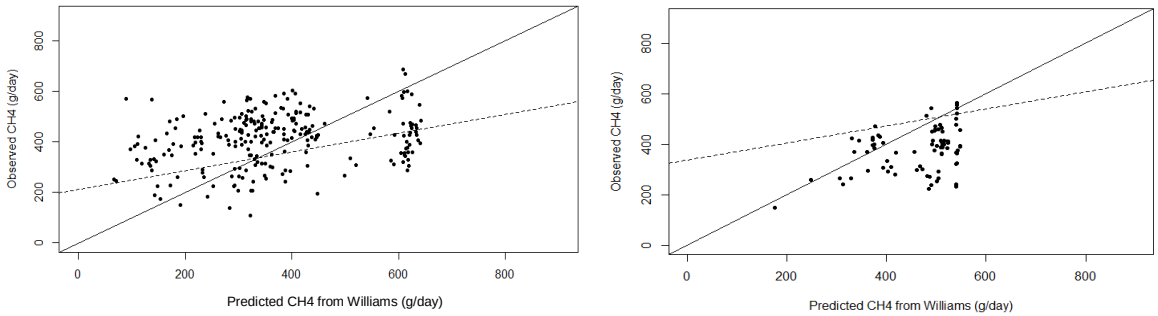


Figure 3 Observed and predicted CH_4 production (g/d) using published equation from Williams et al. (2014) on the internal (left) and external (right) validation datasets. The black and red solid lines represent the fitted regression line for the relationship between predicted and observed values and the identity line (y = x), respectively

- van Lingen et al., 2014

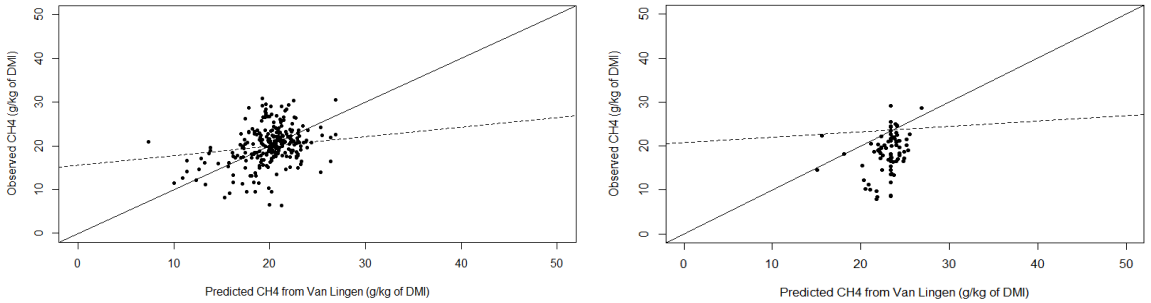


Figure 4 Observed and predicted CH_4 yield (g/kg of DMI) using published equation from van Lingen et al. (2014) on the internal (left) and external (right) validation datasets. The black and red solid lines represent the fitted regression line for the relationship between predicted and observed values and the identity line (y = x), respectively

- van Gastelen et al., 2017

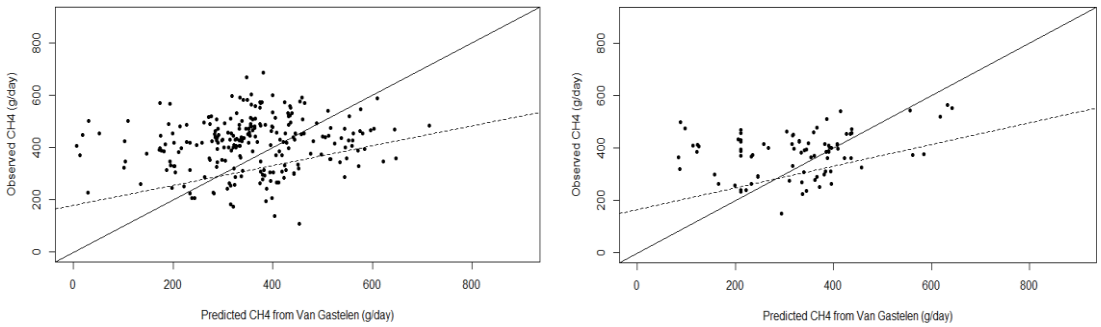


Figure 5 Observed and predicted CH_4 production (g/d) using published equation from van Gastelen et al. (2017) on the internal (left) and external (right) validation datasets. The black and red solid lines represent the fitted regression line for the relationship between predicted and observed values and the identity line (y = x), respectively

- van Gastelen et al., 2018

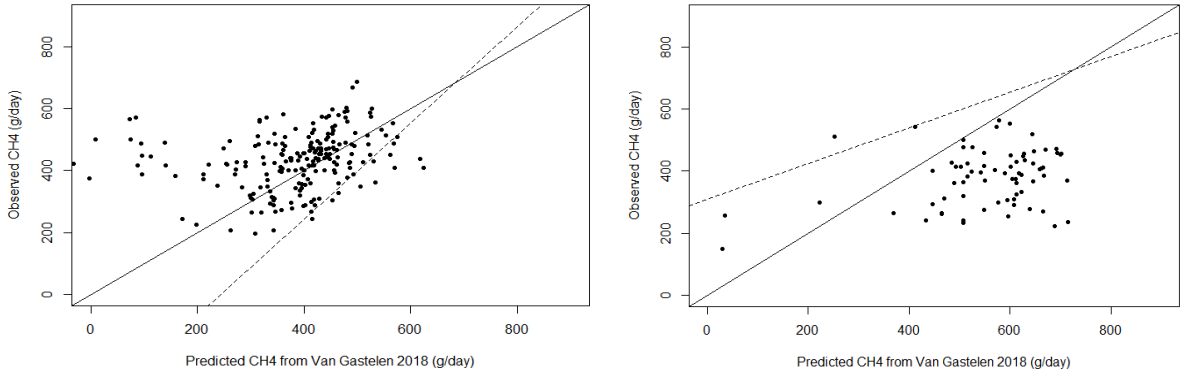


Figure 6 Observed and predicted CH_4 production (g/d) using published equation from van Gastelen et al. (2018) on the internal (left) and external (right) validation datasets. The black and red solid lines represent the fitted regression line for the relationship between predicted and observed values and the identity line (y = x), respectively

Appendix 9 Observed versus predicted CH_4 production (g/d), yield (g/kg of DMI) and intensity (g/kg of milk) by the prediction equations based on milk FA developed in this work.

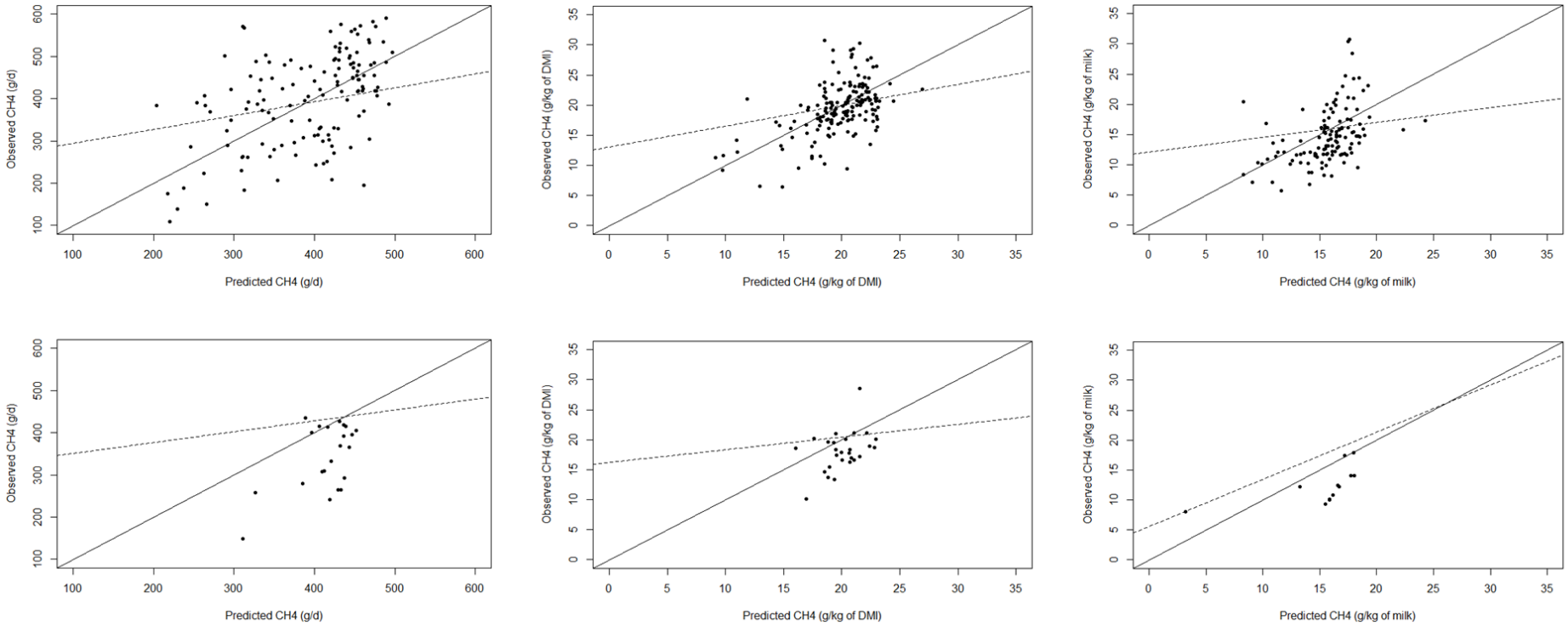


Figure 1 Predicted versus observed plots (upper plots: from Validation dataset; lower plots: from Literature dataset) for the CH_4 emission equations in g/d (equation 1), g/kg of DMI (equation 12), g/kg of milk (equation 18) based on milk fatty acids. The black dashed and black solid lines represent the fitted regression line for the relationship between predicted and observed values and the identity line (y = x), respectively

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