



Manipulation of the hydrogen pool available in the rumen to reduce methane emissions from ruminants

Jessie Guyader

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Jessie GUYADER

Manipulation du pool d'hydrogène disponible dans le rumen
pour limiter les émissions de méthane par les ruminants

Manipulation of the hydrogen pool available in the rumen to
reduce methane emissions from ruminants

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Résumé

La réduction des émissions de méthane (CH_4) des ruminants permet de limiter les impacts environnementaux négatifs de leur élevage et d'améliorer leur efficacité digestive. Dans le rumen, le CH_4 est majoritairement produit par les méthanogènes à partir de l'hydrogène (H_2). La disponibilité de l' H_2 pour ces micro-organismes est réduite en limitant sa production par les protozoaires (*via* un apport de lipides ou extraits de plantes dans la ration) ou en stimulant des voies utilisatrices d' H_2 compétitives à la méthanogenèse (via un apport alimentaire de nitrate). Aucune étude n'a porté sur l'association de stratégies alimentaires jouant à la fois sur la production et l'utilisation d' H_2 pour diminuer les émissions de CH_4 . Notre objectif était de comprendre l'importance des différentes voies métaboliques de l' H_2 dans le rumen. Nous avons émis l'hypothèse que manipuler simultanément la production et l'utilisation de l' H_2 permet une diminution plus importante des émissions de CH_4 plutôt que d'agir sur un seul niveau. Nos résultats expérimentaux ont montré l'additivité de l'association **lipides du lin-nitrate** sur la méthanogenèse des bovins. Cet effet était persistant mais non bénéfique pour les performances digestives et laitières des animaux. L'association **saponine de thé-nitrate** n'a pas été efficace pour réduire les émissions de CH_4 car l'effet dépressif de la saponine sur les protozoaires n'a pas été observé. Cette thèse ouvre la possibilité d'étudier le potentiel anti-méthanogène de nouvelles associations de stratégies alimentaires ayant des mécanismes d'action différents dans le rumen. Les conditions d'utilisation de ces stratégies en élevage devront être délimitées, et leur rentabilité prouvée, pour être acceptées par l'éleveur.

Mots clés: Fermentation; Hydrogène; Méthane; Microbiote; Ruminants; Stratégies de réduction.

Abstract

Reduction of methane (CH₄) emissions from ruminants may limit the negative environmental impacts of their breeding and may improve their digestive efficiency. In the rumen, CH₄ is mainly produced by methanogens from hydrogen (H₂). Hydrogen availability for these micro-organisms is reduced by limiting its production by protozoa (*via* lipids or plants extracts supplementation in diets) or by stimulating pathways competing with methanogenesis for H₂ consumption (*via* nitrate supplementation in diets). No study tested association of dietary strategies acting on both H₂ production and consumption to reduce CH₄ emissions. Our objective was to understand the importance of the different H₂ metabolic pathways in the rumen. We assumed that simultaneous manipulation of H₂ production and consumption reduces CH₄ emissions to a higher extent than acting on a single pathway. Our experimental results showed the additive CH₄-mitigating effect of the association **lipids from linseed-nitrate** supplemented to bovine. This effect was persistent but not beneficial for digestive and lactating performances of animals. The association **tea saponin-nitrate** was not efficient to reduce CH₄ emissions, as the depressive effect of saponin towards protozoa has not been observed. This PhD thesis opens the possibility to study the anti-methanogenic potential of new association of dietary strategies having different mechanisms of action in the rumen. Conditions of use of these strategies at the breeding scale will have to be delineated, and their cost effectiveness proved to be accepted by farmers.

Keywords: Fermentation; Hydrogen; Methane; Microbiota; Mitigation strategy; Ruminants.

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List of common abbreviations

AA: Amino acid
ADF: Acid detergent fiber
BCM: Bromochloromethane
BES: 2-bromo-ethane sulfonate
BW: Body weight
CP: Crude protein
CV: Coefficient of variation
DM: Dry matter
DMI: Dry matter intake
DNRA: Dissimilatory reduction of nitrate to ammonium
DOM: Digestible organic matter
E₀: Standard reduction potential
Fed: Ferredoxin
ΔG: Gibbs free energy
GE: Gross energy
GEI: Gross energy intake
GHG: Greenhouse gas
GWP: Global warming potential
Hb: Hemoglobin
LCA: Life cycle assessment
MetHb: Methemoglobin
NDF: Neutral detergent fiber
NPN: Non-protein nitrogen
OM: Organic matter
PUFA: Polyunsaturated fatty acid
RMSE: Residual mean square error
SD: Standard deviation
VFA: Volatile fatty acid

General introduction

I. CONSTRAINTS OF RUMINANTS BREEDING IN THE (FUTURE) AGRICULTURAL CHALLENGE: PRODUCE MORE AND BETTER WITH FEWER RESOURCES

Nowadays, the world population is significantly increasing, and is expected to pass from 7 billion (2014) to more than 9 billion in 2050 (Steinfeld et al., 2006). In addition, the individual level of consumption of animal products increased for the last 40 years in developing countries: between 1962 and 2003, meat and milk consumption passed respectively from 10 to 29 kg/person/year, and from 28 to 48 kg/person/year. Consequently, to fulfil the increasing demand of livestock products, a rise of meat and milk production is expected in the future (Figure 1), and development of sustainable systems of animal production that do not directly compete with mankind for foodstuffs is clearly necessary. **In this global context, ruminants play a major role in the human food supply chain by converting non-consumable fibrous feedstuff for humans to highly nutritional products. However, ruminants are criticized for their high contribution to greenhouse gas (GHG) emissions, and their impact on climate change is a major concern worldwide (Steinfeld et al., 2006).**

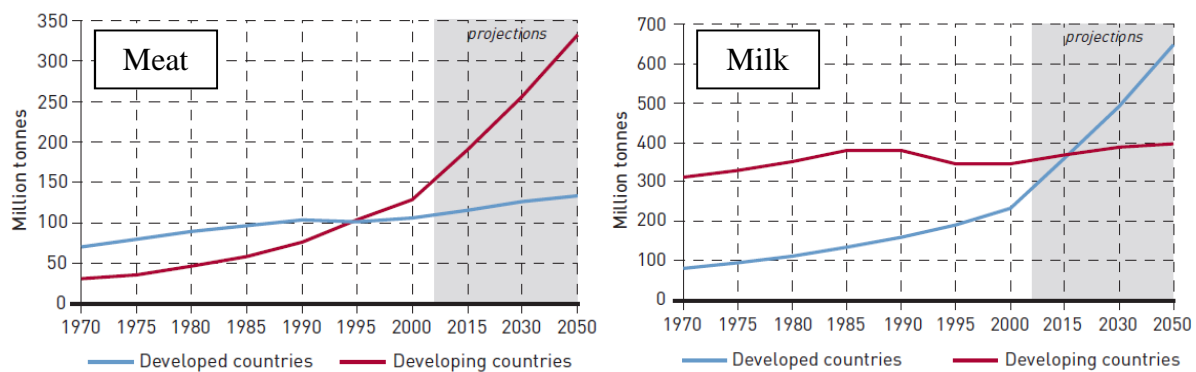


Figure 1 Past and projected meat and milk production in developed and developing countries from 1970 to 2050 (from Steinfeld et al., 2006)

1.1. Interest of ruminants production: valorization of forage to highly nutritional products for human consumption

Ruminants own a specific compartment at the beginning of their digestive tract, the rumen, in which feeds are fermented by microbes. This digestive particularity offers them the ability of producing human food using fibrous feedstuff that cannot be directly used by

humans and mono-gastric animals. On the contrary, pigs and chicken diets based on cereals are competitive with human food. However, among the future human protein sources, their feed conversion ratio (25 kg feed/kilogram edible weight) is the highest compared to pork (9.1), poultry (4.5) and crickets (2.1) (van Huis, 2013). Nowadays, ruminants are almost the sole source of milk for humans, by providing 644 million tons of milk (fat-protein corrected milk), among which dairy cattle is the main producer (Figure 2). Ruminants also provide 77.3 million tons of meat (carcass weight) representing 29% of the overall world meat production (Figure 2) (Gerber et al., 2013b).

Beside this major economic role, ruminants managed in extensive system also have a major role in terms of ecosystem services such as landscape management (Harrison et al., 2010). Among others, they help to maintain herbaceous areas difficult to access such as mountainous areas and prevent the development of weed and shrub species responsible for fire development and losses in plant biodiversity.

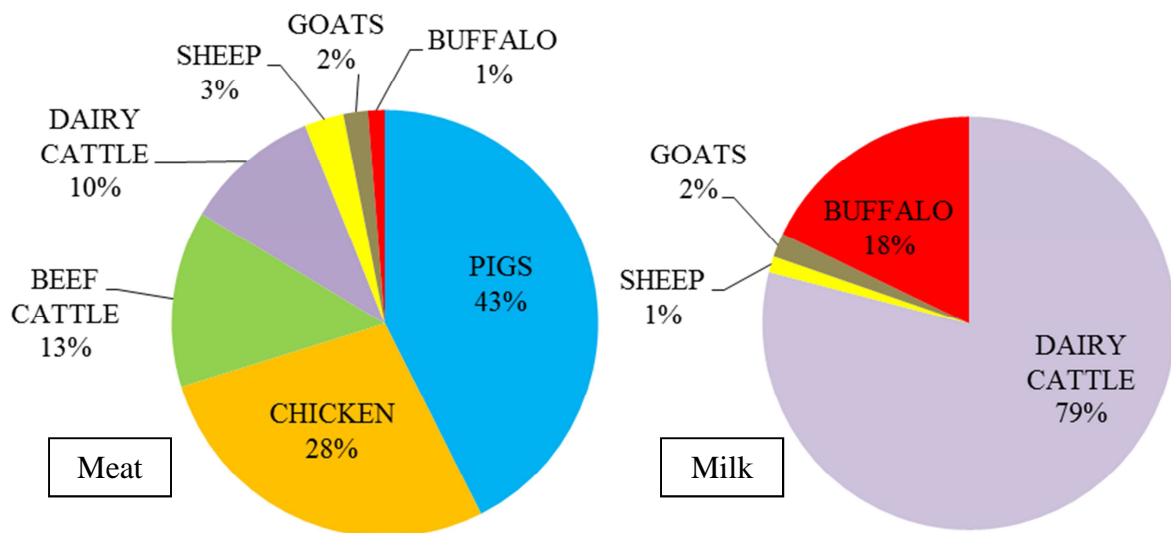


Figure 2 Contribution of ruminants to the overall world meat and milk production (from Gerber et al., 2013b)

1.2. Downside of ruminants breeding: contribution to greenhouse gases emissions via enteric methane production

Ruminants' production is accused of having a significant impact on the environment at the local and global level. Locally, the main issues concern intensive operations that contaminate the air, land or water with nitrogenous compounds and phosphorous releases. Globally, ruminants are pointed out for their contribution to GHG emissions, which occurs in

both intensive and extensive systems (Steinfeld et al., 2006). Carbon dioxide (CO₂), methane (CH₄) and nitrous oxide (N₂O) are the main GHG from anthropic origin (77, 14 and 8% of total GHG produced, respectively), with a global warming potential (GWP) of 1, 25 and 298 (IPCC, 2007). According to latest estimations (Gerber et al., 2013b), contribution of livestock supply chain to total anthropogenic GHG emissions raises at 14.5%, with CH₄, N₂O and CO₂ emissions representing 44, 29 and 27%, respectively (expressed as CO₂-equivalent). Ruminants are mostly involved in CH₄ emissions, which represent 80% of CH₄ emissions from the livestock supply chain, the remaining 20% coming from manure management (Gill et al., 2010). In ruminants, 87% of CH₄ is produced in the rumen and eructated in the atmosphere, the remaining coming from the rest of the digestive tract (Murray et al., 1976). In France, cattle contributes more than 90% to total enteric CH₄ emissions (Figure 3; Vermorel et al., 2008).

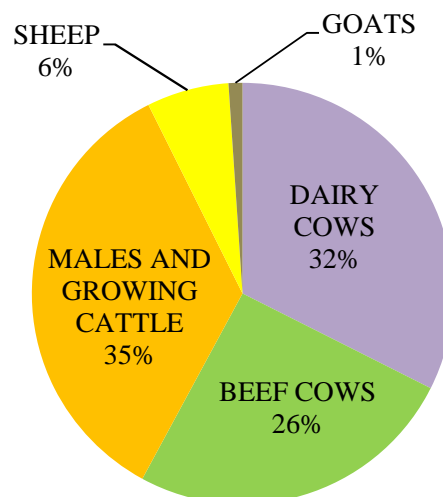


Figure 3 Contribution of cattle, sheep and goat to total methane emissions from ruminants in France (from Vermorel et al., 2008)

In addition to be the main GHG emitted at the farm level, CH₄ released by ruminants constitutes an energetic loss for the animal, ranging from 2 to 12% of gross energy intake (GEI) by the animal (Johnson and Johnson, 1995) (*versus* 0.4% of digestible energy intake for pigs for instance; Noblet and van Milgen, 2004). Consequently, reduction of enteric CH₄ emissions from ruminants is desirable as a strategy to reduce global GHG emissions, without altering their productivity and their feed conversion efficiency.

Several strategies have been tested worldwide to limit methanogenesis (Grainger and Beauchemin, 2011; Gerber et al., 2013a; Knapp et al., 2014). Most of them consist in manipulating rumen parameters *via* feeding (modification of diet composition,

supplementation of dietary additives) or biotechnologies (defaunation, use of probiotics, exogenous microbial products or vaccines). Genetic selection of low CH₄-emitting animals is a more recent strategy. However, none of these strategies reduce CH₄ emissions on the long-term without losses in animals' performances, while being cheap and safe for the animal and the consumers. In this PhD thesis, we chose to work on dietary strategies as they allow getting results in a shorter term than other strategies.

II. HOW TO REDUCE METHANE EMISSIONS FROM RUMINANTS VIA DIETARY STRATEGIES? OBJECTIVES AND SCIENTIFIC APPROACH OF THIS PHD THESIS

In the rumen, microbes find their energy in the form of ATP through dehydrogenation reactions releasing hydrogen (H₂). As soon as produced, H₂ is used by methanogenic archaea, a microbial group distinct from Eubacteria, to reduce CO₂ into CH₄ according to the following equation: $\text{CO}_2 + 4\text{H}_2 \rightarrow \text{CH}_4 + 2 \text{H}_2\text{O}$. Methanogenesis is essential for an optimal performance of the rumen by avoiding H₂ accumulation which would inhibit fermentations. Consequently, H₂ and methanogenic archaea are the two determining parameters of CH₄ production in the rumen.

Recent results suggest that a reduction of methanogenic archaea activity can be achieved by a reduction of H₂ availability for these microorganisms (Popova, 2011). To reduce H₂ availability in the rumen, we classified the different dietary CH₄-mitigating strategies proposed by Gerber et al. (2013a) in two groups:

1/ Strategies reducing H₂ production (Table 1). This can be reached by limiting the number of protozoa in the rumen. Indeed, they are important H₂ producers and they would be involved in 10 to 35% of CH₄ production according to the diets (Morgavi et al., 2010). Addition of lipids or plants extracts (tannins, saponins, essential oils) in diets may reduce the number of protozoa in the rumen.

2/ Strategies stimulating H₂ consumption by other pathways (Table 2). Biochemical pathways using H₂ and/or chemicals directly inhibiting methanogenic archaea would allow reducing the proportion of H₂ directed towards methanogenesis. In this objective, diets including H₂-sinks (nitrate, sulfate), propionate enhancers (organic acids, high concentrate diets) or methanogens' inhibitors (chloroform) have been tested.

Today, a lot of these dietary strategies have been tested individually to reduce methanogenesis, but to our knowledge no studies reported the effects of the association of a strategy acting on H₂ production with a strategy acting on H₂ utilization.

The objective of this PhD thesis was to better understand the importance of the different metabolic pathways of H₂ (production AND utilization) in the rumen, in order to propose and evaluate new dietary strategies to mitigate CH₄ emissions. We assumed that manipulating at the same time production and utilization of H₂ allows a more important reduction of CH₄ emissions than acting on a single pathway (production OR utilization). To deal with this hypothesis, the scientific program of this PhD thesis was based on different approaches:

1/ Bibliographical approach. A literature review detailed the biological processes of H₂ production and consumption in the rumen. In addition, a quantitative analysis of the literature (meta-analysis) aimed at studying the influence of a variation of rumen protozoa concentration on CH₄ emissions.

2/ Experimental approach. We tested *in vivo* the CH₄-mitigating effect of different dietary strategies fed alone or in association to non-lactating and dairy cows. The originality of our approach consisted in combining strategies having different mechanisms of action on the rumen H₂ pool. Measurements of CH₄ emissions were linked with measurements of digestive efficiencies and animals' performances. When possible, rumen fermentations (fermentative and microbial parameters) were also analyzed in order to explain the mechanisms of action of tested strategies. In terms of rumen microbiota analysis, we mainly focused on populations producing (protozoa) and using (methanogens) H₂. To complete this *in vivo* approach, we estimated *in vitro* and in presence of different H₂-sinks, the distribution of H₂ in the fermentation end-products.

A final critical analysis of the overall results was made in the last section of this manuscript.

Table 1 Overview of dietary enteric methane-mitigating strategies tested in ruminants to decrease hydrogen production (adapted from Gerber et al., 2013a)

Active compound	CH ₄ -mitigating effect	Long term effect established	Risk for environment and animal	Effect on digestibility and animals' performances	Reference (<u>Review or meta-analysis</u> ; Experimental studies)
Lipids	Significant effect of medium-chain (lauric, myristic acid) and polyunsaturated (linoleic and especially linolenic acid) fatty acids	Yes	No	Reduction of performances with doses higher than 4% added fat	<u>Rasmussen and Harrison, 2011</u> ; Beauchemin et al., 2009; Machmüller et al., 2000; Martin et al., 2011; Martin et al., 2008
Tannins	Variable effect according to tested source and dose	No	No	Frequent reduction of digestive efficiencies	<u>Goel and Makkar, 2012</u> ; Animut et al., 2008; Grainger, 2009; Pongchompu et al., 2009
Saponins	Variable effect according to tested source and dose	No	No	Variable effect according to tested source and dose	Holtshausen et al., 2009; Zhou et al., 2012
Essential oils	Variable effect according to tested source and dose	No	No	Variable effect according to tested source and dose	<u>Benchaar and Greathead, 2011</u> ; <u>Calsamiglia et al., 2007</u> ; Klevenhusen et al., 2011; Shinkai et al., 2012

Table 2 Overview of dietary enteric methane-mitigating strategies tested in ruminants to modify hydrogen consumption (adapted from Gerber et al., 2013a)

Mechanism of action in the rumen	Active compound	CH ₄ -mitigating effect	Long term effect established	Risks for environment and animal	Effect on digestibility and animals' performances	Reference (<u>Review or meta-analysis</u> ; Experimental studies)
Hydrogen-sinks	Nitrate	Significant and linear dose response effect	Yes	Risks of blood metHb; Nitrogen release poorly studied	No	<u>Lee and Beauchemin, 2014b</u> ; El-Zaiat et al., 2014; Nolan et al., 2010; Van Zijderveld et al., 2011; Veneman et al., 2014
	Sulfate	Significant effect	No	Risks of polioencephalomalacia	Not studied	Van Zijderveld et al., 2010
	Nitroethane	Significant effect	No	Not studied	Not studied	Anderson et al., 2006; Brown et al., 2011
Propionate enhancers	Malic acid, fumaric acid	Variable effect	No	No	No	Bayaru et al., 2001; Foley et al., 2009; Wood et al., 2009
	Ionophores (monensin)	Variable effect. May also have a toxic effect towards protozoa	No	Not studied	No	<u>Appuhamy et al., 2013</u> ; Guan, 2006; McGinn et al., 2004
Methanogens inhibitors	Chloroform, BCM, BES, Cyclodextrin	Significant effect	No	Not studied	No	Abecia et al., 2012; Knight et al., 2011; Mohammed et al., 2004
	Fungal metabolites	Variable effect	No	Not studied	Not studied	Morgavi et al., 2013; Ramírez-Restrepo et al., 2014

BCM: bromochloromethane; BES: 2-bromo-ethane sulfonate; metHb: methemoglobin

Literature review

CHAPTER 1: Ruminal hydrogen production: importance of eukaryotes

In the rumen, dihydrogen (further named hydrogen or H_2) is produced by bacteria, protozoa and fungi during feed fermentation. This process is essential as it allows products reduced during feed fermentation (coenzymes and pyruvate) to be oxidized and used in further fermentative reactions.

Two oxidation-reduction¹ reactions are involved in H_2 production (Figure 4). In the first redox reaction (1: $\text{Prod}_{\text{red}} + 2H^+ + 2e^- + \text{Fed}_{\text{ox}} \rightarrow \text{Prod}_{\text{ox}} + \text{Fed}_{\text{red}} + 2H^+ + 2e^-$), the reduced product (Prod) is oxidized thanks to a ferredoxin (Fed). In the second redox reaction (2: $\text{Fed}_{\text{red}} + 2H^+ + 2e^- \leftrightarrow \text{Fed}_{\text{ox}} + H_2$), the reduced Fed is oxidized leading to H_2 synthesis.

The three following sections will describe i) the mechanisms of Fed reduction in prokaryotes and eukaryotes (first redox reaction in Figure 4), ii) the production of H_2 during the oxidation of Fed (second redox reaction in Figure 4), and iii) the solubility and concentration of H_2 in this digestive compartment.

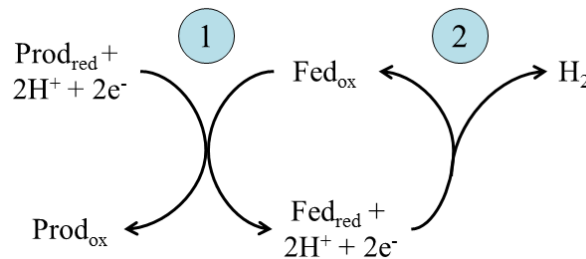


Figure 4 Oxidation-reduction reactions involved in H_2 production (Prod_{red} = reduced product, Prod_{ox} = oxidized product, Fed_{ox} = oxidized ferredoxin and Fed_{red} = reduced ferredoxin) (from Hegarty and Gerdes, 1999)

I. FERREDOXIN REDUCTION IN RUMEN MICROBES

1.1. Definition and microbial distribution of ferredoxin

Ferredoxins are proteins able to shuttle electrons from a donor to an acceptor. This property is achieved thanks to the presence of an iron-sulfur cluster (Fe_2S_2 or Fe_4S_4) at the

¹ Oxidation-reduction reactions (or redox reactions) involve two redox couples exchanging electrons.

core of the protein. The redox state of the iron (Fe) atoms reflects the redox state of the Fed: when Fe is reduced (Fe^{3+}), the Fed is reduced and when Fe is oxidized (Fe^{2+}), the Fed is oxidized (Stiefel and George, 1994).

Ferredoxins have been reported in a wide range of bacteria from various biological environments (review of Yoch and Valentine, 1972). In the rumen, their presence have been reported in methanogenic archaea (Thauer et al., 1977), in several genera of bacteria such as *Ruminococcus*, *Selenomonas*, *Megasphaera* and *Desulfovibrio* (Glass et al., 1977; Michel and Macy, 1990; Valentine and Wolfe, 1963), in the entodiniomorphid and holotrich orders of protozoa (Paul et al., 1990; Yarlett et al., 1985) and in the anaerobic fungus *Neocallimastix* spp. (Rees et al., 1998; Yarlett et al., 1986).

1.2. Ferredoxin production during microbial feed fermentation

Reduced Fed are produced during feed fermentation. As carbohydrates are the predominant components in ruminants' diet, Fed are mostly reduced during the fermentation of sugars into volatile fatty acids (VFA). To a minor extent, Fed are also reduced during protein fermentation (Czerkawski, 1986).

1.2.1. Production of reduced ferredoxins during carbohydrates fermentation

When carbohydrates enter the rumen, they are hydrolyzed by several microbial exogenous enzymes which act in synergy to generate glucose or xylulose. The subsequent fermentation of these two products leads to VFA, which are the main source of energy for the ruminant. In prokaryote, the fermentation of glucose mainly generates acetate, butyrate and propionate, whereas in eukaryote, acetate and butyrate are mainly synthesized (Jarrige et al., 1995; Williams and Coleman, 1997).

The production of reduced Fed during glucose fermentation is different between VFA. In prokaryotes (bacteria; Figure 5), the production of two moles acetate or one mole butyrate from glucose generates 8 and 4 moles reduced Fed. The production of propionate requires 4 moles reduced Fed. In eukaryotes (protozoa and fungi; Figure 6), the production of two moles acetate from one mole glucose leads to the production of 8 or 12 moles reduced Fed, depending on the fermentative route (through malate). The formation of one mole butyrate generates 4 moles reduced Fed. Finally, knowing that the ratio of acetate to propionate to butyrate to valerate is approximately 66:19:11:4 in the rumen (Sauvant et al., 2011), it is clear that glucose fermentation to VFA results in an important production of reduced Fed.

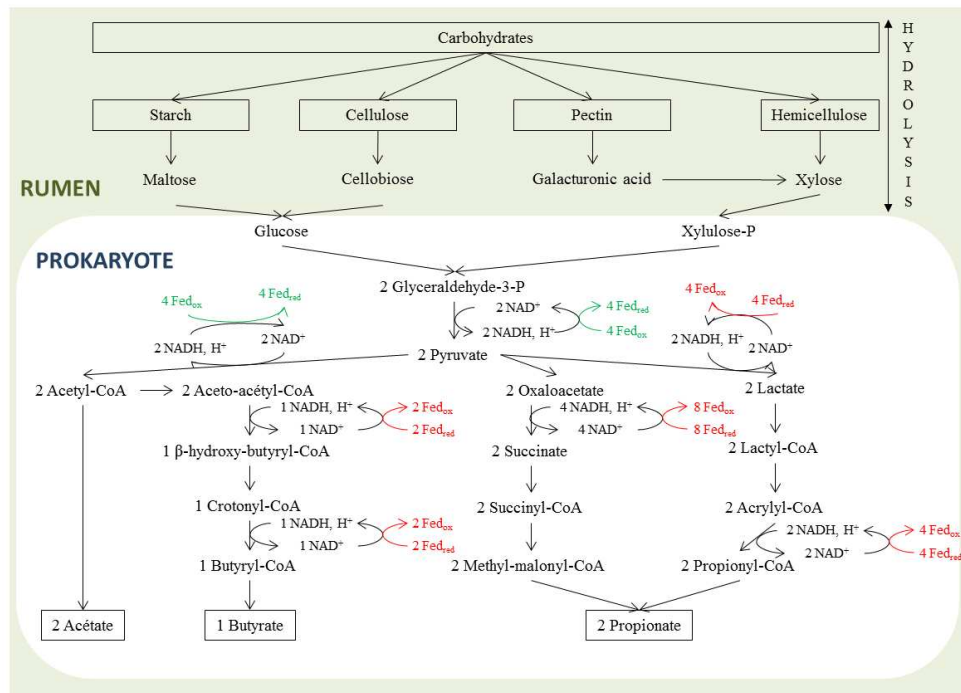


Figure 5 Carbohydrates hydrolysis and glucose fermentation pathway in prokaryotes. Reactions leading to the production of reduced ferredoxins are in green. Reactions leading to the production of oxidized ferredoxins are in red. (from Fonty et al., 1995; Prescott et al., 2010)

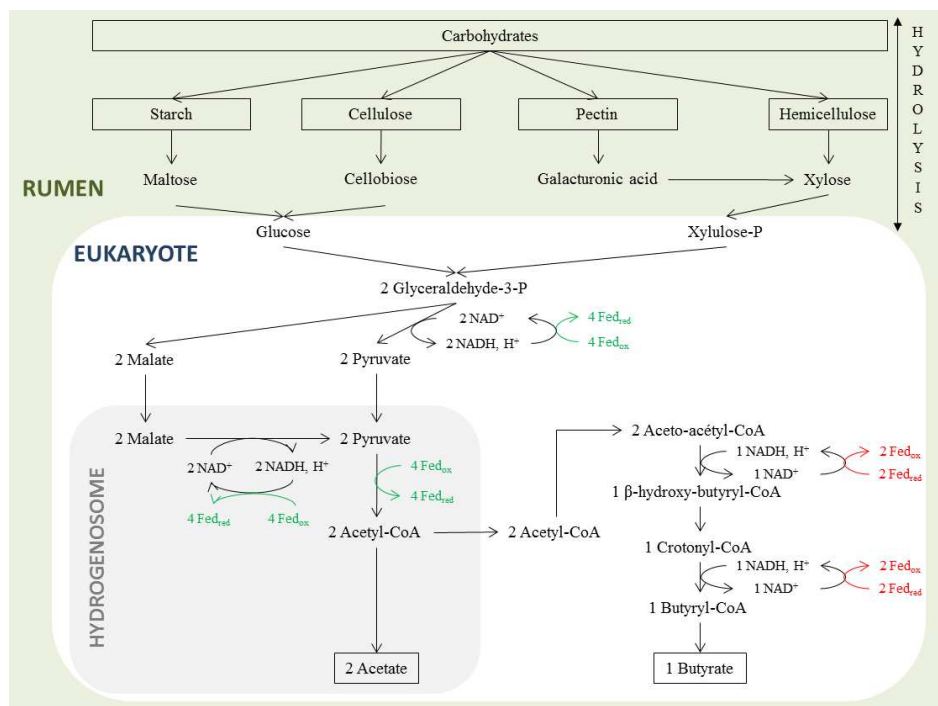


Figure 6 Carbohydrates hydrolysis and glucose fermentation pathway in eukaryotes including hydrogenosome. Reactions leading to the production of reduced ferredoxins are in green. Reactions leading to the production of oxidized ferredoxins are in red. (from Müller, 1993; Williams and Coleman, 1997)

1.2.2. Production of reduced ferredoxins during protein fermentation

Proteins entering the rumen are hydrolyzed by exogenous enzymes to generate amino acids (AA). During further microbial fermentation of AA, Fed are also reduced (Wu, 2013; Figure 7).

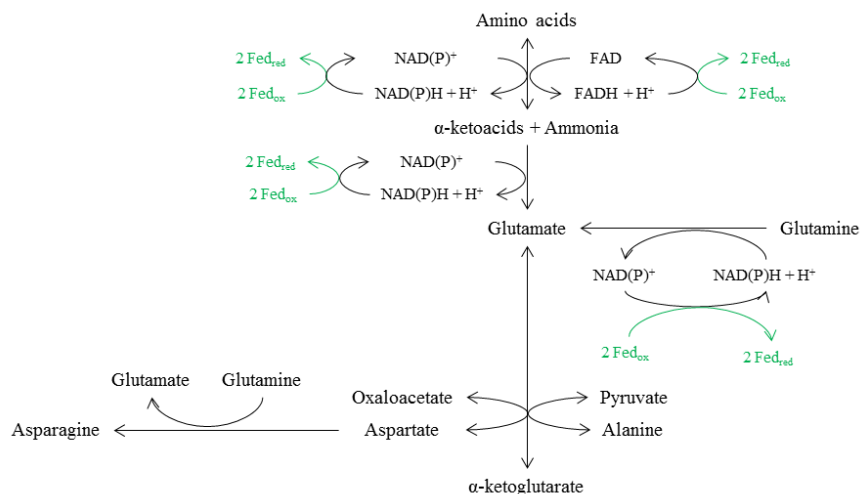


Figure 7 Microbial fermentation of amino acids in the rumen: exemple of glutamine and asparagine, which serve as substrates for the microbial synthesis of all other amino acids. Reactions leading to the production of reduced ferredoxins are in green. (from Wu, 2013)

1.3. Mechanisms of ferredoxin reduction

Ferredoxins are reduced during the oxidation of coenzymes in both prokaryotes and eukaryotes, but also during pyruvate oxidation in eukaryotes only (Figure 5, Figure 6).

1.3.1. Ferredoxin reduction from coenzymes

Ferredoxin reduction from coenzymes² takes place in the cytoplasmic membrane of rumen prokaryotes and in the cytosol or in the hydrogenosome of eukaryotes (more details about hydrogenosome will be given in following sections). The reaction is carried out by a coenzyme dehydrogenase which uptakes the electrons from coenzymes reduced during feed fermentation to the oxidized Fed (Valentine and Wolfe, 1963). The reaction catalyzed by the NAD(P)H dehydrogenase is:

² Coenzymes are organic compounds which include non-vitamin and vitamin derivatives. Adenosine triphosphate (ATP) responsible for phosphate transfer is an example of non-vitamin derivative. Vitamin derivatives include nicotinamide adenine dinucleotide (NAD^+) and nicotinamide adenine dinucleotide phosphate (NADP^+) derivating from vitamin B₃ (niacin) or flavin-adenine dinucleotide (FAD) and flavin mononucleotide (FMN) derivating from vitamin B₂ (riboflavin). These coenzymes serve as reversible carriers of reducing equivalents. (Broderick, J. B. 2001. Coenzymes and cofactors. Encyclopedia of life sciences, Nature Publishing Group.)



And the reaction catalyzed by the FADH dehydrogenase is:



Where $\text{NAD(P)H} + \text{H}^+$ and $\text{FADH} + \text{H}^+$ are the reduced coenzymes, Fed_{ox} is the oxidized Fed, NAD^+ and FAD are the oxidized coenzymes, Fed_{red} is the reduced Fed and e^- is the electron. As the standard reduction potential of NAD and FAD are more positive than the one of Fed (more precisions about thermodynamic laws are given in chapter 2), the NAD(P)H and FADH dehydrogenases can work only in the direction of Fed reduction, and the reverse direction is strongly inhibited by NAD(P)H, H^+ or FADH, H^+ (Gottschalk, 1986).

Whereas eukaryotes and some bacteria such as *Ruminococcus albus* do not require additional electron carriers (Glass et al., 1977), some bacteria require a cytochrome (cytochrome c) which is an intermediate electron carrier between the coenzyme and the Fed (Dolla et al., 1990). This transport of electrons through different electron carriers is named the electron transport chain. The presence of cytochrome b, a sub-unit of cytochrome c, has been detected in different rumen bacterial species such as *Prevotella* (White et al., 1962), *Fibrobacter succinogenes* (Reddy and Bryant, 1977), *Selenomonas ruminantium* (Stewart et al., 1997) and *Wolinella succinogenes* (Kern and Simon, 2009; Kröger et al., 2002).

Then, Fed reduction allows re-generating coenzymes into their oxidized form. As the concentration of coenzymes is fixed in the rumen, this process is essential to let the fermentations going on (Hegarty and Gerdes, 1999). To our knowledge, very few information exists about the concentration of coenzymes in the rumen. Indeed, coenzymes concentration is difficult to measure as they are quickly metabolized and their dosage requires an extraction from the cell followed by purification. In an *in vivo* experiment, the concentration of NAD analyzed from cells pellets from the ruminal fluid of dairy cows fed a barley or an oat based diet averaged 3.21 and 2.29 μM , respectively (Abdouli and Schaefer, 1986).

1.3.2. Direct ferredoxin reduction from pyruvate

In rumen eukaryotes, Fed reduction also occurred during the direct oxidation of pyruvate. This process occurs in the cytosol but may also occur within a specific organelle called the hydrogenosome (Martin and Müller, 2007; Müller et al., 2012).

Structure and occurrence of hydrogenosomes. Hydrogenosomes are membrane-bound organelles (Figure 8) which have only been reported in several anaerobic or microaerophilic unicellular eukaryotes. They share some similarities with mitochondria as they both use

pyruvate as a major substrate leading to the production of acetyl-CoA and ATP (Müller, 1993). However, as they do not co-exist with mitochondria, it was hypothesized that these two organelles come from the same symbiont which would have evolved differently according to its environment. In aerobic environment, this symbiont would have generated the mitochondria and in anaerobic environment, it would have created the hydrogenosome. Genomes comparison validated this assumption, as hydrogenosomal genome appeared to be highly related to mitochondrial genome (Akhmanova et al., 1998; Martin, 2005).

Hydrogenosomes have been reported in several rumen protozoa: *Polyplastron multivesiculatum* (Paul et al., 1990), *Eudiplodinium maggi* and *Epidinium ecaudatum* (Yarlett et al., 1984), *Dasytricha ruminantium* (Yarlett et al., 1981), *Isotricha prostoma* and *Isotricha intestinalis* (Yarlett et al., 1983). This organelle has also been reported in some rumen fungi such as *Neocallimastix patriciarum* (Yarlett et al., 1986). Nevertheless, hydrogenosomes have not been detected in some protozoal species such as *Entodinium caudatum*, *Entodinium simplex* and *Diploplastron affine* (Yarlett et al., 1984). On the contrary, they host a mitosome, a recently discovered organelle which does not produce energy, and whose function has not been clarified (Hackstein, 2010). For these species, H₂ production and associated mechanisms take place in the cytosol of the cell.

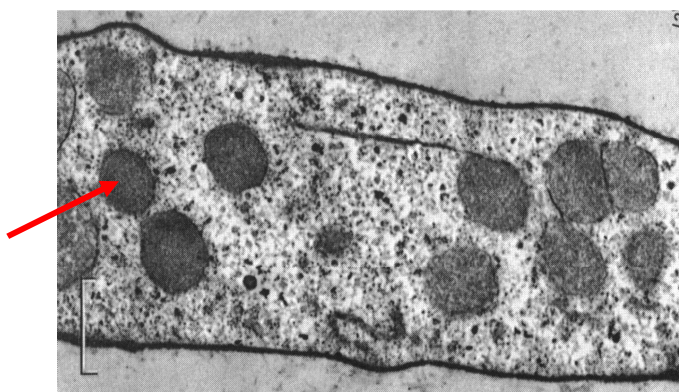


Figure 8 Electron micrograph of rumen fungus (*Neocallimastix patriciarum*) showing hydrogenosome organelles. The scale bar represents 1 µm, the red arrow points out one hydrogenosome. (from Yarlett et al., 1986)

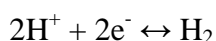
Mechanism of pyruvate oxidation. Eukaryotes directly reduce pyruvate to acetyl-CoA, which is further converted to acetate or butyrate (Yarlett et al., 1985). The conversion of one mole pyruvate to one mole acetyl-CoA is performed by a pyruvate-ferredoxin oxidoreductase through the reduction of two moles Fed:



This reaction is direct as no electron carriers such as cytochromes or coenzymes are required between the pyruvate and the Fed (Müller et al., 2012).

II. HYDROGEN PRODUCTION DURING FERREDOXIN OXIDATION

To ensure continuous fermentations, reduced Fed need to be oxidized. This process is concomitant to the production of H₂ by a hydrogenase. Hydrogenases are present in a large number of prokaryotes (Schwarz and Friedrich, 2003) and eukaryotes (Müller et al., 2012). They are responsible for the reduction or oxidation of H₂:



The direction of this reversible reaction depends on the redox potential of the environment (Vignais and Colbeau, 2004). The rumen being a highly reducing environment (Eh = -150 to -400 mV; Marden, 2007), the reaction is directed towards H₂ production.

2.1. General composition and classification of hydrogenases in anaerobic environments

Most hydrogenases are metallo-enzymes. Their catalytic site consists of a heterodimer, which is a protein complex made of two different sub-units. The first sub-unit is the iron-sulfur cluster [Fe₂-S₂, Fe₃-S₄ or Fe₄S₄] which is responsible for the transport of electrons to the second sub-unit, or active site (Beinert et al., 1997). Hydrogenases can be sorted into three classes according to the metal atoms of their active site (general reviews about hydrogenases: Vignais et al., 2001; Vignais and Colbeau, 2004):

- The [Ni-Fe] hydrogenases are the most numerous ones and are found in both bacteria and archaea. They are divided into four groups. The first group gathers respiratory hydrogenases which are responsible for H₂ oxidation coupled to the reduction of electron acceptors (NO₃⁻, SO₄²⁻, CO₂, O₂...). Hydrogenases of the second group are responsible for the activation of the expression of hydrogenase structural genes (Barz et al., 2010). The third group of hydrogenases is associated to the coenzymes dehydrogenase in charge of the reduction of H₂ and the oxidation of reduced cofactors (NAD(P)H, H⁺). The last and fourth group of hydrogenases is mostly involved in the disposal of reducing equivalents produced during carbon monoxide or formate oxidation.

- The [Fe-Fe] hydrogenases active site consists of a [Fe-Fe] subunit, also called H-cluster. These hydrogenases are found in anaerobic prokaryotes, but they also are the only type of hydrogenases found in eukaryotes such as protozoa or fungi. In these microorganisms, they are exclusively located in the hydrogenosomes. These enzymes are mostly involved in H_2 production. Due to their occurrence in very diverse microbes, they can be associated to various electron acceptors and donors.
- The [Fe-S] cluster free hydrogenases are found in some specific methanogenic archaea. These enzymes do not contain nickel as they mostly grow under nickel limited environment. They also differ from the [Ni-Fe] and [Fe-Fe] hydrogenases by their primary and tertiary structures and, by the fact that, iron is not redox active. Consequently, they have specific cofactors and they do not catalyze the oxidation or the reduction of H_2 . On the contrary, they are mostly involved in the reduction of methylene groups.

2.2. *Hydrogenases involved in ruminal hydrogen production*

The rumen anaerobic environment offers good conditions for the production and activity of hydrogenases, as oxygen (O_2) negatively affects most of hydrogenases activity (La Penna, 2010; Stripp et al., 2009). Indeed, O_2 would react with the active site of the enzyme, creating a superoxide ($E_0 = +0.9V$) which may be released only in the presence of an electron acceptor with a higher standard reduction potential. More detailed thermodynamics approach will be given in Chapter 2.

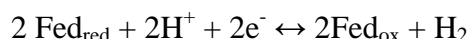
Hydrogenases have been purified and detected in several rumen bacterial species such as *Bacteroides clostridiiformis*, *Butyrivibrio fibrisolvens*, *Eubacterium limosum*, *Fusobacterium necrophorum*, *Megasphaera elsdenii*, *Ruminococcus albus* and *Ruminococcus flavefaciens* (Joyner et al., 1977; Van Dijk et al., 1979). The presence of hydrogenases has also been reported in ruminal protozoa and fungi (Paul et al., 1990; Yarlett et al., 1981; Yarlett et al., 1986).

To our knowledge, hydrogenases composition has poorly been studied. Using radioactivity, it was reported that *Wolinella succinogenes* owns a [Ni-Fe] hydrogenase (Unden et al., 1982). Recent sequencing of cDNA coding for a small piece of hydrogenase (“H-cluster”) showed that *Megasphaera elsdenii*, several species of the genus *Desulfovibrio* and rumen eukaryotes host [Fe-Fe] hydrogenases. Phylogenetic analyses also revealed that

there is few relationship between [Fe-Fe] hydrogenases from prokaryotes and eukaryotes (Boxma et al., 2007).

2.3. Balance of hydrogen production in the rumen during microbial feed fermentation

Within rumen microbes, hydrogenases are responsible for the oxidation of two moles reduced Fed while producing one mole H₂ (Gottschalk, 1986):



Consequently, we can now calculate the molar production of H₂ during carbohydrates fermentation (Table 3). The production of one mole acetate or one mole butyrate from one mole glucose generates 2 moles H₂ whereas 1 mole H₂ is required to produce one mole propionate. These results are similar to Sauvante et al., 2011. As eukaryotes preferentially ferments glucose to acetate and butyrate (Williams and Coleman, 1997), they are considered as important H₂-producers.

Table 3 Molar H₂ production during fermentation of one mole glucose

VFA	Moles from one mole glucose	Reduced ferredoxin production (moles)	H ₂ production (moles)
Acetate	2	+8	+4
Butyrate	1	+4	+2
Propionate	2	-4	-2

Concerning protein fermentation, the balance of H₂ production is less evident to calculate as it is dependent on AA profiles. However, it has been estimated that when microbes grow on AA as the sole N source, H₂ would be produced at a rate of 0.58 moles per kilogram of microbes, assuming a microbial composition of 53 g protein/100g dry microbial matter (Mills et al., 2001).

III. RUMEN HYDROGEN SOLUBILITY AND CONCENTRATION

After its production, H₂ diffuses through the cell cytoplasmic membrane to the ruminal environment in a dissolved form. The diffusion rate is dependent on the microbial cell physiology (cell size and form) and on the external H₂ concentration: the higher the external dissolved H₂ concentration, the lower is the diffusion rate of H₂ out of the cell. This maintains an equilibrated gradient between the cell and its environment (Boone et al., 1989). The

external H_2 concentration is in turn an equilibrium between dissolved and gaseous H_2 concentrations.

3.1. Dissolved hydrogen concentration in the rumen liquid phase

3.1.1. Hydrogen solubility and maximum theoretical concentration

The theoretical maximum H_2 concentration in the liquid phase of the rumen (dissolved H_2) is related to its solubility. Hydrogen solubility in water ($\mu M/atm$) is a function of temperature (T, K) and salinity (S, ‰). Its calculation involves the determination of Bunsen solubility coefficient (β , ml dissolved H_2 in 1 mL H_2O ; Wiesenburg and Guinasso, 1979):

$$\ln \beta = A1 + A2 \times \frac{100}{T} + A3 \times \ln\left(\frac{T}{100}\right) + S \times (B1 + B2 \times \frac{T}{100} + B3 \times \frac{T}{100^2})$$

Where $A1 = -47.8948$, $A2 = 65.0368$, $A3 = 20.1709$, $B1 = -0.082225$, $B2 = 0.049564$ and $B3 = -0.0078689$.

Then, at ruminal temperature (39°C or 312K) and assuming a null salinity in the rumen, β is equal to 0.0166 ml H_2 /ml H_2O . By applying the equation of ideal gas law in normal pressure (1.01325×10^5 Pa) and temperature (273K) conditions, the β solubility of H_2 is 740.9 μM . Consequently, the maximum concentration of dissolved H_2 in the rumen is 740.9 μM assuming there is no other dissolved gas in the liquid. This result is coherent, knowing the standard H_2 solubility (759 μM) at 30°C in water with zero salinity (Wiesenburg and Guinasso, 1979).

3.1.2. Observed rumen dissolved hydrogen concentration

Measure of dissolved H_2 concentrations. Owing to the high volatility of H_2 and its high turnover time (0.08 sec; Smolenski and Robinson, 1988), the dosage of dissolved H_2 concentrations is not easy. In the literature, two studies succeeded to measure *in situ* dissolved H_2 concentrations in the rumen. In the first one (Hillman et al., 1985), dissolved H_2 diffused in a Clark-type oxygen electrode placed within the rumen. Hydrogen concentration was determined *via* a mass spectrometer. In the second one (Smolenski and Robinson, 1988), dissolved H_2 was uptaken by a carrier gas (helium) passing through a probe immersed into the rumen. The gas mixture was then heated in order to separate helium from H_2 , and H_2 concentration was measured with a gas chromatograph.

Other developed methods are based on point-by-point analysis by gas chromatography of gas extracted from rumen juice. Rumen fluid was sampled in a syringe and H_2 was extracted *via* two methods: i) H_2 was gasified by heating the sample (Hungate, 1967) or by injecting the sample into a basic solution (Robinson et al., 1981); ii) Nitrogen (N) was diffused into the sample and after mixing and collection of upper gas, H_2 concentration was determined according to N dilution (Czerkawski and Breckenridge, 1971; Wang et al., 2014).

Observed ruminal dissolved H_2 concentrations. In a normal functioning rumen and outside feeding time, the basal concentration of dissolved H_2 is low, ranging between 0.6 and 3.4 μM (Table 4). This corresponds to a range between 0.081 and 0.459% of its maximal solubility. Two factors induce variations in these concentrations: the diet composition and the feeding time (Janssen, 2010). Dissolved H_2 concentrations increased from 2 to 3 hours postfeeding due to the increase in fermentation (Figure 9; Czerkawski and Breckenridge, 1971). This postfeeding rise is all the more important as diets are rich in quickly and readily fermentable feed (e.g. high grain diets).

Table 4 Dissolved hydrogen concentration in the rumen of ovine or cattle.

Reference	Animal species	Diet	Dissolved H_2 concentrations
Hungate (1967)	Bovine	100% lucerne hay	0.6-1.3 μM
Hillman et al. (1985)	Ovine	100% grass hay	0.6-3.4 μM
Smolenski et Robinson (1988)	Bovine	High forage diet (composition not mentioned)	1-1.4 μM (20 μM 10 min postfeeding)
Robinson et al. (1981)	Bovine	75% grain + 25% hay	1 μM (15 μM 1 h postfeeding)
Czerkawski et al. (1971)	Ovine	Molassed sugar beet before H_2 measurement (complete diet not mentioned)	48 μM (20 min postfeeding) 1-2 μM (5 h postfeeding)
Morgavi et al. (2012)	Ovine	58% lucerne pellet + 25% cracked maize grain + 17% prairie hay	22.6 μM (3 h postfeeding)

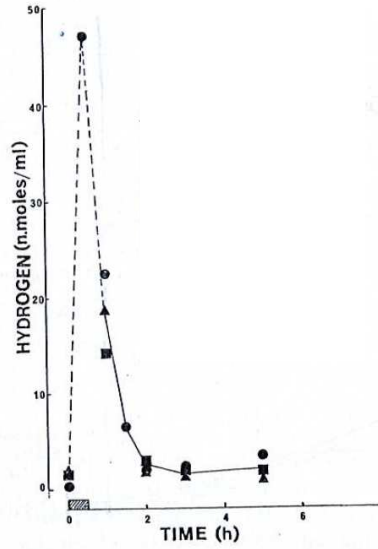


Figure 9 Ruminal dissolved hydrogen concentrations of sheep given 500 g molassed sugar beet pulp at time 0 (from Czerkawski and Breckenridge, 1971)

3.2. Equilibrium between dissolved and gaseous hydrogen in the dorsal sac

3.2.1. Theoretical equilibrium between dissolved and gaseous hydrogen

The presence of a dissolved gas in a liquid phase necessarily involves the presence of its gaseous form. Then, according to the dissolved H_2 concentration in the rumen, it may be possible to calculate the theoretical partial pressure of gaseous H_2 in the dorsal sac of this digestive compartment according to the Henry's law (Sander, 1999):

$$K_H = \frac{c_a}{p_g}$$

Where K_H is the Henry's law constant (M/atm), c_a is the concentration of H_2 in the liquid phase (M) and p_g is the partial pressure of H_2 in the gaseous phase (atm).

The Henry's law constant K_H depends on the medium temperature as the equilibrium between dissolved and gaseous phase is dependent on this parameter:

$$K_H = K_H^\theta \times \exp \left[-A \left(\frac{1}{T} - \frac{1}{T^\theta} \right) \right]$$

Where K_H^θ is K_H at standard temperature conditions ($K_H^\theta = 7.8 \times 10^{-4} M/atm$), A is a constant depending on the enthalpy of the solution ($A = 500K$), T is the temperature in the medium and T^θ is the standard temperature ($T^\theta = 298K$). Finally, at rumen temperature ($T = 312K$), K_H is equal to $0.000841 M/atm$. Consequently, if dissolved H_2 concentrations reach its maximum ($c_a = 740.9 \mu M$), the theoretical partial pressure of H_2 would be $0.88 atm$ (88% H_2).

However, a recent *in vitro* ruminal study showed that an increase of dissolved H_2 concentrations is not necessarily linked with an increase of gaseous H_2 (Wang et al., 2014). In that study, the authors concluded that the equilibrium between dissolved and gaseous H_2 may not completely respect Henry's law, probably because of mass-transfer³ limitation. Indeed, the transfer of H_2 from the rumen liquid phase to the rumen gaseous phase may be affected by the diffusivity coefficient of this gas and by the mixing efficiency of this digestive compartment (Pauss et al., 1990). Then, H_2 may accumulate in certain part of the rumen, limiting the possibility to calculate gaseous H_2 concentrations from dissolved H_2 concentrations measured in one part of the rumen, and *vice versa*. This also highlights the importance of *in vivo* measurement of H_2 concentrations in both phases. Nonetheless, to our knowledge, such experiment has still not been carried out.

3.2.2. Observed hydrogen concentrations in the rumen gaseous phase

Measure of gaseous H_2 concentration in the dorsal sac of the rumen. Several methods have been applied to measure H_2 concentrations in the rumen gaseous phase. With non-cannulated cows, gas has always been sampled by rumenocentesis, and gas composition was analyzed by gas chromatography (Jouany and Senaud, 1979; McArthur and Miltimore, 1961; Moate et al., 1997; Moate et al., 2013; Moate et al., 2014) or by the Orsat gas analyzer⁴ (Olson, 1940). With cannulated cows, gas has been collected with a bag attached to the cannula and filled thanks to rumen contraction (Barry et al., 1977) or with a syringe inserted through the plug of the rumen cannula (Moate et al., 2013). Gas composition was analyzed by gas chromatography.

Observed gaseous H_2 concentrations. Partial pressure of H_2 in the gaseous phase of bovine and ovine rumen ranges between 0.023 and 26.5% (Table 5). Several factors may explain the within-experiment variability. Gaseous H_2 concentrations are higher during the 2 h following meals (Barry et al., 1977; Jouany and Senaud, 1979) and when rapidly-degradable substrates are fed (Barry et al., 1977). Bloated animals after legumes feeding may have higher gaseous H_2 proportions, probably linked with a rumen dysfunction (Olson, 1940). However, Moate et al. (1997) did not observe differences in gaseous H_2 between bloated and non-

³ Mass transfer is defined as the movement of a mass from one phase to another.

⁴ The Orsat gas analyser system is based on absorption of gases of interest by specific chemical solutions.

bloated dairy cows. Between-experiments variability in gaseous H_2 concentrations may be explained by the presence or absence of rumen cannula: cannulated animals have lower H_2 proportions than non-cannulated animals, probably because of air exchange *via* the cannula between the rumen and its external environment (Moate et al., 2013).

Table 5 Composition of rumen headspace gas (adapted from Hegarty and Gerdes, 1999)

Reference	Animal species	Diet	Rumen headspace gas composition (%)		
			CO ₂	CH ₄	H ₂
Olson, 1940	Bovine	Sweet clover			
		- Non bloated	60.7	0.14	9.36
		- Bloated	62.0	15.3	0.31
		Alfalfa			
		- Non bloated	53.5	0.05	26.5
		- Bloated	59.8	18.4	0.05
McArthur and Miltimore, 1961	Bovine	Unspecified	65.4	26.8	0.18
Barry et al., 1977	Ovine	100% hay			
		- before feeding	47.1	36.2	0.033
		- feeding time	24.5	12.0	0.046
		- 2 h postfeeding	47.5	33.0	0.062
		80% hay, 20% concentrate			
		- before feeding	54.3	26.1	0.023
		- feeding time	35.7	16.3	0.319
		- 2 h postfeeding	68.4	24.8	0.135
Jouany and Senaud, 1979	Ovine	40% dehydrated lucerne, 9% wheat straw, 51% concentrate			
		- 1 h postfeeding	62.6	32.0	1.68
		- 5 h postfeeding	57.3	32.8	1.10
		- 10 h postfeeding	45.0	35.7	0.05
Moate et al., 1997	Bovine	White clover pasture			
		- Non bloated	75.8	23.1	<0.10
		- Bloated	75.0	23.5	<0.10
Moate et al., 2013	Bovine	54% grain, 46% alfalfa hay			
		- Non-cannulated	49.8	26.1	0.14
		- Cannulated	13.4	3.8	0.03
Moate et al., 2014	Bovine	Alfafa hay (AH), grain, dry or ensiled grape marc (DGM or EGM)			
		- 76% AH, 24% grain	62.8	37.1	0.11
		- 50% AH, 27% DGM, 23% grain	60.1	39.8	0.13
		- 50% AH, 27% EGM, 23% grain	61.3	38.6	0.11
Average			54.4	23.9	2.1
SD			15.31	12.29	6.27

SD: Standard deviation

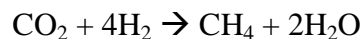
CHAPTER 2: Methanogenesis, not a unique pathway using hydrogen in the rumen

Hydrogenases activity can be inhibited by an accumulation of H₂ in their environment, with bacterial hydrogenases ([Ni-Fe] hydrogenases) being even more sensitive than protozoal hydrogenases ([Fe-Fe] hydrogenases) (Fourmond et al., 2013). Consequently, to ensure continuity of fermentation in the rumen, it is essential to maintain a low H₂ concentration *via* efficient mechanisms of removal and uptake of H₂.

I. METHANE PRODUCTION

Methane production is the main pathway using H₂. Czerkawski (1986) estimated that 48% of produced H₂ would be used towards this pathway. With a different approach, a more recent mechanistic model even increased this percentage to 80% with the assumption that methanogenesis uses the excess of H₂ which has not been used by other H₂ using pathways (Mills et al., 2001).

In the rumen, hydrogenotrophic methanogens use H₂ as an energy source for their growth while producing CH₄:



The linear and positive relationship between rumen H₂ concentration and CH₄ production has been emphasized in four *in vitro* experiments (Czerkawski et al., 1972; Hungate, 1967; Wang et al., 2014; Zaiß and Kaltwasser, 1979): correlation between dissolved H₂ and CH₄ concentration in headspace would average 0.92 (Wang et al., 2014) and Zaiß and Kaltwasser (1979) reported a correlation of 0.90 between hydrogenase activity and methanogenesis.

The microbial mechanisms under CH₄ production involve interspecies H₂ transfer between H₂-producers and methanogens (Wolin et al., 1997). The most studied example of this H₂ transfer is the symbiotic relationship between methanogens and protozoa (Finlay et al., 1994; Newbold et al., 1995; Stumm et al., 1982; Ushida and Jouany, 1996; Vogels et al., 1980): methanogens are positioned on the protozoa to reduce the distance for diffusion of H₂ from the hydrogenosome. These methanogens associated with protozoa would be responsible for between 9 and 25% of methanogenesis in rumen fluid (Newbold et al., 1995). A recent analysis of the literature highlighted a positive relationship between protozoa and CH₄

emissions: a reduction of 0.12 log₁₀ protozoa cells/mL would reduce CH₄ by 1 g/kg DMI (Morgavi et al., 2010). By an *in vitro* approach, *Entodinium* species were found to be the protozoal genus contributing the most to CH₄ emissions, followed by *Epidinium caudatum*. *Polyplastron* had the lowest contribution (Newbold et al., 1995).

II. VOLATILE FATTY ACIDS SYNTHESIS

Volatile fatty acids synthesis would be responsible for 19-33% of the H₂ uptake (Czerkawski, 1986; Mills et al., 2001). Only propionate and valerate formation uses H₂, with one mole H₂ required per mole produced propionate or valerate.

Two propionate precursors have been tested to reduce CH₄ emissions. Firstly, based on stoichiometry, the conversion of one mole fumarate to propionate would reduce CH₄ emissions by 5.6L (Newbold et al., 2005). However, fumaric acid tested *in vivo*, showed a low and variable anti-methanogenic effect which is not dose-dependent (4% CH₄ reduction per percent added fumaric acid, on average). A reduction of CH₄ emissions (g/kg DMI) of 21.8% was reported when supplying 2.0% of fumaric acid to male steers (Bayaru et al., 2001). In beef cattle, CH₄ emissions (g/kg DMI) raised by 10.2% while feeding 2.4% of fumaric acid (Beauchemin and McGinn, 2006). In dairy cattle, 2.5% of fumaric acid did not affect CH₄ emissions (g/kg DMI; Van Zijderveld et al., 2011a). In sheep supplied with 10% of fumaric acid, CH₄ emissions (g/kg DMI) were reduced by 57% (Wood et al., 2009). The contradictory CH₄ mitigating effect of fumarate was also reported when analyzing several *in vitro* experiments by a meta-analysis approach (Ungerfeld et al., 2007). These authors calculated that only 48% of added fumarate would be converted into propionate, confirming previous results (Newbold et al., 2005). They assumed that this incomplete conversion of fumarate may be caused by its rapid disappearance in the rumen.

Secondly, the anti-methanogenic effect of malic acid seems to be low (2% CH₄ reduction per percent malic acid, on average), but more repeatable with doses equal or higher than 2%. Malic acid at a dose of 1.2% did not affect CH₄ emissions (g/kg DMI) of dairy cows (Doreau et al., 2014b). With a dose of 2.0% fed to male steers, malic acid reduced CH₄ emissions (g/kg DMI) by 17.3% (Lila et al., 2004). Using malic acid at doses of 3.5 and 7.5%, CH₄ emissions (g/kg DMI) of heifers were linearly reduced from 2.7% to 9.2%, respectively (Foley et al., 2009).

III. MICROBIAL BIOMASS SYNTHESIS

According to calculations, bacteria and protozoa would be composed of 6.23 H atoms/100 g cells (Reichl and Baldwin, 1975; Table 6). Then, H₂ is essential for microbial synthesis, but their requirement level is variable in the literature. Czerkawski (1986) estimated that 12% of produced H₂ is used for microbial growth. In the model of Mills et al. (2001), this percentage is much lower, considering that 0.6% of H₂ would be directed towards microbial growth. This important difference between the two studies must come from the different ways of calculation of microbial composition. Mills et al. (2001) estimated that microbes require H₂ only when they grow with non-protein nitrogen (NPN), and this requirement was assessed at 0.41 moles H₂ per kilogram of microbes. This requirement level has been set considering polysaccharide-free microbial dry matter, whereas previous studies took into account the storage polysaccharide (Benchaar et al., 1998). Consequently, in order to precisely assess the amount of H₂ used for microbial biomass synthesis, it will be necessary to standardize the methods of calculation.

The between-experiment variability in H₂ requirement for microbial growth may also come from the level of nutrients deficiency in the diets. Indeed, when the crude protein (CP) content of the diet is low, microbes have to use NPN source, which increases microbial growth efficiency and then, H₂ uptake (Leng, 2014).

Table 6 Bacterial composition (from Reichl and Baldwin, 1975)

	Protein	Nucleic acid	Polysaccharide	Lipid	Ash
Bacteria (g/100g dry cells)	54.46	9.08	20.16	11.54	4.76
Bacteria (mol/100g cells)	0.474	0.028	0.124	0.019	--
Hydrogen (atoms/mol)	7.59	12	10	55.8	--

IV. BIOHYDROGENATION OF POLY-UNSATURATED FATTY ACIDS

Czerkawski (1986) and Mills et al. (2001) estimated that only between 1 and 2.6% of H₂ is uptaken for biohydrogenation, which consists in H saturation of double bonds of unsaturated fatty acids. This means that the reduction in CH₄ emissions observed in several experiments testing polyunsaturated fatty acids (PUFA) in ruminants diets (Beauchemin and McGinn, 2006; Beauchemin et al., 2009; Chung et al., 2011; Martin et al., 2008) cannot be solely explained by biohydrogenation.

For instance, we can assume that a complete saturation of oleic acid (C18:1), linoleic acid (C18:2) and linolenic acid (C18:3) requires 1, 2 and 3 moles H_2 , respectively. When applying these coefficients to the experiment of Martin et al. (2008), feeding 5.8% linseed oil (49.2% C18:3; 21.3% C18:2; 15.1% C18:1) to lactating cows eating 14.7 kg DM would reduce CH_4 emissions by 25.1 g/day. However in this experiment, CH_4 was reduced by 268.9 g/day which was 10 times more than theoretically calculated, showing the absence of relationship between the quantity of saturated double bonds and the extent of CH_4 inhibition. In other words, this difference highlights that the CH_4 mitigating effect of PUFA is only partly due to H_2 uptake for biohydrogenation. Other reasons may explain the negative effects of lipids on methanogenesis. PUFA must have a toxic effect on protozoa which are important H_2 producers (Doreau and Ferlay, 1995). The degradation of diet digestibility with high doses of PUFA (more than 5% added fat in Martin et al., 2008) must reduce H_2 production and availability for methanogens. As lipids are mostly digested in the intestine, H_2 production in the rumen is reduced when fed in substitution of carbohydrates.

V. OTHER HYDROGEN-SINKS COMPETING METHANOGENESIS

In aerobic environment, oxygen (O_2) is the most important H_2 -sink, due to its high affinity for H_2 ($O_2 + 2H_2 = 2H_2O$). Inversely, in anaerobic environment, CO_2 , propionate precursors (Hattori and Matsui, 2008; Henderson, 1980; Reddy and Peck, 1978), nitrate, sulfate (Laverman et al., 2012; Van Zijderveld et al., 2010), iron or manganese (Lovley, 1991; Nealson and Saffarini, 1994) can play the role of H_2 -sink. When all these electrons acceptors are present in an anaerobic environment, thermodynamic laws define the ranking of molecules reduction.

5.1. Thermodynamic laws governing the affinity of electrons acceptors for hydrogen

Reactions between H_2 and electrons acceptors are oxidation-reduction reactions, which involve two redox couples exchanging electrons. Each couple is composed of an oxidant (Ox) and a reducer (Red):

Couple 1 (Ox_1/Red_1): $Red_1 = Ox_1 + ne^-$ (Oxidation)

Couple 2 (Ox_2/Red_2): $Ox_2 + ne^- = Red_2$ (Reduction)

Final equation balance: $Red_1 + Ox_2 = Ox_1 + Red_2$

Each redox couple is characterized by an equilibrium constant between the oxidant and the reducer, named the “standard reduction potential” (E_0 , V) which measures the tendency of the reducing agent to lose electrons (

Table 7). The exchange of electrons between two couples is spontaneously possible if the variation ΔE_0 between their standard reduction potential is positive (exergonic reaction):

$$\Delta E_0 = E_0 (\text{Reduction}) - E_0 (\text{Oxidation}) > 0$$

Should this not be the case ($\Delta E_0 < 0$), the reaction would require energy (endergonic reaction).

The affinity between two redox couples is determined by the “Gibbs free energy” (ΔG) liberated during their reaction:

$$\Delta G = -nF \times \Delta E_0$$

Where n = number of electrons involved in the process, F = Faraday constant (96.500 kJ/V/mol) and ΔE_0 = the difference of standard reduction potentials between the two redox couples (V). In spontaneous process, ΔG is negative and the lower it is, the higher will be the free energy liberated. This means that redox couples with negative E_0 will tend to give electrons to redox couples with the more positive E_0 . Then, in a given environment, H_2 will have a decreasing affinity for O_2 , NO_3^- , MnO_4^- , Fe^{3+} , Fumarate, SO_4^{2-} and CO_2 (Table 8).

Table 7 Standard reduction potentials of several common redox couples at pH = 7 (Prescott et al., 2010; Tratnyek and Macalady, 2000)

Redox couples	Reduction half-reaction	E_0 (V)
H^+/H_2	$2H^+ + 2e^- \rightarrow H_2$	-0.42
Fed_{ox}/Fed_{red}	$Fed_{ox} + e^- \rightarrow Fed_{red}$	-0.42
$NAD(P)^+/NAD(P)H$	$NAD(P)^+ + 2H^+ + 2e^- \rightarrow NAD(P)H + H^+$	-0.32
CO_2/CH_4	$CO_2 + 8H^+ + 8e^- \rightarrow CH_4 + 2H_2O$	-0.25
SO_4^{2-}/HS^-	$SO_4^{2-} + 9H^+ + 8e^- \rightarrow HS^- + 4H_2O$	-0.21
$FAD/FADH_2$	$FAD + 2H^+ + 2e^- \rightarrow FADH_2$	-0.18
Fumarate/Succinate	$HOOCCH=CHCOOH + 2H^+ + 2e^- \rightarrow HOOC(CH_2)_2COOH$	+0.03
NO_3^-/NO_2^-	$NO_3^- + 2H^+ + 2e^- \rightarrow NO_2^- + H_2O$	+0.42
Fe^{3+}/Fe^{2+}	$Fe^{3+} + e^- \rightarrow Fe^{2+}$	+0.77
O_2/H_2O	$O_2 + 4H^+ + 4e^- \rightarrow 2H_2O$	+0.82
MnO_4^-/Mn^{2+}	$MnO_4^- + 8H^+ + 5e^- \rightarrow Mn^{2+} + 4H_2O$	+0.84
NO_2^-/NH_4^+	$NO_2^- + 8H^+ + 6e^- \rightarrow NH_4^+ + 2H_2O$	+0.90

Table 8 Gibbs free energy liberated between H₂ and several electron acceptors

Redox couples	ΔG (kJ)
O ₂ /H ₂ O	-239
NO ₃ ⁻ /NO ₂ ⁻	-162
MnO ₄ ⁻ /Mn ²⁺	-122
Fe ³⁺ /Fe ²⁺	-115
Fumarate/succinate	-87
SO ₄ ²⁻ /HS ⁻	-41
CO ₂ /CH ₄	-33

5.2. Electrons acceptors tested in rumen

The rumen being an anaerobic environment, O₂ cannot be used to oxidize H₂. Others electrons acceptors have been tested in the rumen to reduce methanogenesis with the hypothesis that they can efficiently compete for H₂, reducing its availability for CH₄ production. To our knowledge, fumarate (mentioned above), nitrate and sulfate are the only other electrons acceptors which have been tested *in vivo*.

Four moles H₂ would be used in the reduction of 1 mole nitrate to 1 mole ammonia (*via* nitrite production by a periplasmic reductase; Iwamoto et al., 2001; Simon et al., 2003) or in the reduction of 1 mole sulfate to 1 mole hydrogen sulfide. Consequently, knowing that 4 moles H₂ are also required to produce 1 mole CH₄, theoretically, 1 mole nitrate or sulfate in diets would reduce CH₄ production by 1 mole (22.4 L). In *in vivo* experiments, nitrate or sulfate effectively reduced CH₄ production (Table 9). Methane reduction efficiency, calculated as the ratio between observed CH₄ emissions and expected CH₄ emissions based on stoichiometry, ranged between 42 and 119%. The inefficient use of nitrate and sulfate may be explained by the higher proportion of acetate in the rumen of animals supplemented with nitrate, which synthesis produces H₂ counteracting the reduction of H₂ availability caused by these two chemicals (Nolan et al., 2010; Hulshof et al., 2012; Veneman et al., 2014).

Table 9 Efficiency of methane reduction when supplementing ruminants with electrons acceptors.

Reference	Animal species	Electron acceptor	Dose (% DM)	CH ₄ reduction (g/kg DMI)		Efficiency ¹ (%)
				Expected	Observed	
Van Zijderveld et al., 2010	Ovine	Nitrate	2.6	6.7	5.9	89
Van Zijderveld et al., 2011	Cattle	Nitrate	2.1	5.4	3.0	56
Hulshof et al., 2012	Cattle	Nitrate	2.2	5.7	6.1	107
Nolan et al., 2012	Ovine	Nitrate	2.5	6.5	4.8	74
Veneman et al., 2014	Cattle	Nitrate	2.0	5.2	4.6	89
Veneman et al., 2014	Cattle	Nitrate	2.0	5.2	6.1	119
Van Zijderveld et al., 2010	Ovine	Sulfate	2.6	6.7	2.8	42

¹ Efficiency was calculated as the ratio between observed *in vivo* CH₄ emissions and expected CH₄ emissions based on stoichiometry.

Supplementation of animals with nitrate or sulfate presents risks for their health, which explain why large scale use of these two chemicals is still not authorized in animal nutrition. Indeed, rapid ingestion by animals of high doses of nitrate may induce nitrite accumulation in the rumen which enters blood through the rumen wall, leading to the conversion of hemoglobin (Hb) to methemoglobin (metHb; Lewis, 1951). Contrary to Hb, metHb cannot transport oxygen and its accumulation may become life-threatening. Hydrogen sulfide coming from sulfate reduction may be eructated by the animal and re-enter the body during respiration. Inhalation of this gas by ruminants may induce polyoencephalomalacia which is a neurologic disorders characterized by necrosis of the cerebral cortex (Gould, 1998). Consequently, to counter the negative effects of nitrate and sulfate, it would be interesting to test novel electron acceptors.

Knowing the Gibbs free energy liberated during the redox reaction between H₂ and iron (Table 8), we assumed that iron III (Fe³⁺) can also be an efficient electrons acceptor in the rumen, by diverting one electron from methanogenesis. We tested this hypothesis (unpublished data) using an automated *in vitro* rumen batch culture system (Muetzel et al., 2014). Five sources of iron (4 mM; iron II sulfate, iron II chloride, iron II acetate, iron III sulfate and iron III chloride) were incubated for 48 h with a substrate made of hay and concentrate (50:50) and a pasture-fed bovine inoculum. The iron II sources were used to know the outcome of iron II coming from iron III reduction. Both iron II and iron III sources reduced methanogenesis. Iron CH₄-mitigating efficiencies, calculated as the ratio between

expected CH₄ emissions based on stoichiometry and observed CH₄ emissions, ranged between 84 and 93% for iron II sources and averaged 84% for iron III sources.

As iron II presented the same CH₄-mitigating efficiency than iron III, we made two assumptions. Firstly, we assumed that iron may enhance another pathway using H₂ such as microbial biomass. To test this effect, iron II acetate (4 mM) and iron II chloride (4 mM) were incubated again for 48 h with glucose as the sole protein-free substrate to quantify the effect of iron on microbial growth. We observed that iron increased the concentration in insoluble proteins (Figure 12), indicating that iron may enhance H₂ uptake *via* a better microbial biomass synthesis. An additional dose-response study may highlight to which extent microbes are sensitive to iron availability. Anyway, knowing the low contribution of microbes in the use of H₂, other mechanisms must be involved in the CH₄-mitigating effect of iron.

Then, owing to the change in color of the medium within the first 10 h incubation (from green to dark black, Figure 10), we assumed that iron III and II are reduced in another form of iron while using electrons. In the rumen, knowing the average pH ([5.5;6.5]; Lettat, 2012) and Eh ([-150;-350]mV; Marden, 2007; personal database), diagrams of iron minerals indicate that iron should be in the form of vivianite (Fe₃(PO₄)₂·8(H₂O)) and/or magnetite (Fe₃O₄) (Figure 11). Then, to reduce iron III to iron, it may be 3 rather than 1 electron which would be deviated from methanogenesis.



Figure 10 Color of the medium after 48 h incubation with hay and concentrate (50:50) supplemented with (left bottle) or without (right bottle) iron sources.

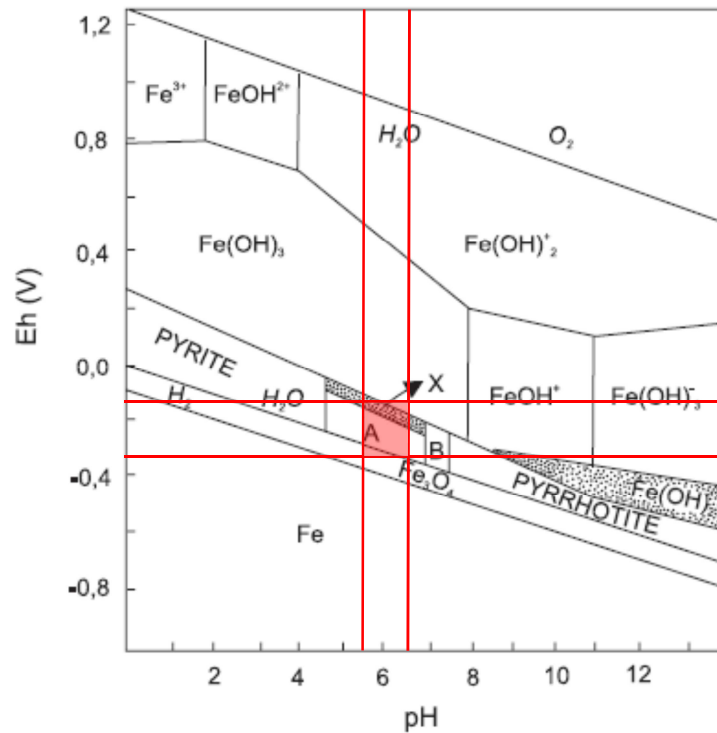


Figure 11 Forms of iron minerals according to Eh and pH (A: vivianite; B: siderite; Lemos et al., 2007). Vertical and horizontal lines respectively correspond to pH and Eh ranges commonly found in ruminal conditions. The red square represents all the possible combinations of pH and Eh in the rumen, with associated forms of iron.

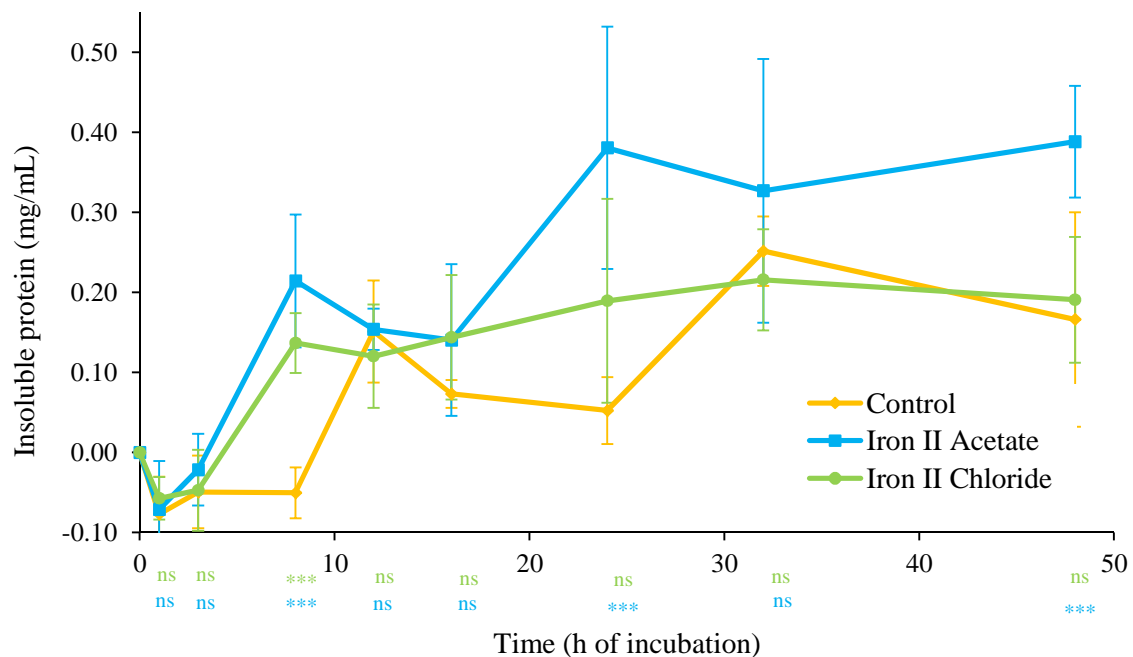


Figure 12 Insoluble protein concentration during 48 h incubation with hay and concentrate (50:50) supplemented with iron II acetate (4 mM) and iron II chloride (4 mM). Error bars indicate standard deviation.

CHAPTER 3: Emissions of gaseous hydrogen from the rumen: small energetic losses

Rumen stoichiometric models aiming to predict CH₄ emissions generally assume that the amount of H₂ produced is equal to the amount of H₂ used on a molar basis (Alemu et al., 2011; Benchaar et al., 1998; Mills et al., 2001). This hypothesis means that the H₂ recovery in CH₄, VFA and microbial synthesis would be equal to 100%, with no H₂ gas emitted from the animal. Consequently, few *in vivo* studies measured concentrations of H₂ emissions. However, results from these studies showed that H₂ emissions occur, even if they generally remain low, hardly detectable and represent a low percentage of GEI (less than 1% GEI).

I. FACTORS OF VARIATION OF GASEOUS HYDROGEN EMISSIONS

1.1. Measurement of hydrogen emissions

In the literature, two methods have been used to quantify gaseous H₂ emissions. In both of them, animals were placed in respiratory chambers but these methods differed in terms of gas sampling method. The first one consisted in manual sampling of gas with a syringe in the chamber air intake and exhaust ducts (Van Zijderveld et al., 2011). In the second method, gas was automatically sampled *via* a shunt from the air intake and exhaust duct going directly to a gas analyzer (Pinares-Patiño et al., 2012a).

Gas composition was then analyzed by gas chromatography. Two detectors have been used, having different detection levels: an electrochemical H₂ detector with a detection level of 5 to 10 ppm (Pinares-Patiño et al., 2012a) and a Quintron Breathtracker with a detection level of 1 to 2 ppm (Van Zijderveld et al., 2011).

1.2. Factors influencing hydrogen emissions

1.2.1. Intake level and meals frequency

The higher the amounts of DMI, the lower are H₂ and CH₄ emissions (% GEI). Indeed, the comparison of gaseous emissions of sheep fed increasing amounts of forage (DMI ranging from 0.40 kg forage/day to 1.60 kg/day) showed that CH₄ and H₂ emissions were linearly

reduced: from 8.39% GEI to 6.02% GEI for CH₄ and from 0.052% GEI to 0.034% GEI for H₂ (Hammond et al., 2013).

A low frequency of meals distribution induces higher postfeeding peaks of H₂ emissions associated to lower CH₄ emissions. One study compared H₂ emissions of two groups of sheep fed the same diet (60:40 mixture of lucerne hay and wheat grain) distributed either two or eight times per day (Swainson et al., 2011; Figure 13). Daily CH₄ emissions were lower for sheep fed twice daily (3.47 vs 6.35% GEI) whereas H₂ emissions were similar between the two groups (0.061% GEI). However, sheep fed twice daily presented high peaks of H₂ emissions till 40 ppm one hour postfeeding, directly followed by peaks of CH₄ emissions (up to 180 ppm). Gaseous emissions recovered lower and basal value within 3 h for H₂ (0 ppm) and 7 h for CH₄ (40 ppm). Inversely, sheep fed eight times a day presented more regular gaseous emissions within a day, which never reached values higher than 15 ppm for H₂ and which ranged between 80 and 160 ppm for CH₄.

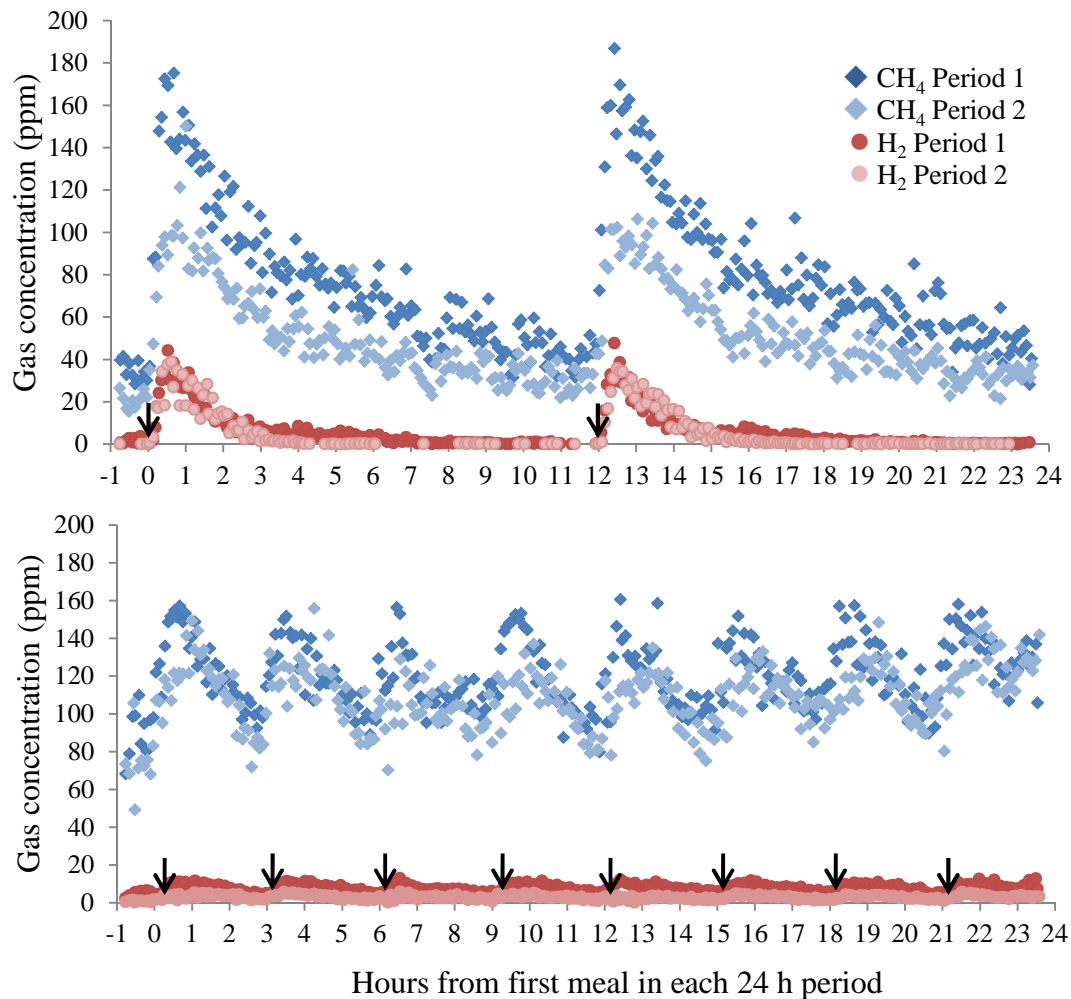


Figure 13 Daily methane and hydrogen emissions kinetics (ppm) of sheep fed 2 (upper graph) or 8 (lower graph) times daily. The arrows indicate times of feeding. (from Swainson et al., 2011)

1.2.1. Diet composition and additives supplementation

High starch diets reduce CH₄ emissions without necessarily reducing H₂ emissions. A comparison between sheep fed either grass or a 60:40 mixture of lucerne hay and wheat grain showed that H₂ emissions from animals fed the high concentrate diet represented 0.115% GEI, which was six times more than the sheep fed grass (0.019% GEI) (Pinares-Patiño et al., 2010). Inversely, CH₄ emissions were lower for sheep fed the high concentrate diet (7.31 vs 11.66% GEI). However, it was recently shown that steers fed 92.5% of concentrates significantly emitted less H₂ than steers fed a mixed diet with 52% concentrates (1.47 vs 1.79% GEI) whereas CH₄ emissions (% GEI) were reduced by 37% with the high concentrate diet (Rooke et al., 2014).

Some chemicals supplemented in the diet of ruminants for reducing CH₄ emissions give rise to H₂ emissions. While inhibiting methanogenesis by 4.6% GEI with 0.2% of

hemiacetal of chloral and starch in diet, H₂ emissions were detected in rams up to 1.7% GEI (Johnson, 1972). Similarly, using the same inhibitor with half of previous dosage (CH₄ reduction of 3.94% GEI), H₂ emissions represented 0.8% GEI (Johnson, 1974). More recently, using 2.1% nitrate to reduce CH₄ by 1% GEI, dairy cows emitted more than 0.017% GEI, which was 2.5 times more than control cows (0.006% GEI; Figure 14; Van Zijderveld et al., 2011). When having a closer look to the kinetics, one may observe that the peak of gaseous H₂ caused by nitrate is situated 2 h postfeeding and 1 h before the postprandial peak of CH₄ emissions from control cows. Hydrogen release with nitrate supplementation may be explained by the punctual inhibiting effect of this chemical towards hydrogenotrophic methanogens (Iwamoto et al., 2001; Van Zijderveld et al., 2010).

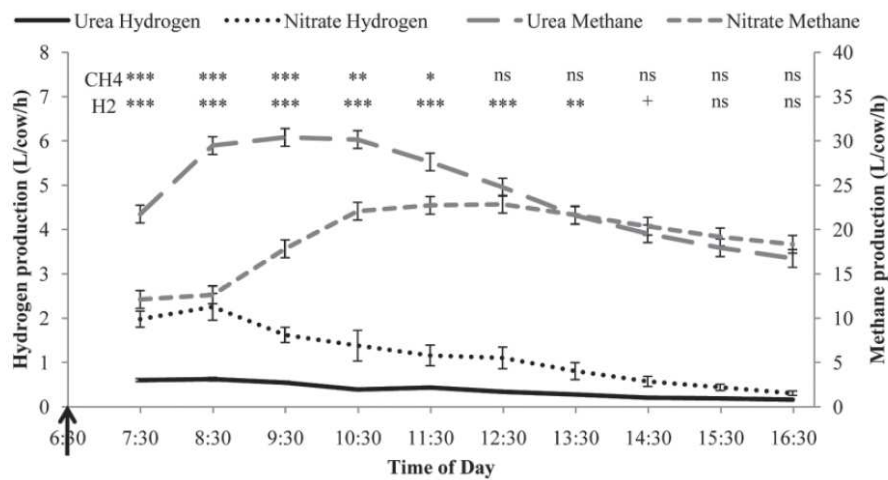


Figure 14 Methane and hydrogen emissions kinetics of dairy cows supplemented with nitrate. The arrow indicates time of feeding. (from Van Zijderveld et al, 2011b)

Finally, H₂ emissions represent a low spoilage of energy (from 0.006 to 1.8% GEI) which is not used by the animal to produce VFA or microbial biomass. These low H₂ levels point out that the molecule is quickly metabolized in the rumen. The relationship between H₂ and CH₄ emissions is different between *in vitro* and *in vivo* experiments, as a positive and linear relationship has been reported *in vitro* between these two factors (chapter 2). *In vivo*, this relationship would be dependent on H₂ concentrations: above 0.1% GEI, a rise of H₂ emissions may be associated with a reduction of CH₄ emissions (Johnson, 1974, 1972; Pinares-Patiño et al., 2010; Van Zijderveld et al., 2011). Inversely, under 0.1% GEI, H₂ and CH₄ emissions are either not correlated (Swainson et al., 2011) or positively correlated (Hammond et al., 2013).

II. CAUSES OF HYDROGEN EMISSIONS AND CONSEQUENCES

2.1. Two potential causes of hydrogen emissions

To our knowledge, no *in vivo* experiment reported simultaneous measurements of dissolved H_2 in the liquid phase of the rumen, gaseous H_2 in the dorsal sac of the rumen and emissions of H_2 from the rumen. However, knowing the relationship between dissolved and gaseous H_2 , we assume that an increase of H_2 emissions is linked to an evacuation of excessive gaseous H_2 coming from high dissolved H_2 concentrations in the liquid phase. Different scenarii may explain a build-up of H_2 in the rumen liquid phase: i) an increase in H_2 production with a constant H_2 use, ii/ a constant H_2 production with a lower H_2 use.

The first scenario may be applied in the case of an increase of DMI, a higher percentage of starch in diet or a lower feed frequency inducing the arrival of a large amount of feed in the rumen quickly fermented to H_2 . In that case, the rate of production of H_2 may overload the capacity of methanogens to use H_2 (Rooke et al., 2014), therefore resulting in H_2 emissions (Swainson et al., 2011). Conversely, the second scenario may be applied while using anti-methanogenic strategies such as nitrate. Nitrate was shown to reduce the quantity of methanogens (Van Zijderveld et al., 2011), which are consequently not sufficient enough to compensate for the arrival of H_2 following ingestion.

2.2. Consequences of hydrogen emissions

Hydrogen is an indirect GHG: it does not interact with solar and terrestrial radiations, but it perturbs the global distribution of important GHG such as CH_4 and ozone (O_3), by reacting with hydroxyl radicals. However, before considering H_2 emissions as a new source of pollution from ruminants, two factors have to be kept in mind. Firstly, despite some variations, H_2 emissions from ruminants remain at very low levels. Secondly, the GWP for H_2 is 5.8, which is much lower than CH_4 (GWP = 21) (Derwent et al., 2006).

CHAPTER 4: Influence of rumen protozoa on methane emissions from ruminants: a meta-analysis approach

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Abstract

A meta-analysis was conducted to evaluate the effects of protozoa concentration on methane emission from ruminants. A database was built from 59 publications reporting data from 76 *in vivo* experiments. The experiments included in the database recorded methane production and rumen protozoa concentration measured on the same groups of animals. Quantitative data such as diet chemical composition, rumen fermentation and microbial parameters, and qualitative information such as methane mitigation strategies were also collected. In the database, 31% of the experiments reported a concomitant reduction of both protozoa concentration and methane emission (g/kg dry matter intake). Nearly all of these experiments tested lipids as methane mitigation strategies. By contrast, 21% of the experiments reported a variation in methane emission without changes in protozoa numbers indicating that methanogenesis is also regulated by other mechanisms not involving protozoa. Experiments that used chemical compounds as an antimethanogenic treatment belonged to this group. The relationship between methane emission and protozoa concentration was studied with a variance-covariance model, with experiment as a fixed effect. The experiments included in the analysis had a within-experiment variation of protozoa concentration higher than $5.3 \log_{10}$ cells/ml corresponding to the average standard error of the mean of the database for this variable. To detect potential interfering factors for the relationship, the influence of several qualitative and quantitative secondary factors was tested. This meta-analysis showed a significant linear relationship between methane emission and protozoa concentration: CH_4 (g/kg dry matter intake) = $-30.7 + 8.14 \times \text{protozoa} (\log_{10} \text{ cells/ml})$ with 28 experiments (91 treatments), root mean square error = 1.94 and adjusted $R^2 = 0.90$. The proportion of butyrate in the rumen positively influenced the least square means of this relationship.

Keywords: methane, protozoa, meta-analysis, ruminant, volatile fatty acids

Implications

Our meta-analysis allows the effect of a variation in rumen protozoa concentration on methane emission to be quantified when protozoa ranged between 4.5 and $7.3 \log_{10}$ cells/ml. From selected experiments, a reduction of $0.12 \log_{10}$ protozoa cells/ml induced a significant reduction of 1g methane/kg dry matter intake. Among the experiments of the database, 31% reported a reduction of both protozoa concentration and methane emission, most of these using lipids. However, a reduction of methane emission with no change in protozoa was

reported in 21% of the experiments, showing that protozoa are not the only factor responsible for reduced methanogenesis.

Introduction

In the rumen, methanogens produce methane (CH₄) mainly from carbon dioxide (CO₂) and hydrogen (H₂) released during fermentation of feeds by bacteria, protozoa and fungi. Protozoa are involved in methanogenesis through their high production of butyrate (C₄) and acetate (C₂), two volatile fatty acids (VFA) whose biosynthesis liberates 2 and 4 moles of H₂ respectively, per mole of fermented glucose (Sauvant et al., 2011). Half of this H₂ is used by methanogens inside or in close association with protozoa cells, to produce CH₄ (Czerkawski, 1986; Williams and Coleman, 1992). Hence it was hypothesized that the reduction of rumen protozoa concentration might be an efficient way to decrease CH₄ emission (Finlay et al., 1994). Previous experiments testing experimental defaunation reported CH₄ reduction ranging from 13% to 35% *in vivo* (Hegarty, 1999; Morgavi et al., 2008; Morgavi et al., 2012) and from 9% to 25% *in vitro* (Newbold et al., 1995). However, the relationship between protozoa concentration and CH₄ emission is not precisely quantified. Preliminary work on a limited number of publications indicated that these two parameters were positively correlated (Morgavi et al., 2010). This finding prompted us to carry out a deeper analysis of the effects of a variation in protozoa concentration on CH₄ emission, by applying a meta-analysis approach with a variance-covariance model (Sauvant et al., 2008). To this end, we exhaustively gathered evidence from experiments reporting simultaneous measurements of CH₄ emission and rumen protozoa concentrations on the same groups of animals. To refine the study, we also tested the influence of qualitative and quantitative interfering factors for this relationship.

Material and methods

Selection of publications

We included in the database only publications reporting *in vivo* data of both CH₄ emission and rumen protozoa concentration measured on the same groups of animals. To find publications, bibliographical databases of editorial platforms (Web of Knowledge, ScienceDirect and Google Scholar) were interrogated, with methane, protozoa and ruminants as keywords. Unpublished experiments from our research group (INRA, UMR1213 Herbivores) were also added. Quantitative factors (intake, chemical composition of the diet, total tract digestibility,

rumen VFA concentrations, rumen pH, rumen bacteria and methanogen concentrations, and rumen sampling time relative to feeding time) were added to the database when available, with standard errors (s.e.) and statistical differences between treatments. Reported data of rumen parameters in kinetics were averaged. Qualitative factors (animal species, CH₄ mitigation strategies, and techniques for measuring CH₄ emission and protozoa concentration) were also collected. Publications using CH₄ emission calculated from equations instead of actual measures were excluded.

When relevant, treatments testing an additive or supplement were characterized by the main active compound in the additive (e.g. C18:1n-9 for rapeseed, diallyl disulfide for garlic, or tannin for *Quillaja saponaria*), by the quantity of the additive and of the main active compound in dry matter, and by the physical form of the additive (grain, powder, oil). For linseed, sunflower, rapeseed, soya, coconut and cottonseed, when the lipid values were not available, the quantity of the main fatty acid was calculated from tables of composition and nutritive value of raw ingredients (Sauvant et al., 2004). An experiment was defined as one control treatment and at least one experimental treatment testing one or several CH₄ mitigation strategies with the same basal diet. When relevant, one publication could supply different experiments, if controls were different. The final curated database contained 59 papers (number of experiments $N_{exp} = 76$, number of treatments $N_t = 219$) including 6 unpublished experiments ($N_t = 24$) from our research group. The list of published papers used is given in Supplementary material S1.

Coding of experiments

Experiments were first classified into four groups according to their CH₄ mitigation strategy. The group “biotechnology” ($N_{exp}=13$, $N_t=35$) consisted of experiments testing experimental defaunation, probiotics (*Lactobacillus*, *Propionibacterium*, *Saccharomyces*, *Trichosporon*), prebiotics (galacto-oligosaccharides) or exogenous microbial products (fibrolytic enzyme, secondary metabolites from *Monascus*). The group “additives” ($N_{exp}=26$, $N_t=64$) consisted of experiments testing chemical compounds (iodopropane, nitrate, sulfate), organic acids (malate, fumarate) or plants rich in tannin, saponin or essential oil (anacardic acid, diallyl disulfide, carvacrol, allyl isothiocyanate). The group “feed components” ($N_{exp}=25$, $N_t=74$) consisted of experiments testing lipids (C12:0, C14:0, C18:1n-9, C18:2n-6, C18:3n-3), forages (*Cichorium intybus*, *Lolium perenne*, *Trifolium repens*, *Trifolium pratense*, *Medicago sativa*, *Vigna unguiculata*) or cereal grains (wheat, maize, barley). The group “association” ($N_{exp}=12$, $N_t=46$) grouped experiments associating several strategies.

Experiments were further coded according to the distribution of the additive. Experiments with a “dose-response effect” tested different amounts of an additive (Nexp=41, Nt=105). Experiments with a “source effect” tested different sources of an active compound given at equal doses (e.g. the comparison between tannins originating from chestnut tree or acacia, Nexp=21, Nt=62). Experiments with a “form effect” tested different forms of an additive given at equal doses (e.g. the comparison between linseed fatty acids supplied as seed or oil, Nexp=2, Nt=6). Experiments testing experimental defaunation were considered as having a dose-response effect with protozoa as the active compound (Nexp=7, Nt= 17).

Experiments were then sorted into four classes according to their variations in CH₄ or protozoa: no variation of either parameters, variation in protozoa concentration only, variation in CH₄ emission only or variation in both parameters. Protozoa concentration was expressed as log₁₀ cells/ml, to ensure normal distribution of residues. Three experiments reporting protozoa concentration as proportion of protozoal 18S rDNA per total bacterial 16S rDNA, or as log gene copies of protozoal 18S rRNA/g of fresh matter, could not be used, as conversion to log₁₀ cells/ml was not possible. Methane emission were expressed in g per kg dry matter intake (DMI) to allow interpretation of data from animals with different levels of DM intake, i.e. large and small ruminants. Two papers had to be excluded, as DMI was not mentioned. Experiments were considered as reporting a significant variation in protozoa or CH₄ if the within-experiment variation of the parameter was respectively higher than one or two times the database average standard error of the mean (s.e.m.) for the parameter. The threshold levels for protozoa and CH₄ were then 5.3 log₁₀ cells/ml (2.2×10^5 /ml) and 1.1 g/kg DMI, respectively.

Statistical analysis

Description of the meta-design. The relationship between the four CH₄ mitigation strategies and their effects on protozoa concentration or CH₄ emission was assessed with three chi square tests. The effect of the following classes on CH₄ mitigation strategies was tested: (i) variation in protozoa vs. no variation in protozoa, (ii) variation in CH₄ vs. no variation in CH₄, and (iii) variation in protozoa and/or CH₄.

In addition, the relationship between quantitative factors (see further) and rumen protozoa concentration (log₁₀ cells/ml) or CH₄ emission (g/kg DMI) was examined to gain a better understanding of the effect of these factors on the relationship between CH₄ and protozoa. This analysis was performed using all the experiments in the database except for those testing defaunation, as they presented a high leverage effect. A one-way ANOVA was used to test

wether protozoa or CH₄ varied according to qualitative factors. In order to elucidate the relationship between protozoa or CH₄ and quantitative factors, various and complementary approaches were taken. Firstly, global correlation was calculated using all treatments irrespective of the experiment. Secondly, the between-experiment correlation was calculated using for each experiment, the mean of each factor and the mean of the protozoa concentration or of the CH₄ emission. Thirdly, the within-experiment correlation was calculated with a general linear model (GLM) using experiments with a reliable within-experiment variation of protozoa concentration or CH₄ emission:

$$Factor = \alpha + \alpha_i + \beta \times protozoa + \beta_i \times protozoa + e \quad [1]$$

$$Factor = \alpha + \alpha_i + \beta \times CH_4 + \beta_i \times CH_4 + e \quad [1']$$

where α = the overall intercept, α_i = the fixed effect of the experiment i on the overall intercept α , β = the overall slope, β_i = the fixed effect of the experiment i on the slope and e = the random residual error.

Response of CH₄ emission to a variation in rumen protozoa concentration. The average response law was sought using experiments that had a sufficient variation in rumen protozoa concentration between control and treatment (average within-experiment variation of 5.3 log₁₀ cells/ml). Five experiments using defaunated animals were excluded, as justified above. A GLM was applied to determine the relationship between CH₄ (g/kg DMI) and rumen protozoa concentration (log₁₀ cells/ml):

$$CH_4 = \alpha + \alpha_i + \beta \times protozoa + \beta_i \times protozoa + e \quad [2]$$

where α , α_i , β , β_i , and e were as defined in equation 1. A quadratic adjustment was also tested and compared with the linear one. The experiment effect was included in the model as a fixed factor. Given that quantitative and qualitative factors differed between experiments and that they were not documented for all treatments, one of the major aims of this work was to study and explain how these factors might affect the relationship between protozoa and CH₄ emission. Normality of residuals was tested (Anderson-Darling test) and normalized residuals were calculated. Treatments with high normalized residuals (Nout, less than -3 or greater than +3) were identified and discarded from the model as statistical outliers if they also had a high leverage effect based on Hi calculation and Cook distance (Sauvant et al., 2008).

Determination of factors influencing the response law. Potential interfering factors for the response of CH₄ to protozoa were investigated. The interfering quantitative factors tested were: intake level (g DMI/day per kg BW), total tract digestibility of organic matter (OM),

NDF, starch and CP (%), rumen total concentration of VFAs (mmol/l), proportions of C2, C4 and propionate (C3) (mol/100mol), C2/C3 and (C2+C4)/C3 ratios (mol/mol), pH and concentrations of bacteria and methanogens (cells/ml) in rumen fluid. The interfering qualitative factors tested were: method of CH₄ measurement (SF₆, chamber), CH₄ mitigation strategy (biotechnology, additives, feed components, association), animal species (large or small ruminants), method of distribution of the additive (dose-response, source, form) and rumen sampling time (before feeding, i.e. more than six hours after last feeding; after feeding, i.e. less than six hours after last feeding; and average of before and after feeding).

The influence of these factors on the response law of CH₄ to protozoa was tested in a three-step process as described previously (Loncke et al., 2009). The first step consisted in highlighting the interfering factors influencing the three parameters of the model: slopes, least square means (LSMeans) and residuals (i.e. the difference between observed CH₄ emission and emission predicted by the response law). A factor influencing the slopes or residuals may explain differences in variations of CH₄ emission between experiments for a similar variation in protozoa concentration. A factor influencing the LSMMeans may explain the differences in CH₄ emission between experiments for a same level of protozoa. Slopes and LSMMeans of each experiment used in the determination of the response law were calculated and their correlation with quantitative factors was tested. Residuals (observed minus predicted CH₄ emission) were calculated for all the treatments in the database, except for experiments testing defaunation, in order to ensure a normal distribution of the residuals. The relationship between residuals and quantitative factors was tested using the GLM procedure with experiment as a fixed factor:

$$\text{Residuals} = \alpha + \alpha_i + \beta \times \text{factor} + \beta_i \times \text{factor} + e \quad [3]$$

where α , α_i , β , β_i and e were as defined in equation 1. A reliable within-experiment response is achieved only with a minimal variation of the factor. Thus for each factor, the within-experiment variation was calculated and the experiments presenting the 25% lowest variations were not included in the GLM. The influence of qualitative factors on the model parameters was tested with a one-way ANOVA.

In the second step of the analysis, the significant interfering factors were included individually in equation 2. Quantitative factors were tested as additional covariable, either in substitution of the experiment effect (equation [4]) or in addition to the experiment effect (equation [4']):

$$CH_4 = \alpha + \beta \times \text{protozoa} + \gamma \times \text{factor} + e \quad [4]$$

$$CH_4 = \alpha + \alpha_i + \beta \times \text{protozoa} + \beta_i \times \text{protozoa} + \gamma \times \text{factor} + \gamma_i \times \text{factor} + e[4']$$

where α , α_i , β , β_i , and e were as defined in equation 1, γ = the linear term for the factor and γ_i = the fixed effect of the experiment i on the factor slope. This approach allows the identification of factors able to replace the experiment effect while explaining a part of the variability between experiments not explained by the model. Qualitative factors were added as fixed effects to the equation 2 with the experiment effect nested within the factor:

$$CH_4 = \alpha + \alpha_i(\text{factor}) + \beta \times \text{protozoa} + \text{factor} + \text{factor} \times \text{protozoa} + e \quad [5]$$

where α , β and e were as defined in equation 1 and α_i = the fixed effect of the experiment i (nested within the qualitative factor) on the overall intercept α . In a third step, significant interfering quantitative factors were included simultaneously in equation 2 to rank them in terms of how much they contributed to the relationship between CH_4 and protozoa.

At each step of the meta-analysis process, graphical observations were made to check the coherence of relationships, and to identify obviously abnormal values. All statistical analyses were carried out using Minitab, Version 16. Statistical significance was considered at $P \leq 0.05$ and a trend was declared at $P < 0.1$.

Results

Description of the meta-design

A summary of the main database parameters is given in Table 1. Information is presented separately for large and small ruminants, represented by 37 experiments with dairy and beef cattle and 39 experiments with sheep and goats. No statistical difference was observed between animal species for CH_4 emission (g/kg DMI, $P=0.707$; g/kg LW, $P=0.207$), intake level (g DMI/day per kg BW, $P=0.492$), gross energy of the diet (MJ/kg DM, $P=0.452$) or diet CP and OM content (g/kg DM, $P=0.103$ and $P=0.645$, respectively). In contrast, small ruminants had a more fibrous diet with a higher NDF content (g/kg DM, $P<0.001$) and a lower diet OM digestibility (% , $P=0.001$), inducing a higher proportion in the rumen of C2 and lower proportions of C3 and C4 (mol/100mol, $P<0.001$) than in large ruminants. Rumen protozoa concentration (\log_{10} cells/ml) tended to be lower in small than in large ruminants ($P=0.075$).

The CH_4 emission tended to be higher when expressed in g/kg digestible OM intake ($P=0.074$), and lower when expressed as a percentage of gross energy intake ($P=0.097$), in small compared to large ruminants. On these reduced datasets presenting measurements of OM digestibility or gross energy intake, CH_4 emission expressed in g/kg DMI did not differ between small and large ruminants ($P=0.899$ and $P=0.481$, respectively).

Table 1 Description of the complete database: methane emission, intake, diet composition and rumen parameters in large and small ruminants

	Large ruminants					Small ruminants					Species effect
	Nt	Mean	s.d.	Min	Max	Nt	Mean	s.d.	Min	Max	<i>P</i> -value
Methane emission (g/kg DMI)	96	18.7	6.4	2.4	36.3	115	19.0	5.7	7.9	40.5	0.707
Methane emission (g/kg DOMI)	49	27.9	10.2	3.7	51.9	72	30.7	6.9	13.0	50.9	0.074
Methane emission (g/kg LW)	73	0.44	0.21	0.09	1.17	104	0.48	0.23	0.10	1.39	0.207
Methane emission (% of GE intake)	78	5.99	1.68	2.30	10.80	67	5.50	1.86	2.36	10.41	0.097
Intake (g DMI/day per kg BW)	77	24.9	9.3	11.5	43.2	100	25.9	9.6	11.5	46.4	0.492
Dietary composition (g/kg DM)											
OM	69	916.0	30.0	800.0	966.0	62	913.4	33.3	804.0	949.0	0.645
NDF	77	367.0	89.4	169.0	671.0	97	441.1	83.4	239.0	678.0	<0.001
Starch	33	227.9	129.0	22.3	472.0	6	224.0	44.2	158.0	253.0	0.943
CP	81	155.3	33.0	59.0	230.0	101	146.6	37.2	25.1	256.0	0.103
Gross energy (MJ/kg DM)	44	18.4	1.2	16.7	21.7	46	18.5	1.0	16.4	20.0	0.578
Concentrate: Forage (%)	92	46.2	16.6	0.0	90.0	117	23.5	26.0	0.0	83.0	<0.001
OM total tract digestibility (%)	59	69.2	5.5	52.0	83.0	68	64.1	10.6	39.9	83.3	0.001
Rumen parameters											
Protozoa (log ₁₀ cells/ml)	100	5.58	0.80	0.00	6.80	107	5.22	1.86	0.00	7.31	0.075
Total VFA (mmol/l)	85	108.0	25.0	44.7	165.3	112	90.9	31.6	22.9	171.1	<0.001
C2 (mol/100mol)	89	62.1	4.8	48.2	74.3	112	69.5	4.4	60.3	79.1	<0.001
C3 (mol/100mol)	89	21.8	4.2	12.2	36.0	112	18.4	4.1	9.5	27.5	<0.001
C4 (mol/100mol)	89	11.5	2.0	6.7	16.1	112	8.9	2.3	5.4	16.0	<0.001
C2/C3	89	2.99	0.79	1.34	6.07	112	4.02	1.20	2.34	8.22	<0.001
(C2+C4)/C3	89	3.54	0.91	1.54	6.89	112	4.54	1.35	2.54	9.35	<0.001
pH	81	6.40	0.40	5.06	7.33	92	6.60	0.33	5.66	7.16	<0.001

Min = Minimum; Max = Maximum; Nt = number of treatments; DMI = dry matter intake; LW = live weight; GE = gross energy; DOMI = digestible organic matter intake; OM = organic matter; VFA = volatile fatty acids; C2 = acetate; C3 = propionate; C4 = butyrate.

To measure CH₄ emission, 31 experiments (Nt=88) used the SF₆ tracer technique and 45 experiments (Nt=131) used open or closed chambers. Protozoa concentration was determined from rumen samples taken before feeding (Nexp=19, Nt=64), after feeding (Nexp=34, Nt=89) and both before and after feeding (Nexp=17, Nt=47). This information was unclear or not reported in six experiments (Nt=19). To determine protozoa concentrations, counting chambers were used in 70 experiments (Nt=201) and six experiments (Nt=18) used qPCR. Information on CH₄ emission (g/kg DMI) and protozoa concentration (log₁₀ cells/ml) was collected for 70 experiments (Nt=198). The distribution of these experiments according to their variation in CH₄ or protozoa is presented in Table 2.

Table 2 Number of experiments without or with significant within-experiment variation of protozoa concentration (log₁₀ cells/ml) or methane emission (g/kg dry matter intake)

	No protozoa variation		Protozoa variation	
	No CH ₄ variation	CH ₄ variation	No CH ₄ variation	CH ₄ variation
Biotechnology				
Defaunation	2	0	3	2
Pro/Prebiotics, Microbial products	2	3	1	0
Additives				
Chemicals	0	2	0	0
Organic acids	2	2	0	0
Plant extracts				
Tannins	1	0	2	2
Saponins	5	1	2	0
Essentials oil	1	3	0	1
Feed components				
Lipids	1	4	1	10
Forages	5	0	1	1
Concentrates	0	0	1	1
Association	0	0	3	5

The chi square tests showed that no variation in protozoa was mostly observed in experiments from the “additives” strategy, whereas all the experiments in the “association” strategy reported variation in protozoa ($P=0.004$). Conversely, if the effect on protozoa was not considered, no specific strategy affected CH₄ emission ($P=0.376$). Looking simultaneously at their effects on protozoa concentration and/or CH₄ emission, strategies were statistically related to specific effects on these parameters ($P=0.032$). No variation in either protozoa or CH₄ was observed in 19 experiments, in particular those testing different forages. Conversely, 15 experiments reported a variation in CH₄ with no variation in protozoa, mostly experiments

testing chemicals or essential oils. Fourteen experiments reported a reduction of protozoa concentration with no change in CH₄ emission. A reduction of protozoa concentration was associated with a reduction of CH₄ emission in 22 experiments. Experiments testing tannins and lipids were the most numerous in this last group.

Table 3 reports the correlations between rumen protozoa concentration and quantitative factors. With a global analysis approach, rumen protozoa were negatively correlated to OM and CP total tract digestibility ($P=0.001$), rumen total VFA concentration ($P=0.001$), C3 proportion ($P<0.001$) and bacteria concentration ($P=0.005$). Using the same approach, rumen protozoa concentration was positively correlated to rumen pH ($P=0.019$), proportion of C2 ($P<0.001$) and the ratios C2/C3 and (C2+C4)/C3 ($P<0.001$). Similar trends were observed with the between-experiment analysis approach, except for rumen pH and bacteria which were no longer correlated to protozoa. With the within-experiment approach, intake, NDF digestibility, rumen proportion of C2 and the ratios C2/C3 and (C2+C4)/C3 were positively correlated with protozoa ($P<0.001$, $P=0.018$, $P=0.009$, $P=0.047$ and $P=0.039$, respectively) whereas rumen proportion of C3 was again negatively correlated to this parameter ($P=0.003$). In none of these approaches were rumen proportion of C4 and number of methanogens correlated to protozoa. The rumen protozoa were significantly affected by animal species, CH₄ mitigation strategy and rumen sampling time ($P=0.027$, $P=0.031$, $P=0.006$, respectively; data not shown) and a tendency was observed with the method of distribution (dose-response, source, form) of the additive ($P=0.061$; data not shown).

Table 4 reports the correlations between CH₄ emission and quantitative factors. With a global approach, CH₄ emissions were negatively correlated with intake ($P=0.016$), C3 proportion ($P<0.001$) and rumen methanogens ($P=0.012$) and positively correlated with OM and NDF digestibility ($P<0.001$ and $P=0.002$), C2 and C4 proportions ($P=0.012$ and $P<0.001$), C2/C3 and (C2+C4)/C3 ratios ($P=0.007$ and $P=0.001$), rumen pH ($P<0.001$) and bacteria concentration ($P=0.017$). With the between-experiment approach, OM digestibility, C4 proportion and rumen pH were also positively correlated with CH₄ ($P=0.008$, $P=0.030$ and $P=0.013$, respectively) and C3 proportion and methanogen concentration were negatively correlated with CH₄ ($P=0.009$ and $P=0.016$). The within-experiment approach yielded the same information as the between-experiment approach, except that C2 proportion was positively correlated with CH₄ ($P<0.001$) unlike C4 proportion ($P=0.169$). Methane emission was not significantly affected by animal species, CH₄ mitigation strategy, CH₄ method of measurement or the method of distribution of the additive (dose-response, source, form) ($P=0.131$, $P=0.431$, $P=0.084$, $P=0.331$, respectively; data not shown).

Table 3 Global correlation, between and within-experiment (equation 1) relationship between rumen protozoa concentration and quantitative factors

Quantitative factors	Rumen protozoa concentration (log ₁₀ cells/ml)									
	Global			Between experiment			Within-experiment			
	Nt	r	P-value	Nexp	r	P-value	Nexp	Nt	Slope	P-value
Intake (g DMI/day per kg BW)	151	-0.068	0.405	55	-0.079	0.564	27	83	2.618	<0.001
Total tract digestibility (%)										
OM	125	-0.299	0.001	43	-0.326	0.033	21	68	1.254	0.382
NDF	125	-0.032	0.725	45	-0.089	0.563	18	57	4.305	0.018
Starch	31	-0.221	0.233	10	-0.253	0.480	4	13	-0.537	0.355
CP	71	-0.495	<0.001	28	-0.561	0.002	11	32	-0.009	0.997
Rumen parameters										
Total VFA (mmol/l)	164	-0.249	0.001	57	-0.243	0.068	25	80	-0.372	0.921
C2 (mol/100mol)	168	0.365	<0.001	59	0.361	0.005	26	82	2.310	0.009
C3 (mol/100mol)	168	-0.435	<0.001	59	-0.452	<0.001	26	82	-2.432	0.003
C4 (mol/100mol)	168	-0.035	0.656	59	-0.062	0.643	26	82	0.665	0.159
C2/C3	168	0.462	<0.001	59	0.528	<0.001	26	82	0.426	0.047
(C2+C4)/C3	168	0.460	<0.001	59	0.525	<0.001	26	82	0.512	0.039
pH	154	0.188	0.019	54	0.217	0.116	23	74	0.116	0.065
Methanogens (cells/ml)	28	0.117	0.555	12	-0.010	0.975	3	8	4.090	0.717
Bacteria (cells/ml)	67	-0.340	0.005	22	-0.320	0.146	13	45	-6.200	0.725

Nexp = number of experiments; Nt = number of treatments; DMI = dry matter intake; OM = organic matter; VFA = volatile fatty acids; C2 = acetate; C3 = propionate; C4 = butyrate.

Table 4 Global correlation, between and within-experiment (equation 1') relationship between methane emission and quantitative factors

Quantitative factors	Methane emission (g/kg DMI)									
	Global			Between experiment			Within-experiment			
	Nt	r	P-value	Nexp	r	P-value	Nexp	Nt	Slope	P-value
Intake (g DMI/day per kg BW)	159	-0.191	0.016	59	-0.196	0.138	34	99	0.038	0.382
Total tract digestibility (%)										
OM	121	0.369	<0.001	42	0.404	0.008	21	69	0.292	0.002
NDF	121	0.278	0.002	44	0.269	0.077	22	65	0.356	0.001
Starch	31	0.082	0.661	10	0.019	0.958	7	25	0.053	0.809
CP	67	0.085	0.492	27	0.162	0.420	12	32	-0.176	0.285
Rumen parameters										
Total VFA (mmol/l)	172	0.018	0.819	61	0.008	0.951	33	102	0.046	0.879
C2 (mol/100mol)	176	0.188	0.012	63	0.174	0.171	35	106	0.280	<0.001
C3 (mol/100mol)	176	-0.333	<0.001	63	-0.328	0.009	35	106	-0.312	<0.001
C4 (mol/100mol)	176	0.269	<0.001	63	0.274	0.030	35	106	0.049	0.169
C2/C3	176	0.204	0.007	63	0.188	0.139	35	106	0.075	<0.001
(C2+C4)/C3	176	0.238	0.001	63	0.225	0.077	35	106	0.087	<0.001
pH	160	0.293	<0.001	57	0.328	0.013	28	84	0.004	0.399
Methanogens (cells/ml)	28	-0.468	0.012	12	-0.673	0.016	9	22	0.262	0.097
Bacteria (cells/ml)	67	0.291	0.017	22	0.373	0.088	10	34	-0.770	0.910

Nexp = number of experiments; Nt = number of treatments; DMI = dry matter intake; OM = organic matter; VFA = volatile fatty acids; C2 = acetate; C3 = propionate; C4 = butyrate.

Effects of a variation of rumen protozoa concentration on CH₄ emission

The within-experiment relationship between rumen protozoa concentration and CH₄ emission is presented in Figure 1. When protozoa concentration ranged between 4.5 and 7.3 log₁₀ cells/ml (0.3 and 206×10⁵ cells/ml), the response law relating CH₄ emission (g/kg DMI) to rumen protozoa concentration (log₁₀ cells/ml) was linear (equation 2):

$$\text{CH}_4 = -30.74 (\text{s.e. } 5.09)^{***} + 8.14 (\text{s.e. } 0.85)^{***} \times \text{protozoa}$$

Where Nt = 91, Nexp = 28, residual mean square error (r.m.s.e.) = 1.94, R² = 0.93, adjusted R² = 0.90 and Nout = 0.

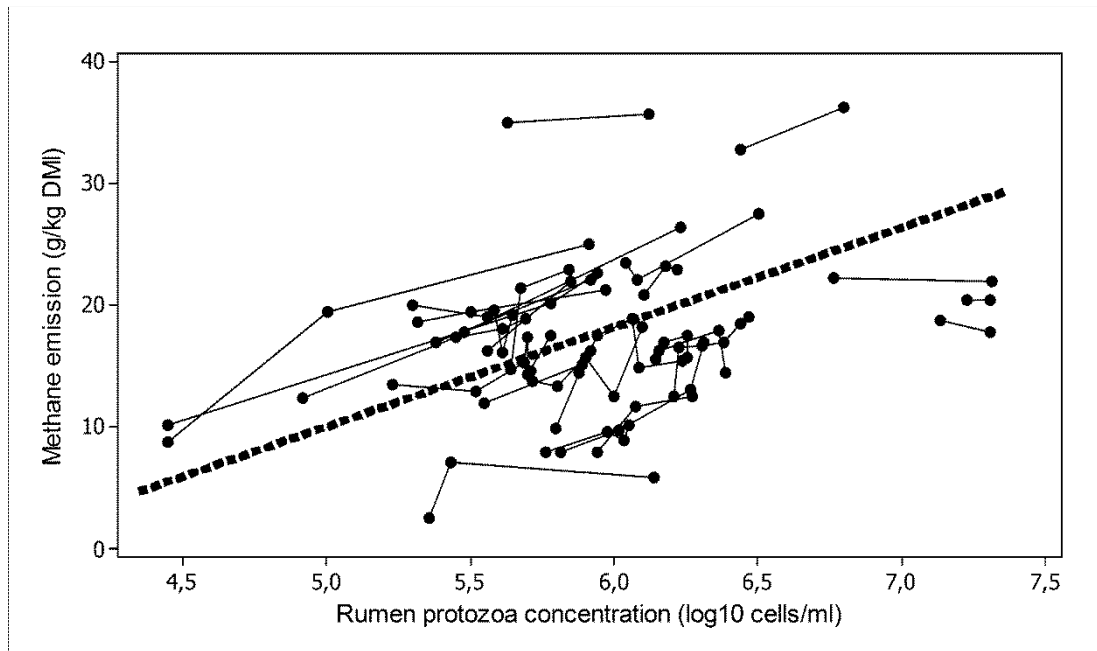


Figure 1 Relationship between methane emission and rumen protozoa concentration (raw data). The black dashed line represents the average within-experiment relationship (equation 2).

Effects of interfering factors for the response law relating CH₄ to protozoa

Table 5 presents the correlations between slopes and LSMeans of experiments from equation 2 with quantitative factors. One experiment presenting a very high slope value had to be excluded to get a normal distribution of slopes ($P=0.210$) and LSMeans ($P=0.141$). The digestibility of OM and CP and the rumen proportion of C4 were positively correlated to LSMeans ($P=0.013$, $P<0.001$ and $P=0.017$, respectively) and slopes were correlated with intake ($P=0.018$) and CP digestibility ($P=0.016$). No other significant correlation was observed.

Table 5 Correlations between slopes and LSMeans of experiments from equation 2 with quantitative factors

Quantitative factors	Nexp	Slope		LSMeans	
		r	P-value	r	P-value
Intake (g DMI/day per kg BW)	26	-0.460	0.018	-0.320	0.111
Total tract digestibility (%)					
OM	20	0.113	0.635	0.544	0.013
NDF	17	0.151	0.564	-0.080	0.759
Starch	4	-0.126	0.874	0.657	0.343
CP	10	0.731	0.016	0.911	<0.001
Rumen parameters					
Total VFA (mmol/l)	25	-0.369	0.069	0.062	0.770
C2 (mol/100mol)	25	-0.153	0.465	-0.122	0.560
C3 (mol/100mol)	25	0.192	0.357	0.003	0.988
C4 (mol/100mol)	25	0.021	0.919	0.474	0.017
C2/C3	25	-0.257	0.216	-0.196	0.348
(C2+C4)/C3	25	-0.259	0.211	-0.158	0.450
pH	22	-0.096	0.670	-0.083	0.712
Methanogens (cells/ml)	3	0.890	0.301	-0.753	0.458
Bacteria (cells/ml)	13	0.161	0.600	-0.183	0.550

Nexp = number of experiments; LSMeans = least square means; DMI = dry matter intake; OM = organic matter; VFA = volatile fatty acids; C2 = acetate; C3 = propionate; C4 = butyrate.

Table 6 gives the within-experiment correlation between quantitative factors and residuals determined from equation 2 for all the experiments in the database. The distribution of calculated residuals did not significantly differ from normality ($P=0.054$). They were positively correlated to rumen proportion of C2 ($P=0.008$) and the ratios C2/C3 and (C2+C4)/C3 ($P<0.001$) and negatively correlated to rumen proportion of C3 ($P=0.013$). No qualitative factors influenced slopes or LSMeans but residuals were influenced by method of CH₄ measurement, CH₄ mitigation strategy, distribution of additive, animal species and rumen sampling time ($P=0.003$, $P=0.021$, $P=0.003$, $P=0.018$ and $P=0.006$, respectively; data not shown).

Table 6 Within-experiment relationship between the residuals (observed CH₄ minus predicted CH₄ with equation 2) and quantitative factors (equation 3)

Quantitative factors	Var	Nexp	Nt	Nout	Intercept (s.e.)	<i>P</i> -value	Slope (s.e.)	<i>P</i> -value	r.m.s.e.	R ²
Intake (g DMI/day per kg BW)	0.3	41	119	2	13.5 (5.60)	0.019	-0.402 (0.213)	0.062	2.970	0.88
Total tract digestibility (%)										
OM	1.8	32	94	3	-10.7 (7.49)	0.157	0.202 (0.113)	0.078	3.118	0.86
NDF	2.3	31	92	3	-1.2 (4.73)	0.806	0.096 (0.085)	0.265	3.206	0.80
Starch	0.3	8	27	0	-9.5 (31.75)	0.769	0.123 (0.337)	0.719	4.205	0.68
CP	1.2	20	50	2	5.2 (5.44)	0.350	-0.029 (0.090)	0.752	2.492	0.91
Rumen parameters										
Total VFA (mmol/l)	4.0	43	128	4	2.3 (3.48)	0.514	0.009 (0.035)	0.800	3.131	0.88
C2 (mol/100mol)	1.1	43	126	1	-23.2 (9.97)	0.023	0.420 (0.154)	0.008	3.183	0.87
C3 (mol/100mol)	1.1	43	125	1	12.4 (3.65)	0.001	-0.436 (0.172)	0.013	3.325	0.84
C4 (mol/100mol)	0.7	46	135	3	3.4 (2.96)	0.257	0.020 (0.283)	0.945	3.147	0.86
C2/C3	0.2	43	123	1	-5.3 (2.37)	0.028	2.544 (0.667)	<0.001	3.086	0.88
(C2+C4)/C3	0.3	42	121	1	-5.5 (2.44)	0.027	2.216 (0.603)	<0.001	3.164	0.87
pH	0.1	40	119	3	6.6 (17.62)	0.709	-0.466 (2.717)	0.864	3.219	0.87
Methanogens (cells/ml)	0.2	10	23	0	1.1 (1.92)	0.582	0.756 (0.370)	0.064	3.435	0.81
Bacteria (cells/ml)	1.0	14	48	1	0.7 (0.93)	0.485	-0.002 (0.004)	0.647	2.777	0.92

Var = minimum within-experiment variation level of the tested factor; Nexp = number of experiments; Nt = number of treatments; Nout = number of outliers; s.e. = standard error; r.m.s.e. = residual mean square error; DMI = dry matter intake; OM = organic matter; VFA = volatile fatty acids; C2 = acetate; C3 = propionate; C4 = butyrate.

Table 7 Relationship between methane emission (g/kg dry matter intake) and rumen protozoa concentration (log₁₀ cells/ml) with quantitative factors in addition (equation 4') or in substitution (equation 4) of the experiment effect

Quantitative factors	Nexp	Nt	Nout	Intercept (s.e.)	P-value	Protozoa		Factor		r.m.s.e	R ²
						Slope (s.e.)	P-value	Slope (s.e.)	P-value		
Equation 2	28	91	0	-30.7 (5.1)	<0.001	8.14 (0.85)	<0.001	-	-	1.94	0.93
With experiment effect (equation 4')											
Intake (g DMI/day per kg BW)	24	74	0	-28.3 (6.5)	<0.001	8.51 (0.99)	<0.001	-0.202 (0.223)	0.371	2.02	0.94
OM digestibility (%)	18	59	0	-38.7 (7.8)	0.001	8.14 (0.87)	<0.001	0.101 (0.098)	0.309	1.82	0.92
CP digestibility (%)	10	30	0	-15.0 (9.5)	0.131	8.12 (1.12)	<0.001	-0.273 (0.108)	0.020	1.61	0.89
C2 (mol/100mol)	23	73	0	-42.9 (10.3)	<0.001	5.97 (0.93)	<0.001	0.376 (0.164)	0.027	1.68	0.95
C3 (mol/100mol)	23	73	1	-0.7 (7.2)	0.918	4.98 (0.90)	<0.001	-0.578 (0.142)	<0.001	1.53	0.96
C4 (mol/100mol)	23	73	0	-24.0 (5.3)	<0.001	6.13 (0.91)	<0.001	0.527 (0.226)	0.024	1.68	0.95
C2/C3	23	73	1	-24.3 (4.8)	<0.001	5.50 (0.86)	<0.001	2.437 (0.619)	<0.001	1.54	0.96
(C2+C4)/C3	23	73	1	-23.6 (4.8)	<0.001	5.39 (0.85)	<0.001	2.110 (0.515)	<0.001	1.53	0.96
Without experiment effect (equation 4)											
Intake (g DMI/day per kg BW)	-	74	1	-7.1 (8.1)	0.385	5.01 (1.38)	0.001	-0.229 (0.099)	0.023	6.11	0.18
OM digestibility (%)	-	59	1	-30.4 (8.0)	<0.001	5.16 (1.00)	<0.001	0.253 (0.059)	<0.001	4.22	0.38
CP digestibility (%)	-	30	0	-30.6 (11.7)	0.015	5.67 (1.27)	<0.001	0.247 (0.076)	0.003	3.00	0.43
C2 (mol/100mol)	-	73	1	-8.2 (9.0)	0.362	4.19 (1.54)	0.008	0.009 (0.115)	0.937	6.07	0.12
C3 (mol/100mol)	-	73	1	1.04 (11.2)	0.927	3.33 (1.57)	0.038	-0.182 (0.156)	0.245	6.01	0.14
C4 (mol/100mol)	-	73	2	-21.9 (8.2)	0.010	4.77 (1.25)	<0.001	1.093 (0.274)	<0.001	5.48	0.28
C2/C3	-	73	1	-9.2 (8.8)	0.303	4.58 (1.65)	0.007	-0.214 (0.570)	0.709	6.06	0.12
(C2+C4)/C3	-	73	1	-8.0 (8.8)	0.368	4.25 (1.65)	0.012	-0.002 (0.530)	0.996	6.07	0.12

Nexp = number of experiments; Nt = number of treatments; Nout = number of outliers; s.e. = standard error; r.m.s.e. = residual mean square error; DMI = dry matter intake; OM = organic matter; C2 = acetate; C3 = propionate; C4 = butyrate.

None of the qualitative factors appeared significant when included in the model with the experiment effect nested within the factor (equation 5, data not shown). Table 7 shows the response law relating CH₄ to protozoa with significant quantitative factors added to or substituted for the experiment effect. Added to experiment effect, CP digestibility and C3 proportion were negatively correlated to CH₄ emission ($P=0.020$ and $P<0.001$), whereas C2 and C4 proportions and the ratios C2/C3 and (C2+C4)/C3 were positively correlated to CH₄ emission ($P=0.027$, $P=0.024$, $P<0.001$ and $P<0.001$, respectively). Substituted for experiment effect, OM and CP digestibility and rumen C4 proportion were positively correlated to CH₄ emission ($P<0.001$, $P=0.003$ and $P<0.001$, respectively), whereas intake was negatively correlated to CH₄ emission ($P=0.023$). When simultaneously including these four quantitative factors in equation 2 together with protozoa concentration (data not shown), protozoa concentration ($P=0.028$) and C4 proportion ($P=0.018$) explained 39% and 48% of the variability, experiment effect excluded. Intake and digestibility of OM and CP digestibility were not significant.

Discussion

The database was well-balanced for animal species, with almost the same number of treatments between small and large ruminants. A confounding effect between diet composition and animal species was noteworthy, with large ruminants having a diet richer in energy than small ruminants. This led to differences in rumen fermentation profiles, with lower proportion of C2 and higher proportion of C3 in large ruminants. However, CH₄ emission (expressed in g/kg DMI or g/kg LW) and protozoa concentration (log₁₀ cells/ml) were homogeneously distributed between animal species. Consequently, it appears unlikely that any potential animal species effect would be revealed in further analyses.

Influence of CH₄ mitigation strategy on CH₄ and protozoa

Although the database was not built to evaluate mitigation strategies for their effect on CH₄ emission and rumen protozoa, the chi square tests highlighted that most experiments testing lipids or tannins reduced both protozoa concentration and CH₄ emission. This information confirmed that a potential mode of action of these compounds on methanogenesis is through protozoal inhibition. These additives may change protozoa membrane permeability, leading to cell lysis (Doreau and Ferlay, 1995; Goel et al., 2005). As reported in a previous review, the effect of these compounds is variable depending on the source, the mode and the length of administration (Popova et al., 2011). This could explain the variability of the effects of these

additives on protozoa concentration. As an example, lipid effect on protozoa is dependent on the fatty acid profile, with a higher effect of medium chain fatty acids than polyunsaturated ones, which was confirmed by our data: lauric acid tended to reduce protozoa more markedly than polyunsaturated fatty acids (Jordan et al., 2006).

Defaunation studies did not necessarily observe a reduction of CH₄ emission. Difference in diets may explain this variable effect as removal of protozoa has a more marked effect on methanogenesis with high concentrate diets (Hegarty, 1999). However, this effect was not clearly seen in our database, as two out of four experiments reporting no variation in CH₄ emission after defaunation used a diet with 83% of concentrate. Conversely, in the two experiments showing a reduction of CH₄ emission after defaunation, animals were fed a diet with more than 60% of concentrate.

Chemicals, essential oils and organic acids were identified as methanogenesis reducers without affecting protozoa. Two different mechanisms can be pointed out for these additives. On the one hand, some essential oils are known to directly inhibit growth and activity of methanogens inducing a direct reduction of CH₄ emission (Calsamiglia et al., 2007). On the other hand, some chemicals and organic acids divert H₂ from methanogenesis to other pathways. For example, nitrate and sulfate are reduced to ammonia and hydrogen sulfide, respectively, with the consumption of four moles of H₂ (Ungerfeld and Kohn, 2006). Enhancing C₃ synthesis with malate or fumarate, which are precursors of C₃, is another way to divert H₂ from methanogenesis (Ungerfeld et al., 2007). However, experiments testing organic acids in our database were inconclusive, as already reported in a previous review (Hook et al., 2010). A part of added fumarate may be used for C₂ production, balancing the effect on C₃ production (Ungerfeld et al., 2007).

Finally, in our dataset, forage modification, addition of probiotics, prebiotics or exogenous microbial products had a weak influence on protozoa concentration, while their effect on CH₄ emission was variable. The mechanisms of action of these additives on CH₄ emission remain to be clarified. Probiotics and prebiotics may either enhance specific microbial groups able to use excess H₂ for C₃ synthesis, or stimulate microbial growth leading to a higher H₂ consumption for microbial biomass synthesis (Jeyanathan et al., 2014). However, in one experiment testing probiotics, CH₄ yield was reduced by 25% with no changes in ruminal fermentation and protozoa (Lettat, 2012).

Effects of a variation in protozoa concentration on CH₄ emission

To our knowledge, only one publication has established a quantitative relationship between numbers of protozoa and CH₄ emission (Morgavi et al., 2010). In that work using a dataset of 21 experiments, the number of protozoa explained 47% of the variability in CH₄ emission (r.m.s.e. = 3.25). Methane was reduced by 1g CH₄/kg DMI by every decrease of 0.12 log₁₀ protozoa cells/ml. In agreement with these findings, we showed that rumen protozoa concentration explained 93% of the variability in CH₄ emission, and that a reduction of 0.12 log₁₀ protozoa cells/ml induced a reduction of 1g CH₄/kg DMI (r.m.s.e. = 1.94). Our analysis is more reliable than the previous work as it included seven additional experiments and presented a lower r.m.s.e. In addition, our approach distinguished between intra and inter-experiment effects, and focused more specifically on experiments with a significant within-experiment variation of protozoa concentration. The equation 2 can be used to quantify with a good accuracy the impact of changes in protozoa concentration (in the range 4.5-7.3 log₁₀ cells/ml) on CH₄ emission in the wide diversity of intake level and diet composition defined by the meta-design. However, the significant experiment effect implies that this equation cannot accurately estimate the absolute CH₄ emission from a measured protozoa concentration. Consequently, the study of interfering factors is required.

Interfering factors for the response law relating CH₄ to protozoa

One aim of this study was to improve our understanding of the relationship between CH₄ and protozoa by testing different quantitative and qualitative potential interfering factors. A reliable interfering factor can be accepted if its inclusion into the response law does not lead to a large variation in the initial equation slope (protozoa linear term). When including the experiment effect, the slopes associated with CP digestibility, VFA proportions and the ratios C2/C3 and (C2+C4)/C3 were significant, but the r.m.s.e. of the overall equations were only slightly improved. However, the change in the mean slope (or its s.e.) associated with protozoa demonstrated confounding effects between quantitative interfering factors and experiment effect. A positive relationship between the C2/C3 ratio and CH₄ emission has already been quantified by a meta-analysis approach (Sauvant et al., 2011). With the present database, a similar relationship was observed ($P < 0.05$, data not shown), and the residuals of this relationship were evidenced to be positively correlated to rumen protozoa ($P < 0.001$, data not shown).

When substituting for the experiment effect, intake, OM and CP digestibility, and rumen proportion of C4 significantly influenced the response law relating CH₄ to protozoa, but

strongly modified the slope associated with protozoa, and markedly increased the r.m.s.e. This result shows that taking into account experimental effects provides the most precise estimate of the influence of protozoa on CH₄ production.

When simultaneously adding intake, OM and CP digestibility and rumen proportion of C4 in equation 2, C4 proportion was the main interfering quantitative factor, with a strong contribution to the explained variability. It is known that protozoa preferentially ferment OM to C4 rather than to C2 or C3 (Williams and Coleman, 1992; Brossard et al., 2004). Surprisingly, in our database, we did not find any significant relationship between protozoa and C4, showing that C4 concentration cannot be considered as a direct indicator of rumen protozoa activity. Other microbial populations may be responsible for C4 production, such as *Butyrivibrio fibrisolvens* (Stewart et al., 1997). Unfortunately, our database contained limited information about quantity or diversity of other rumen microbes, precluding further analyses. The response law relating CH₄ to protozoa is independent of qualitative factors such as method of CH₄ measurement, animal species or CH₄ mitigation strategy. No effect of mitigation strategies was observed on the relationship between protozoa and CH₄, as only experiments showing a relevant within-experiment variation of protozoa concentration were included in the analysis, which strongly oriented the selection towards experiments testing lipids (nearly half of the eligible experiments).

Conclusion

By building an exhaustive database from experiments with data for CH₄ emission and rumen protozoa concentration on the same groups of animals, we showed that a reduction of protozoa concentration was in most cases indicative of a reduction of CH₄ emission. We also quantitatively assessed the effect of a variation in protozoa concentration on CH₄ emission. We showed that this relationship was positively influenced by the proportion of butyrate in the rumen.

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References

Brossard L, Martin C, Chaucheyras-Durand F and Michalet-Doreau B 2004. Protozoa involved in butyric rather than lactic fermentative pattern during latent acidosis in sheep. *Reproduction Nutrition Development* 44, 195-206.

- Calsamiglia S, Busquet M, Cardozo PW, Castillejos L and Ferret A 2007. Invited review: essential oils as modifiers of rumen microbial fermentation. *Journal of Dairy Science* 90, 2580-2595.
- Czerkawski JW 1986. An introduction to rumen studies. Pergamon Press, Oxfordshire and New York, USA.
- Doreau M and Ferlay A 1995. Effect of dietary lipids on nitrogen metabolism in the rumen: a review. *Livestock Production Science* 43, 97-110.
- Finlay BJ, Esteban G, Clarke KJ, Williams AG, Embley TM and Hirt RP 1994. Some rumen ciliates have endosymbiotic methanogens. *FEMS Microbiology Letters* 117, 157-161.
- Goel G, Puniya A, Aguilar C and Singh K 2005. Interaction of gut microflora with tannins in feeds. *Naturwissenschaften* 92, 497-503.
- Hegarty RS 1999. Reducing rumen methane emissions through elimination of rumen protozoa. *Australian Journal of Agricultural Research* 50, 1321-1327.
- Hook SE, Wright ADG and McBride BW 2010. Methanogens: methane producers of the rumen and mitigation strategies. *Archaea* 2010, 1-11.
- Jeyanathan J, Martin C and Morgavi DP 2014. The use of direct-fed microbials for mitigation of ruminant methane emissions: a review. *Animal* 8, 250-261.
- Jordan E, Lovett DK, Monahan FJ, Callan J, Flynn B and O'Mara FP 2006. Effect of refined coconut oil or copra meal on methane output and on intake and performance of beef heifers. *Journal of Animal Science* 84, 162-170.
- Lettat A 2012. Efficacité et mode d'action des bactéries propioniques et/ou lactiques pour prévenir l'acidose latente chez le ruminant. Thesis PhD, Blaise Pascal University, Clermont Ferrand, France.
- Loncke C, Ortigues-Marty I, Vernet J, Lapierre H, Sauvant D and Nozière P 2009. Empirical prediction of net portal appearance of volatile fatty acids, glucose, and their secondary metabolites (b-hydroxybutyrate, lactate) from dietary characteristics in ruminants: A meta-analysis approach. *Journal of Animal Science* 87, 253-268.
- Morgavi DP, Jouany JP and Martin C 2008. Changes in methane emission and rumen fermentation parameters induced by refaunation in sheep. *Australian Journal of Experimental Agriculture* 48, 69-72.
- Morgavi DP, Forano E, Martin C and Newbold CJ 2010. Microbial ecosystem and methanogenesis in ruminants. *Animal* 4, 1024-1036.

- Morgavi DP, Martin C, Jouany JP and Ranilla MJ 2012. Rumen protozoa and methanogenesis: not a simple cause-effect relationship. *British Journal of Nutrition* 107, 388-397.
- Newbold CJ, Lassalas B and Jouany JP 1995. The importance of methanogens associated with ciliate protozoa in ruminal methane production in vitro. *Letters in Applied Microbiology* 21, 230-234.
- Popova M, Morgavi DP, Doreau M and Martin C 2011. Production de méthane et interactions microbiennes dans le rumen. *INRA Productions Animales* 24, 447-460.
- Sauvant D, Perez J and Tran G 2004. Tables de composition et de valeur nutritive des matières premières destinées aux animaux d'élevage. INRA Editions, Paris, France.
- Sauvant D, Schmidely P, Daudin JJ and St-Pierre NR 2008. Meta-analyses of experimental data in animal nutrition. *Animal* 2, 1203-1214.
- Sauvant D, Giger-Reverdin S, Serment A and Broudiscou L 2011. Influences des régimes et de leur fermentation dans le rumen sur la production de méthane par les ruminants. *INRA Productions Animales* 24, 433-446.
- Stewart CS, Flint HJ and Bryant MP 1997. The rumen bacteria. In *The rumen microbial ecosystem* (eds PN Hobson and CS Stewart), pp. 10-72. Blackie Academic & professional, London, United Kingdom.
- Ungerfeld EM and Kohn RA 2006. The role of thermodynamics in the control of ruminal fermentation. In *Ruminant physiology: Digestion, metabolism and impact of nutrition on gene expression, immunology and stress* (eds K Sejrsen, T Hvelplund and MO Nielsen), pp. 55-85. Wageningen Academic Publishers, Wageningen, The Netherlands.
- Ungerfeld EM, Kohn RA, Wallace RJ and Newbold CJ 2007. A meta-analysis of fumarate effects on methane production in ruminal batch cultures. *Journal of Animal Science* 85, 2556-2563.
- Williams AG and Coleman GS 1992. *The rumen protozoa*. Springer-Verlag, New York, USA.

Experimental strategy & materials and methods

Table 10 Steps and associated experiments conducted during the PhD thesis and justification for selection of animals and dietary treatments

Steps	Associated experiment (approach)	Animal type Number Experimental design	Dietary treatments	Justification		Perspective
				Animals	Dietary treatments	
1 & 4	1 (In vivo)	Non-lactating cows n = 4 2 × 2 factorial design	1/ CON: 50% hay + 50% concentrate 2/ NIT: CON + 2.3% nitrate (from calcium nitrate) 3/ LIN: CON + 2.6% added lipids (from linseed oil) 4/ LIN+NIT: CON + 2.3% nitrate + 1.0% added lipids	Physiologically stable animals	<ul style="list-style-type: none"> • High-starch diet to favor protozoa • NIT and LIN doses calculated to reach 15-20% CH₄ reduction when fed individually and 30-40% reduction when fed in association 	Fundamental and mechanistic study
2	2 (In vivo)	Lactating cows n = 8 Randomized block design	1/ CON: 54% maize silage + 6% hay + 40% concentrate 2/ LIN+NIT: CON + 1.8% nitrate (from calcium nitrate) + 3.5% added lipids (from extruded linseed)	Animals farm of interest	<ul style="list-style-type: none"> • Basal diet close to farm conditions • Lower NIT dose to avoid health issues with producing animals • Extruded linseed chosen as favored in animal feed production (pelleting process is more difficult with oil) 	On-farm applicability
3 & 4	3 (In vivo)	Non-lactating cows n = 4 2 × 2 factorial design	1/ CON: 50% hay + 50% concentrate 2/ NIT: CON + 2.3% nitrate (from calcium nitrate) 3/ TEA: CON + 0.5% saponin (from tea) 4/ TEA+NIT: CON + 2.3% nitrate + 0.5% saponin	Physiologically stable animals	<ul style="list-style-type: none"> • High-starch diet to favor protozoa • NIT and TEA doses calculated to reach 15-20% CH₄ reduction when fed individually and 30-40% reduction when fed in association 	Fundamental and mechanistic study
3		Lactating cows n = 7 2 × 2 crossover design	1/ CON: 54% maize silage + 6% hay + 40% concentrate 2/ TEA: CON + 0.5% saponin (from tea)	Animals farm of interest	<ul style="list-style-type: none"> • Basal diet close to farm conditions • TEA dose similar to the experiment with non-lactating cows 	On-farm applicability
5	4 (In vitro)	Non-lactating cows n = 2 2 repeated incubations	1/ CON: 50% hay + 50% concentrate 2/ CON + 1, 2, 4 or 6 mM nitrate (from ammonium nitrate)	Physiologically stable animals	<ul style="list-style-type: none"> • Basal diet close to diet fed in experiments 1 and 3 to non-lactating cows • Nitrate doses chosen from literature review 	Fundamental and mechanistic study
		Non-lactating cows n = 2 2 repeated incubations	1/ CON: 100% glucose 2/ CON + 1, 2, 4 or 6 mM nitrate (from ammonium nitrate)	Physiologically stable animals	<ul style="list-style-type: none"> • Basal diet chosen to favor microbial biomass synthesis • Nitrate doses chosen from literature review 	

I. EXPERIMENTAL STRATEGY OF THE PHD THESIS

The literature review highlighted the importance of ruminal H₂ pool in methanogenesis. Nowadays, dietary CH₄-mitigating strategies aimed at reducing its availability for methanogens *via* a reduction of its production **or** a modification of its utilization. In the meta-analysis, we reported that lipids and plant extracts would be the most pertinent strategies to reduce H₂ production *via* a reduction of protozoa, whereas nitrate would be the best user of H₂ competing with methanogenesis. However, these strategies have been tested individually to reduce methanogenesis, but no studies reported the CH₄-mitigating effect of their association.

We assumed that simultaneous manipulation of H₂ production AND utilization allows a more important reduction of CH₄ emissions than when acting on a single pathway (production OR utilization). Consequently the originality of our experimental approach consisted in associating lipids or plant extract with nitrate, in order to combine dietary strategies having different mechanisms of action on the rumen H₂ pool. Then, this PhD thesis was divided into 5 steps, corresponding to 4 experiments (Table 10), which objectives were:

Step 1. 1/ To evaluate the effect of association of feeding strategies acting on H₂ production (**lipids from linseed**, toxic effect towards protozoa) and H₂ utilization (**nitrate from calcium nitrate**, H₂-sink through nitrate reduction to nitrite and ammonia) on CH₄ emissions, diet digestibility and N balance of non-lactating cows. 2/ To understand the CH₄-mitigating effect of these feeding strategies fed alone or in association by focusing on rumen H₂ pool and fermentation parameters.

Step 2. 1/ To evaluate the long-term effect of **linseed plus nitrate** on CH₄ emissions, lactating performances of dairy cows and animal health (blood metHb, nitrate and nitrite residues in milk and processed milk products). 2/ To check the effect of linseed plus nitrate on total tract digestibility, N balance and rumen fermentation after long-term supplementation.

Step 3. 1/ To evaluate the CH₄-mitigating effect and associated ruminal mechanisms of another feeding strategy acting on H₂ production (**saponin from tea**, toxic effect towards protozoa) fed alone or in association with nitrate to non-lactating cows. 2/ To assess effect of tea saponin on diet digestibility, N balance and lactating performances.

Step 4. To understand the effect of tested CH₄-mitigating strategies fed alone (linseed, tea saponin, nitrate) or in association (linseed plus nitrate or tea saponin plus nitrate) on the quantity, activity and diversity of rumen microbiota from non-lactating cows.

Step 5. 1/ To study the dose response effect of nitrate on *in vitro* production of rumen fermentation end-products such as gas (CH₄ and H₂), VFA and microbial biomass (estimated from insoluble protein). 2/ To understand the CH₄-mitigating mechanisms of nitrate by estimating metabolic H₂ distribution between rumen fermentation end-products.

II. MATERIALS AND METHODS

During this PhD thesis, two new techniques have been developed in the team and will be detailed in the next sections: i) continuous and *in vivo* measurement of enteric CH₄ emissions with open chambers; ii) continuous and *in situ* measurement of dissolved H₂ concentration in the rumen.

2.1. Continuous and *in vivo* measurement of enteric methane emissions: open chambers

Quantification of individual CH₄ emissions is an essential measurement in this work. Currently, our team has the skills and expertise in the quantification of CH₄ emissions using the SF₆ tracer technique. However, this method does not give indications about daily kinetics of emissions (Johnson et al., 1994). Inversely, the chamber technique is considered as the reference technique, and has the advantage to continuously quantify CH₄ (and CO₂) emissions of ruminants, which provides interesting information to explain fermentation pattern (Pinares-Patiño and Waghorn, 2012). Consequently, four open chambers for cattle were built by the team in 2012 and were firstly used during this PhD thesis.

2.1.1. Description of the system and measuring principle

To measure kinetics of enteric CH₄ (and CO₂) emissions of cattle, our system comprised 3 main components:

1/ **The open chamber** was 2.2 m high, 3.6 m long and 2.1 m wide, giving a volume of 16.6 m³. Floor dimensions gave the animal a 2 m² movement area, which was close to its stall condition. The chambers were made of steel with transparent polycarbonate walls allowing

sight contact between animals and with the farm personnel. Chambers had front and rear doors, with the front doors used for animal feeding and the rear doors used to enter or milk the animals, or to remove feces and urine collected once daily in a wheeled box.

2/ **The ventilation system** produced an airflow between 500 and 1000 m³/h. There was no automated controller to adjust the airflow to the size and type of animal or to the gas concentrations in chambers. In our experiments, airflow was manually set and averaged 750±50 m³/h (approximately 45 air changes per h) in each chamber. Air entered the chamber through an aperture at the bottom of the rear door (20 cm high, 2.1 m long). The air exited the chamber thanks to the air extractor *via* the exhaust duct situated at the top of the chamber, above the head of the animal. Airflow in the exhaust duct of each chamber was continuously measured (CP300, KIMO, Montpon-Ménestérol, France) and recorded with one data point every 5 min (KT-210-AO, KIMO, Montpon-Ménestérol, France).

3/ **The gas analyzer** (Ultramat 6, Siemens, Karlsruhe, Germany) alternatively measured concentration (ppm) of gases (CH₄ and CO₂) in the barn (ambient air) and in the four chambers at a 0.1 Hz sample frequency (one data every 10 sec) for 5 min every 25 min. Gas sample from ambient air was taken at the bottom of the rear doors from the four chambers, where entered the airflow. Gas samples from each chamber were taken from the exhaust duct. When entering the analyzer, gas samples were dried with a filter. The analyzer was fitted with a data recording system (Nanodac Invensys, Eurotherm Automation SAS, Dardilly, France).

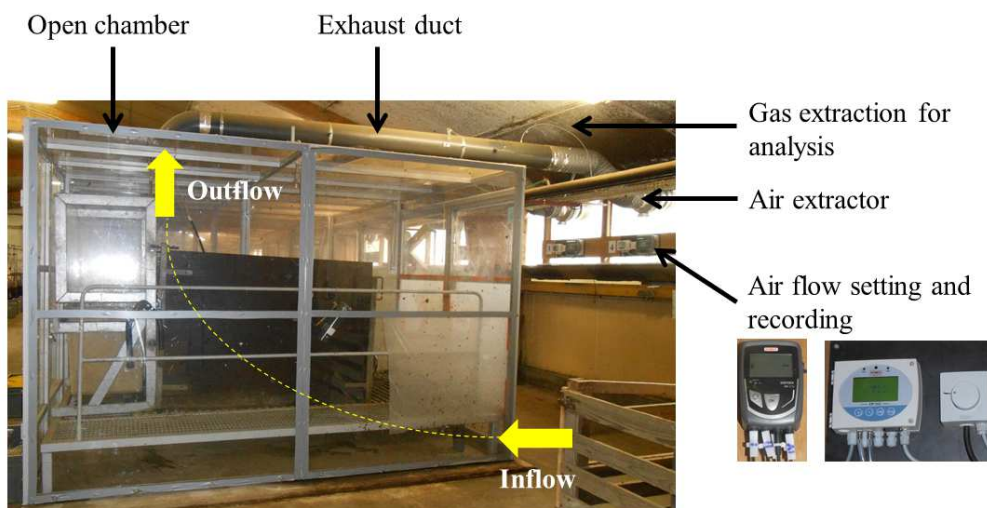


Figure 15 Description of the system for continuous monitoring of enteric CH₄ emissions from cattle. The yellow arrows linked with the dotted line indicate the direction of the air flow within the open chamber, from the inflow to the outflow.

The gas analyzer operated with an infrared (IR) detector, using the principle that some gases are able to absorb specific wavelengths of IR rays (**Figure 16**). A transmitter sent an infrared radiation which was divided into two beams: i) the reference beam which passed through a reference cell with nitrogen gas (N_2) resistant to IR rays; ii) the measurement beam which passed through the measurement cell with the gas sample to analyze. As CH_4 and CO_2 absorb IR radiation (CH_4 : 3-9 μm ; CO_2 : 14 μm), the concentration of CH_4 and CO_2 was positively correlated with the amount of absorbed IR rays. Then, the reference and measurement beams arrived in the receiving cell with the detector. They were compared using the reference beam as a baseline, and the amount of exiting IR rays was quantified. According to the calibration curve, the concentration of CH_4 and CO_2 were finally calculated.

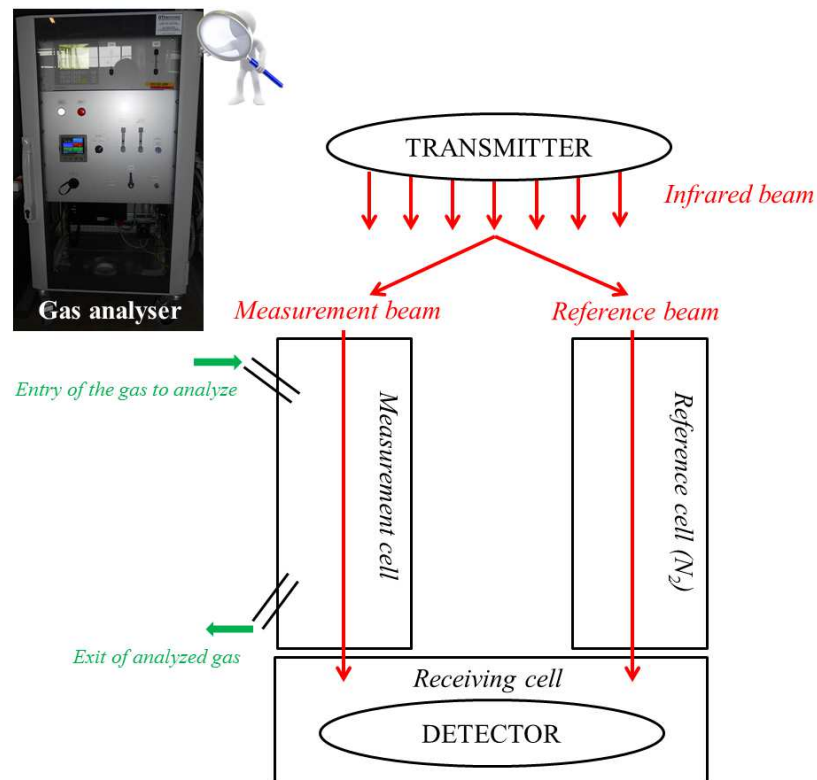


Figure 16 Functional schematic of the methane and carbon dioxide gas analyzer

2.1.2. System setup and functioning

The week before starting measurement, the gas analyzer was calibrated with a defined gas mixture of CH_4 (650 ppm) and CO_2 (700 ppm), and with a pure gas (N_2) which allowed blank calibration. During the measurement week, airflow and gas data were collected daily, and treated with an home-made Excel macro to calculate CH_4 emissions (L/day):

- 1/ For each chamber and ambient, CH₄ data were averaged over the 5-min interval and interpolated by linear regression to get one data point every 5 min.
- 2/ For each data point and for each chamber, ambient CH₄ concentration was subtracted to CH₄ concentration of each chamber.
- 3/ For each chamber, CH₄ emissions (L/day) were calculated from CH₄ concentration (ppm) and airflow (L/h):

$$CH_4 \text{ (L/day)} = CH_4 \text{ (ppm)} \times 10^{-6} \times \text{airflow} \times 24$$

Since the gas going into the analyzer was dried, we assumed that to obtain CH₄ emissions in the environmental sampling conditions, it was necessary to apply the Wexler equation on airflow data (Pinares-Patiño et al., 2012b). This equation required to get the temperature (T), pressure (P) and relative humidity (RH) in the chamber (exhaust duct) to calculate the volume mixing ratio of water vapor (VMR):

$$VMR = \frac{(a_1 + a_2 \times T + a_3 \times T^2 + a_4 \times T^3 + a_5 \times T^4 + a_6 \times T^5 + a_7 \times T^6) \times RH}{P}$$

With a₁, a₂, a₃, a₄, a₅, a₆ and a₇ being the coefficients of water vapor (6.11, 0.44, 1.43 × 10⁻², 2.65 × 10⁻⁴, 3.02 × 10⁻⁶, 2.04 × 10⁻⁸ and 6.39 × 10⁻¹¹, respectively). The VMR was then used to calculate the dry gas flow (DGF), which is the airflow (L/h) corrected for environmental conditions:

$$DGF = \text{Airflow} \times \left(\frac{100 - VMR}{100} \right)$$

The airflow corrected for environmental conditions was converted to have the airflow in standard condition of temperature and pressure (STP, L/h):

$$STP = \frac{P \times DGF}{T + 273.15} \times \frac{273.15}{1013.25}$$

Finally, CH₄ emissions (L/day) were calculated from CH₄ concentrations (ppm) and STP (L/h):

$$CH_4 \text{ (L/day)} = CH_4 \text{ (ppm)} \times 10^{-6} \times STP \times 24$$

However, the difference between uncorrected and corrected CH₄ emissions by environmental parameters was low (~3%), leading us to the conclusion that this correction is not appropriate in our experimental conditions.

2.2. Continuous monitoring of rumen dissolved hydrogen concentration: adaptation of a H_2 -sensor to the rumen environment

According to the literature review (chapter 1), only two methods allow *in situ* and continuous measurement of dissolved H_2 concentrations in the rumen. Hillman et al. (1985) used a Clark-type oxygen electrode placed within the rumen and connected to a mass spectrometer. In the method of Smolenski and Robinson (1988), dissolved H_2 is uptaken by a carrier gas passing through a probe immersed into the rumen and connected to a gas chromatograph. These methods have two disadvantages: i) they require important equipment (mass spectrometer) and large-size probes which may disturb the ruminal environment; ii) the response time is quite long (90% response in 2 min) whereas the turnover time of H_2 in the rumen is much quicker (0.08 sec). Consequently, we chose to adapt a H_2 -sensor commonly used in marine research for *in situ* and continuous measurement of dissolved H_2 concentration in the rumen.

2.2.1. Description of the system and measuring principle

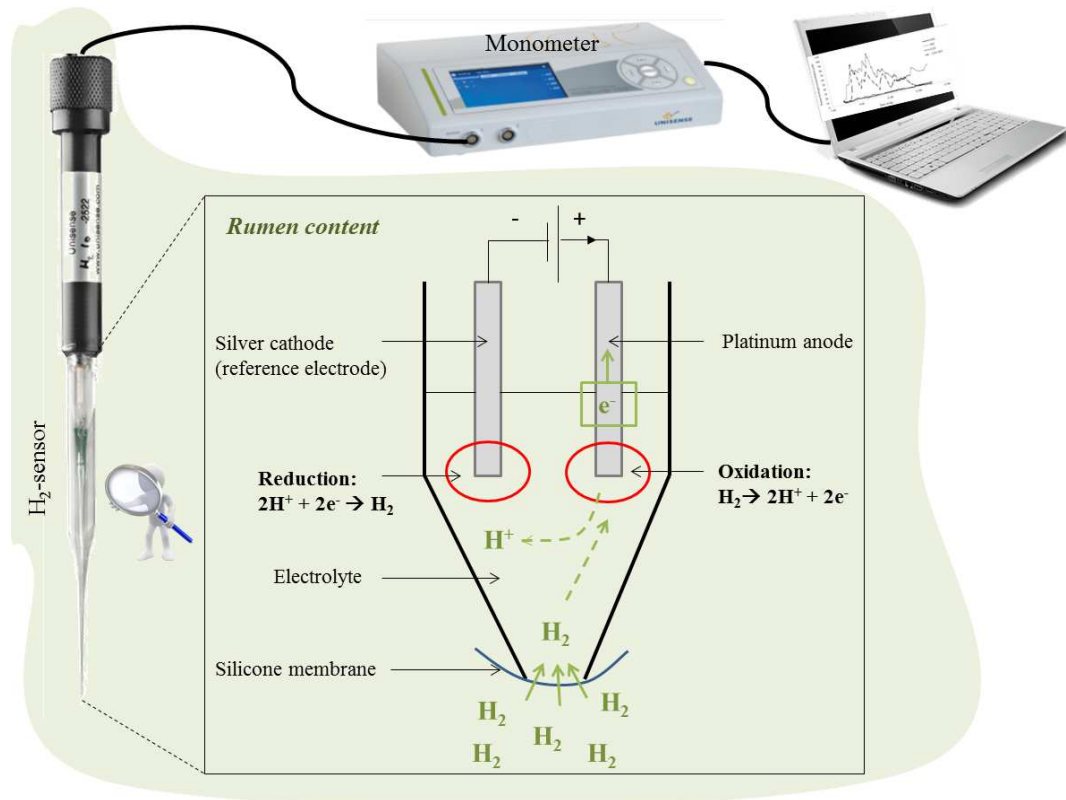


Figure 17 Description of the system for *in situ* and continuous monitoring of dissolved H_2 concentration in the rumen

For *in situ* and continuous measurement of dissolved H_2 concentration in the rumen, the system included 3 components (**Figure 17**):

1/ **The H_2 -sensor** (H_2 -500, Unisense, Denmark) was 17 cm long and diameters were 22 mm at the top and 0.6 mm at the tip. The limit of quantification of H_2 concentration was $0.3 \mu M$ and the sensor gave a 90% response in 3-15 sec. The glass-made tip hosted a Clark-type electrode made of a silver cathode (reference electrode) and a platinum anode, which both bathed into a conductive solution (or electrolyte). The tip was closed by a silicone membrane allowing ruminal dissolved H_2 to diffuse into the sensor.

2/ **The current amplifier or monometer** (Microsensor Monometer Version 1.0, Unisense, Denmark) generated an electric current flowing in the H_2 -sensor in a closed-circuit system, from the cathode to the anode, and from the anode to the cathode through the electrolyte. The electrical voltage (800 mV), dependent on the composition of the gas to analyze, was set according to manufacturer instructions.

3/ **The computer set with the Sensor Trace Basic software** (Version 3.1.3., Unisense, Denmark) calculated and recorded dissolved H_2 concentrations every second.

Concentration of dissolved H_2 was measured in a two-step process:

1/ Dissolved H_2 in rumen content diffused into the sensor through the silicone membrane until reaching an equilibrium concentration.

2/ Dissolved H_2 was oxidized at the anode. Electrons flowed from the anode to the cathode (opposite direction of the electric current), generating a low-intensity electric signal measured by the monometer. Protons remained in the electrolyte until their reduction with electrons coming out of the cathode.

Then, higher was H_2 concentration in the rumen and in the sensor electrolyte, higher was the electric signal generated during electrons flow. In other words, the electric signal measured by the monometer was positively correlated with dissolved H_2 concentration.

2.2.1. System setup and functioning

According to manufacturer instructions, a pre-polarization period was applied before using the sensor, during which it was simply connected to the monometer set to its electrical voltage (800 mV). This process was essential to let the sensor retrieving a stable and weak baseline, *via* elimination of H_2 which could have accumulated in the electrolyte during

storage. Then, longer was the period of non-activity of the sensor, longer was the time required for pre-polarization (from 10 minutes to 8 hours).

After pre-polarization, the sensor was calibrated with a defined gas mixture of H_2 and H_2 -free inert bulk carrier gas (80% H_2 - 20% CO_2). Knowing that H_2 solubility is dependent on salinity and temperature (Wiesenburg and Guinasso, 1979), the sensor was placed in a water bath at $39^\circ C$ in order to reach similar conditions to the rumen. As the sensor linearly detected partial pressure of H_2 , a two-point calibration curve was created as recommended by Unisense: the sensor was immersed into the water bath without bubbling ($0 \mu M H_2$) and the electric signal read by the monometer after stabilization was recorded (first calibration point). Then, the defined gas mixture of H_2 was allowed to bubble until stabilization and recording of the electric signal (second calibration point). Knowing that the maximum concentration of dissolved H_2 in the rumen is $740.9 \mu M$ (see literature review for calculation), the dissolved H_2 concentration is $740.9 \times 0.8 = 592.7 \mu M$ when a 80% H_2 gas is bubbling.

After completing these two steps, the sensor was ready for measurement. Before inserting the sensor into the rumen through the cannula, it was protected with a custom-made plastic cap, and ballasted with a 1-kg weight to ensure continuous measurement of dissolved H_2 concentration at the bottom of the rumen (Figure 18).



Figure 18 Protection cap of the H_2 -sensor

The sensor in its protection was connected to the monometer *via* a 10-m wire extension protected in a plastic tube. After insertion of the sensor into the rumen, the protected wire was attached to the cow with a harness, to make sure the animal cannot move the device. The cannula was closed with a plastic cork to limit rumen liquid and gas leakage (Figure 19).

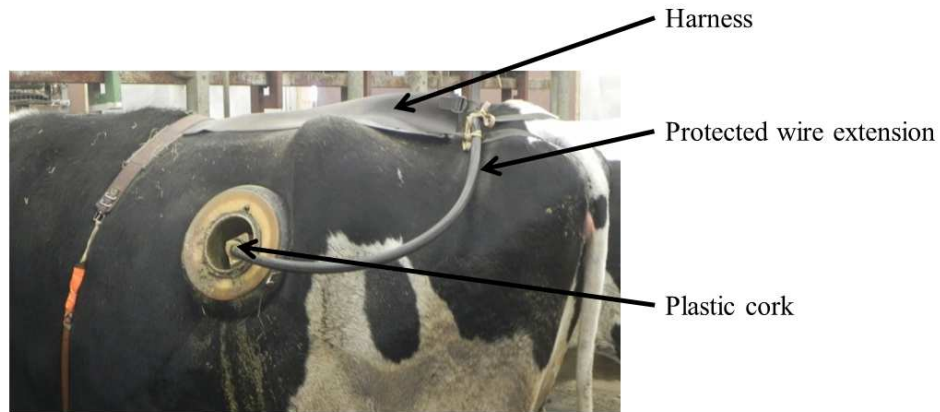


Figure 19 Hydrogen sensor setup on the animal

Results

STEP 1: Additive effect between dietary linseed oil and nitrate as methane emission-reducer in cattle

Objective

1/ To evaluate the effect of association of feeding strategies acting on H₂ production (lipids from linseed, toxic effect towards protozoa) and H₂ utilization (nitrate from calcium nitrate, H₂-sink through nitrate reduction to nitrite and ammonia) on CH₄ emissions, diet digestibility and N balance of non-lactating cows.

2/ To understand the CH₄-mitigating effect of these feeding strategies by focusing on rumen H₂ pool and fermentation parameters.

Experimental approach

4 non-lactating cows → 2 × 2 Factorial design →

CON: 50% hay + 50% pelleted concentrate
NIT: CON + 2.3% nitrate (from calcium nitrate)
LIN: CON + 2.6% added lipids (from linseed oil)
LIN+NIT: CON + 1.0% added lipids + 2.3% nitrate

4 experimental periods of 5 weeks (wk 1 to 2 = Adaptation; wk 3 to 5 = Measurement)

WEEK	1	2	3	4	5
Daily intake					
Blood methHb (3 h after morning feeding, once a week)					
Kinetics of rumen dissolved H ₂ concentrations (during 6 h after morning feeding, one day/cow)					
Total tract digestibility, N balance (6 days)					
Rumen fermentation (0 and 3 h after morning feeding, twice a week)					
Daily kinetics of CH ₄ emissions (4 days)					
Daily kinetics of rumen pH (6 days)					

Main results

	Diet				SEM	P-Value		
	CON	NIT	LIN	LIN+NIT		Nitrate	Linseed	Linseed × nitrate
DM intake (kg/day)	12.4	12.3	12.3	12.2	0.59	0.22	0.35	0.86
CH ₄ emissions (g/kg DM intake)	25.0	19.4	20.7	17.0	0.70	<0.01	<0.01	0.18
DM digestibility (%)	63.7	64.1	64.0	63.3	0.77	0.85	0.65	0.43
N balance (% of N intake)	7.4	11.8	4.0	4.8	2.25	0.20	0.03	0.35
Rumen protozoa (log ₁₀ /mL, 0 h)	5.87	5.71	5.55	5.73	0.060	0.91	0.03	0.02
Rumen C2/C3 (0 h)	4.74	4.68	3.97	4.41	0.221	0.39	0.04	0.26
Rumen H ₂ concentrations (μM)	3.6	45.3	4.0	21.0	14.10	0.07	0.41	0.39

Conclusion

Nitrate plus lipids from linseed have an additive CH₄-mitigating effect without altering digestibility and N balance. These two dietary strategies have different modes of action on the rumen H₂ pool. Further work is necessary to assess the long-term effect of this association on methanogenesis, rumen microbiota and animal performances.

Additive effect between dietary linseed oil and nitrate as methane emission-reducer in cattle

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Abstract

The objective of this study was to test the effect of linseed oil and nitrate fed alone or in combination on methane (CH₄) emissions and diet digestibility in cows. The experiment was conducted as a 2 × 2 factorial design using 4 multiparous non-lactating Holstein cows (initial BW 656 ± 31 kg). Each experimental period lasted 5 weeks, with measures performed in the final 3 weeks (wk 3 to wk 5). Diets given on a DM basis were: 1) control (CON, 50% natural grassland hay and 50% concentrate), 2) CON with 4% linseed oil (LIN), 3) CON with 3% calcium nitrate (NIT), 4) CON with 4% linseed oil plus 3% calcium nitrate (LIN+NIT). Diets were offered twice daily and were formulated to deliver similar amounts (DM basis) of CP (12.2%), starch (25.5%) and NDF (39.5%). Feed offer was restricted to 90% of voluntary intake (12.4 kg DMI/d). Total tract digestibility and N balance were determined from total feces and urine collected separately for 6 d during wk 4. Daily CH₄ emissions were quantified using open chambers for 4 d during wk 5. Rumen fermentation and microbial parameters were analyzed from samples taken before and 3 h after the morning feed. Rumen concentrations of dissolved hydrogen (H₂) were measured continuously up to 6 h post-feeding using a H₂ sensor. Compared with CON, linseed oil and nitrate decreased ($P < 0.01$) CH₄ emissions (g/kg DMI) by 17 and 22%, respectively, when fed alone and by 32% when combined. The LIN diet reduced CH₄ production throughout the day, increased ($P = 0.02$) propionate proportion and decreased ($P = 0.03$) ruminal protozoa concentration compared with CON. The NIT diet strongly reduced CH₄ production 3 h post-feeding, with a simultaneous increase in rumen dissolved H₂ concentration, suggesting that nitrate does not only act as an electron acceptor. As a combined effect, linseed plus nitrate also increased H₂ concentrations in the rumen. Diets had no effect ($P > 0.05$) on total tract digestibility of nutrients, except with linseed oil which tended to reduce ($P < 0.10$) fiber digestibility. Nitrogen balance (% of N intake) was positive for all diets but retention was lesser ($P = 0.03$) with linseed oil. This study demonstrates an additive effect between nitrate and linseed oil for reducing methanogenesis in cows without altering diet digestibility.

Keywords: hydrogen, lipid, methane mitigation, nitrate, ruminant

Introduction

Enteric methane (CH_4) from ruminants is one of the most important greenhouse gas at the farm level (Gerber et al., 2013), and represents an energy loss to the animal (2-12% of GE intake; Johnson and Johnson, 1995). Lipids and nitrate (NO_3^-) emerged as persistent and viable dietary options for mitigating CH_4 emissions from ruminants (Doreau et al., 2014a). Linseed reduced methanogenesis (-5.6% per 1% added fat; Doreau et al., 2011) but this effect was not always reported (Chung et al., 2011; Veneman et al., 2014). Linseed, rich in polyunsaturated fatty acids (PUFA), may improve animal product quality (Scollan et al., 2001; Chilliard et al., 2009), but fat doses greater than 5% may lower animals' performance (McGinn et al., 2004; Martin et al., 2008). In the diet, NO_3^- repeatably reduced CH_4 emissions (-10% per 1% added NO_3^- ; Lee and Beauchemin, 2014), but its use as a urea substitute still requires investigations into its possible impacts on animal health, digestive parameters and residuals in animal products for human consumption.

In the rumen, CH_4 is mainly produced by methanogens using carbon dioxide (CO_2) and hydrogen (H_2). Both are fermentation end-products, but as H_2 is limiting, modulating its concentration could reduce methanogenesis (Hegarty and Gerdes, 1999). Linseed and NO_3^- affect the rumen H_2 pool in unique ways. Linseed reduces H_2 production mainly through its toxic effect against rumen protozoa, which are major H_2 producers (Morgavi et al., 2010). As fat is not fermented in the rumen, substitution of rumen fermentable substrates for lipids may also reduce H_2 production. To a lesser degree, PUFA can reduce H_2 availability in the rumen by consuming H_2 during biohydrogenation (Czerkawski, 1986). Nitrate modifies H_2 consumption by reducing the number of methanogens (Van Zijderveld et al., 2010) and by acting as a H_2 -sink (Lewis, 1951).

As these dietary treatments share different mechanisms of action, we hypothesized that their combination would have an additive effect that leads to lesser net methanogenesis than when they are individually fed. However, as a feeding strategy should reduce CH_4 emissions without adverse effect on animals' digestive efficiency, performance and health, our hypothesis was tested in an in vivo experiment with dry cows designed to evaluate the effect of linseed plus nitrate on: 1) CH_4 emissions and mechanisms involved in methanogenesis (rumen H_2 pool and fermentation); 2) diet digestibility and nitrogen balance.

Materials and methods

The experiment was conducted at the animal facilities of the Experimental Unit UERT at the INRA's Theix Research Centre (Saint-Genès-Champanelle, France) from January to June 2013. Procedures involving animals were performed in accordance with French Ministry of Agriculture guidelines for animal research and with the applicable EU guidelines and regulations on experiments with animals. The experiment was approved by the local Auvergne-region ethics committee on animal experimentation, approval number CE50-12.

Animals, experimental design and diets

Four multiparous non-lactating Holstein cows fitted with rumen cannulas (initial average BW of 656 ± 31 kg and age of 6.7 ± 1.5 years, mean \pm SD) and habituated to handling were housed in individual stalls during the experiment. The cows were randomly assigned to 4 dietary treatments in a 2×2 factorial design, using either calcium nitrate or linseed oil at two different doses (0 and 3% for calcium nitrate; 0 and 4% for linseed oil). Each experimental period lasted 5 weeks, with measures performed in the final 3 weeks (wk 3 to wk 5). The diets, given on a DM basis, were: 1) control (CON), 2) CON with 4% linseed oil (LIN), 3) CON with 3% calcium nitrate (NIT), 4) CON with 4% linseed oil and 3% calcium nitrate (LIN+NIT). The doses of linseed oil (Vandeputte Savonnerie et Huilerie, Mouscron, Belgium) and calcium nitrate (75% NO_3^- in DM; Phytosem, Pont-du-Château, France) were calculated to achieve a theoretical CH_4 reduction of 20% when distributed alone (Martin et al., 2008; Van Zijderveld et al., 2011; Hulshof et al., 2012).

Ingredients and chemical composition of the experimental diets are reported in Table 1. The CON diet consisted of 50% natural grass hay (harvested in semi-mountainous and permanent grassland areas) and 50% concentrate (DM basis). Diets were formulated at the beginning of the experiment to meet at least the ME requirements for maintenance of non-lactating cows (INRA, 2010) and to get sufficient and similar levels of NDF (to avoid any risk of acidosis; Krause and Oetzel, 2006), starch (to favor protozoa development; Jouany, 1989), and CP. Diet levels of fermentable N were kept moderate in order to assess the effect of nitrate on N output. Diets were adjusted to have the same N and Ca concentrations by including urea and calcium carbonate in the non-NIT diets (i.e. CON and LIN). Calcium carbonate was used as it has low solubility in the rumen and thus avoids the formation of calcium salts with lipids (Keyser et al., 1985). A commercial mineral-vitamin premix was added in equal amounts to

all diets. Forage was distributed without further processing. All other ingredients including linseed oil or nitrate or both were pelleted in concentrates (InVivo NSA, Chierry, France).

Table 1 Ingredients and chemical composition of the experimental diets

Item	Diet ¹			
	CON	NIT	LIN	LIN+NIT
Ingredient, % of DM				
Hay	50.00	50.00	50.00	50.00
Pelleted concentrate				
Wheat	25.23	25.23	25.23	25.23
Maize	15.00	15.00	15.00	15.00
Calcium nitrate ²	0	3	0	3
Linseed oil	0	0	4	4
Calcium carbonate	1.7	0	1.7	0
Urea	1.22	0	1.22	0
Dehydrated beet pulp	4.08	4	0.08	0
Molasses beet	1	1	1	1
Binder	1	1	1	1
Mineral-vitamin mix	0.75	0.75	0.75	0.75
Aroma	0.02	0.02	0.02	0.02
Chemical composition				
OM, % of DM	91.3	91.5	91.8	91.8
CP, % of DM	12.7	12.2	12.1	11.7
NDF, % of DM	40.1	40.2	38.8	38.7
ADF, % of DM	23.3	23.1	22.2	22.2
Starch, % of DM	25.4	25.7	25.7	25.3
Ether extract, % of DM	2.08	1.90	4.66	3.12
Total fatty acids, % of DM	1.61	1.24	3.53	2.05
GE, MJ/kg of DM	17.4	16.6	18.3	17.7
Fatty acid, % of total fatty acids				
C16:0	18.56	24.55	14.18	20.38
C18:0	1.98	2.58	4.92	6.56
C18:1 n-9	19.53	22.90	23.13	28.60
C18:2 n-6	47.50	29.33	24.89	21.22
C18:3 n-3	8.01	7.72	29.37	17.63

¹ CON = control; NIT = diet CON containing 3% calcium nitrate; LIN = diet CON containing 4% linseed oil; LIN+NIT = diet CON containing 4% linseed oil and 3% calcium nitrate.

² 5Ca(NO₃)₂.NH₄NO₃.10H₂O; 75% NO₃⁻ in DM.

Feeding and management

Two weeks before starting the experiment, cows were fed CON ad libitum. Then, throughout the experiment, offered feed was restricted to 90% of individual voluntary feed intakes (1.8 times ME requirements for maintenance) to ensure complete consumption. The LIN, NIT and

LIN+NIT concentrates were progressively supplied by replacing the CON concentrate. The LIN concentrate was distributed at maximal dose after a 5-d transition period. The NIT and LIN+NIT concentrates were distributed at their maximal dose after a 10-d transition period.

Throughout the experiment, feed was offered twice daily (66% at 0800 h and 34% at 1600 h for hay; 60% between 0800 and 0930 h in 3 equal portions and 40% between 1600 and 1630 h in 2 equal portions for concentrates). Distribution of concentrates was fractionated to reduce the risk of methemoglobinemia (metHb; Morris et al., 1958). Forage-to-concentrate ratio (50:50) was kept as close as possible to the target ratio by adjusting the amounts of hay and concentrates offered daily. Cows had free access to water throughout the experiment.

Measurements and analyses

Intake. Feed intake was weighed and recorded daily throughout the experiment to estimate DMI. There were no refusals during the experiment. Samples of each feed (200 g of hay and concentrates) were taken on 2 days in wk 4 and wk 5 of each period. One sub-sample was used to determine DM content (103°C for 24 h) and another sub-sample was stored at 4°C before being pooled at the end of the experiment. These pooled samples were ground down using an Ultra Centrifugal Mill (0.75 mm sieve; Retsch GmbH, Haan, Germany) and analyzed for chemical composition.

Organic matter was determined by ashing at 550°C for 6 h (method 942.05; AOAC, 2005). Total N was analyzed by combustion according to the Dumas method (method 968.06; AOAC, 2005), and CP content was calculated as $N \times 6.25$. 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). The GE was analyzed by isoperibolic calorimetry (C200 model, IKA, Staufen, Germany). Ether extract (EE) was determined after acid hydrolysis (method 954.02; AOAC, 2005), and fatty acid (FA) composition was determined by gas chromatography of methyl esters (method 969.33; AOAC, 2005).

Cow liveweights and methemoglobinemia. Cows were weighed at the beginning of the experiment and at the end of each experimental period. Levels of blood metHb were measured on all cows 3 h after morning feeding (1100 h) on the day before the start of the experiment (control blood) and then at d 3 and 5 (1% calcium nitrate in the diet), d 10 (2% calcium nitrate in the diet) and d 12, 17, 19 and 22 (3% calcium nitrate in the diet) of each experimental

period for cows fed NIT and LIN+NIT. Blood from cows fed CON and LIN was not analyzed as we assumed that there was no risk of metHb. Blood (10 mL) was sampled from the jugular vein into K2-EDTA collection tubes (Venosafe, Terumo, Guyancourt, France) and packed on ice for metHb content to be determined by spectrophotometry (UV-160, Shimadzu, Marne-La-Vallée, France) within 1 h at the nearest hospital (CHU Gabriel Montpied, Clermont-Ferrand, France; method of Kaplan, 1965). A metHb threshold value was set at 30% hemoglobin (Hb). Any animal meeting this cut-off would be removed from the experiment and treated with 1% methylene blue (The United States Pharmacopeial Convention, 2008).

Diet digestibility and nitrogen balance. Total tract digestibility and N balance were determined from total and separate collection of feces and urine for 6 days during wk 4 of each experimental period. To separate urine from feces, cows were fitted with flexible pipes (Doreau et al., 2014b) connected to a 30-L flask containing 500 mL of 3 M sulfuric acid to achieve a urine pH < 3 and thus avoid N volatilization. Feces and urine were removed once daily.

Each morning, after weighing and mixing of feces, a 1% fresh aliquot was used for DM determination (103°C for 24 h) and a 0.5% fresh aliquot was pooled across days for each animal and frozen (-20°C). At the end of the experiment, pooled samples were thawed, dried (60°C for 72 h) and ground (1-mm screen) to determine OM, N, NDF and ADF content as previously described.

Each morning, after weighing urine, a 0.5% fresh aliquot was pooled across days for each animal and frozen (-20°C). At the end of the experiment, the N content of thawed urine was determined by the Kjeldahl method (method 2001.11; AOAC, 2005) as it was impossible to apply the Dumas method on fresh urine.

Rumen fermentation parameters. Total rumen contents were sampled (~200 g) from the ventral sac through the cannula before (0745 h) and 3 h after (1100 h) the morning feed on 2 non-consecutive days (d 3 and 5) in wk 4 of each experimental period. The samples were strained through a polyester monofilament fabric (250 µm pore size) and filtrate was subsampled for subsequent analyses. For VFA analysis, 0.8 mL of filtrate was mixed with 0.5 mL of a 0.5 M HCl solution containing 2% (w/v) metaphosphoric acid and 0.4% (w/v) crotonic acid. For ammonia-nitrogen (NH₃-N) analysis, 1 mL of filtrate was mixed with 0.1 mL of 5% orthophosphoric acid. For lactate and nitrate-nitrite concentrations analysis, 3 mL

and 20 mL of filtrate, respectively, were collected without preservative (Sar et al., 2004). All these samples were stored at -20°C until analysis. For protozoa counts, 2 mL of filtrate was mixed with 2 mL of methyl green-formalin solution and stored away from direct light until counting.

Concentrations of VFA and NH₃-N were analyzed by gas chromatography with a flame ionization detector and by colorimetry, respectively (Morgavi et al., 2008). Lactate concentrations were determined by colorimetry (D/L-lactic acid, BioSentec, Auzeville-Tolosane, France). Nitrate and nitrite concentrations were analyzed by colorimetry (method EPA 353.2; SmartChem 200, Unity Scientific, Brookfield, USA; Laboratoire Vétérinaire et Biologique, Lempdes, France). Protozoa were counted by microscopy 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 log₁₀-transformed before statistical analysis.

Monitoring pH and dissolved H₂ concentration in the rumen. Rumen pH was monitored continuously over wk 5 using commercial boluses (eBolus, eCow, Exeter, UK). One day before measurement, the boluses were calibrated using buffer solutions (pH 4 and 7; HM Digital, Culver City, CA). One bolus per cow was immersed in the ventral sac of the rumen. Data were then recorded every 15 min during 6 full days, after which the boluses were removed. At the end of each experimental period, data were uploaded by telemetry to a digital tablet before being transferred to a computer.

The dynamics of dissolved H₂ concentrations in the rumen were successively measured on each cow in wk 3 (one day per cow) with a H₂ sensor (H2-500, Unisense, Denmark). The electrode was connected to a microsensor monometer via a 10-m wire extension (Unisense, Denmark), and the monometer was connected to a portable computer running Sensor Trace Basic software (Version 3.1.3; Unisense, Denmark). The sensor was polarized (800 mV) once in wk 3 (8 h before the start of measurement) and calibrated daily by immersion in a water bath at 39°C bubbling with a 80% H₂/20% CO₂ gas mixture. The sensor and wire extension were protected using a custom-made plastic cap and tube (Figure 1). The system was ballasted with a 1-kg weight and introduced into the cow's ventral sac through the cannula at 30 min before the morning feed (i.e. 0730 h). The setup was fitted taking care to avoid gas and liquid leakage from the rumen. Dissolved H₂ concentration readings were recorded every second for

6 h after the morning feed. For an easier use of the sensor, it was essential to remove it when the rumen was not full i.e. before the afternoon feeding.

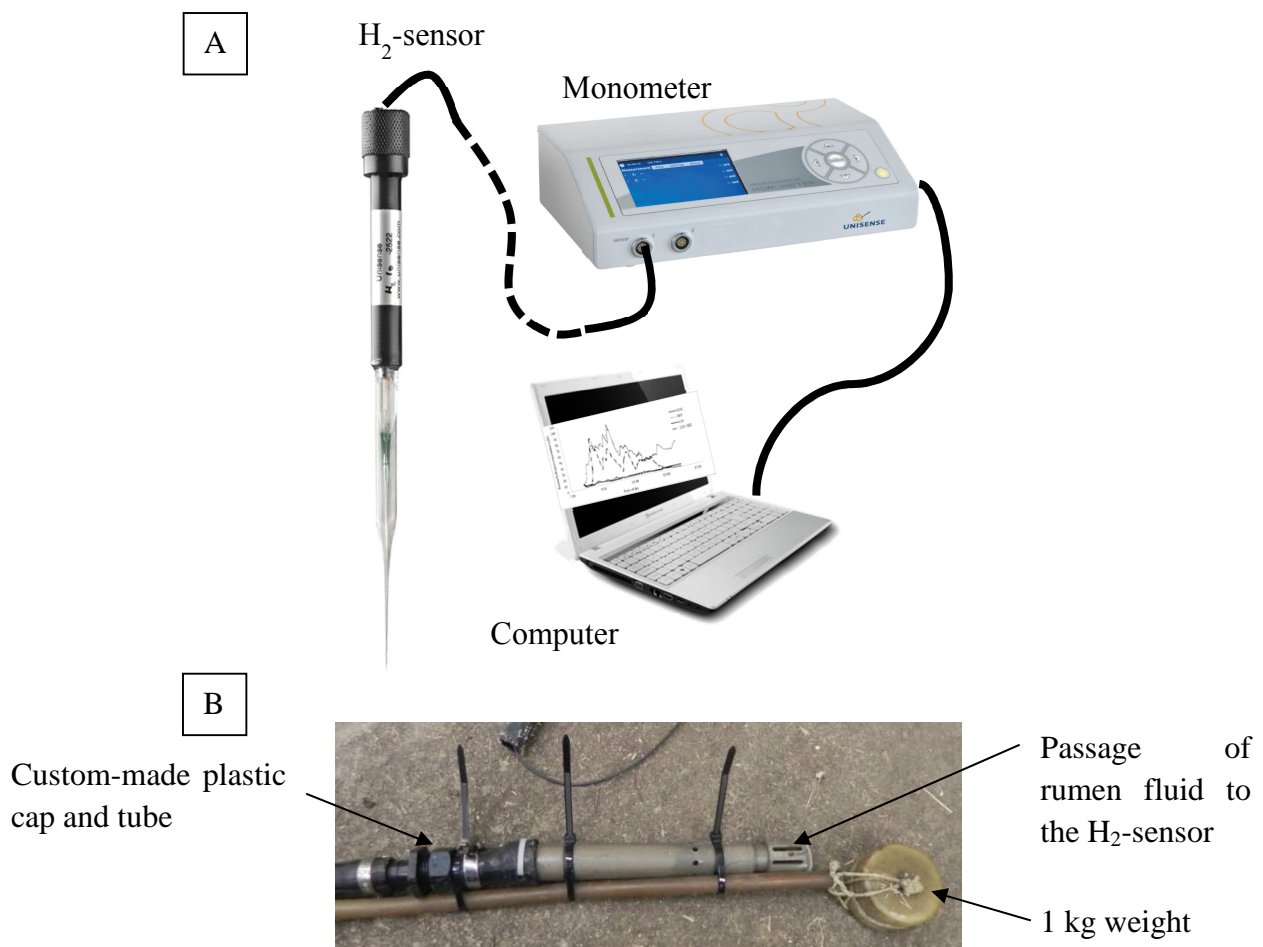


Figure 1. Use of H₂-sensor (Unisense, Denmark): A. Overall setup with sensor, monometer and computer; B. Protection cap of the sensor.

Methane and carbon dioxide emissions. In wk 5, animals were placed in open chambers (1 animal/chamber) for 4 consecutive days. Individual total CH₄ and CO₂ emissions were measured continuously from d 1 (0730 h) to d 5 (0730 h).

Each chamber was 2.2 m high, 3.6 m long and 2.1 m wide, giving a volume of 16.6 m³. The chambers were made of steel with clear polycarbonate walls allowing sight contact between animals and with the farm personnel. Chambers had front and rear doors, with the front doors used for animal feeding and the rear doors used to enter the animals and to remove feces and urine collected in a wheeled recovery box. Front and rear doors were never simultaneously opened in order to avoid an air stream into the chamber. The feces and urine recovery boxes were removed each morning and immediately replaced with new ones in order to minimize

chamber opening time (5 min per chamber on average). When rear doors were closed, front doors were opened (5 min per chamber on average) for morning (1 portion of hay at 0800h, 3 portions of concentrates at 0800, 0830 and 0930h) and afternoon (1 portion of hay at 1600h, 2 portions of concentrates at 1600 and 1630h) feeding.

The chambers operated at a slight negative pressure, with an air flow oscillating between 700 and 800 m³/h (approximately 45 air changes per h). Airflow entered the chamber through an aperture at the bottom of the rear door (0.42 m²) and exited through an exhaust duct situated at the top of the chamber, over the head of the animal. Airflow in the exhaust duct of each chamber was continuously measured (CP300, KIMO, Montpon-Ménestérol, France) and recorded with one datapoint every 5 min (KT-210-AO, KIMO, Montpon-Ménestérol, France). Concentration of gases in the barn and in the 4 chambers was alternatively analyzed at a 0.1 Hz sample frequency for 5 min every 25 min using an infrared detector (Ultramat 6, Siemens, Karlsruhe, Germany) and recorded (Nanodac Invensys, Eurotherm Automation SAS, Dardilly, France). The detector was manually calibrated the day before each measurement period using pure N₂ and a mixture of CH₄ (650 ppm) and CO₂ (700 ppm) in N₂. Missing data between 2 measurement intervals were recovered by linear regression. Chamber doors were never opened during gas analysis, so no data was deleted. Real-time gas emissions in a chamber were calculated by the difference between chamber and ambient gas concentrations multiplied by the airflow corrected for temperature, relative humidity and pressure according to the Wexler equation (Pinares-Patiño et al., 2012).

Statistical analyses

Except for metHb, data were analyzed using the MIXED procedure of SAS (Version 9.2; SAS Institute, 2009). Gaseous emissions (CH₄ and CO₂) and rumen fermentation parameters measured during several days (n = 4 and 2 days, respectively) were averaged per period before being included in the statistical analyses. The model included the random effect of cow (n = 4) and fixed effects of period (n = 4), nitrate (CON and LIN versus NIT and LIN+NIT), linseed (CON and NIT versus LIN and LIN+NIT) and the interaction nitrate × linseed. Rumen fermentation data obtained before and after feeding (VFA, NH₃-N, lactate, protozoa, nitrate and nitrite concentrations) were analyzed using the same model and for the 2 sampling hours separately. Continuous measurements of ruminal pH, dissolved H₂ concentrations and CH₄ emissions were analyzed by repeated time. Several covariance structures were compared, and compound symmetry (CS) was selected as it resulted in the lowest values for the Akaike's

information criteria. The model included the fixed effects of period, hour, nitrate, linseed, nitrate \times linseed and the interactions between hour and dietary treatments (linseed \times hour, nitrate \times hour, linseed \times nitrate \times hour). Differences among treatments were tested using the PDIFF option. Data were considered significant at $P < 0.05$. Least squares means are reported throughout.

Results

Liveweight and blood methemoglobin

Animals gained on average 26.5 kg per experimental period, with a final BW at the end of the trial of 762 ± 47 kg. For diets containing nitrate (NIT and LIN+NIT), blood metHb gradually increased the first 12 d of adaptation period, but no animal exceeded 26.3% metHb (Figure 2).

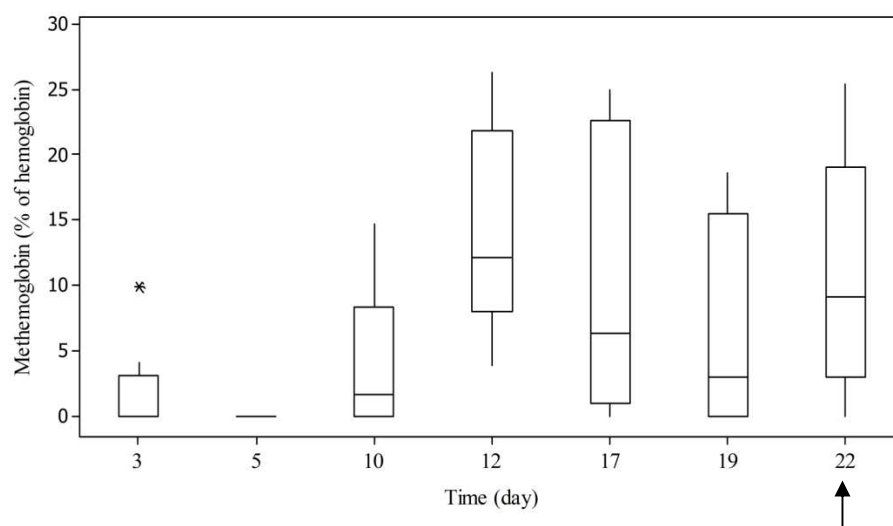


Figure 2 Boxplot of blood metHb levels of non-lactating cows fed diets containing 3% calcium nitrate with or without 4% linseed oil ($n = 8$). The box represents the quartiles with the median at the center and the vertical lines represent the maximum and minimum value within 1.5 interquartile range of the higher and lower quartile, respectively. Values greater than 1.5 interquartile range are considered as outliers and are identified with a star. Blood was analyzed during the 3-wk adaptation period, the arrow indicates the start of the measurement period.

Methane and carbon dioxide emissions

Dry matter intake of cows while in chambers was the same as outside, showing the absence of stress of animals, and that CH_4 determination in our experimental conditions accurately reflected emissions throughout the trial. Methane production was different among diets

irrespective of the unit of expression (Table 2; $P < 0.01$). Compared with CON, CH₄ (g/d) was 18, 23 and 33% lesser for LIN, NIT and LIN+NIT, respectively. An additive CH₄-mitigating effect between linseed and nitrate (linseed \times nitrate, $P > 0.05$) was observed when CH₄ was expressed as a function of DMI, digested DM, digested OM or as a percentage of GE intake. When expressed per kg of digested NDF, CH₄ emissions from cows fed nitrate-containing diets were lesser than emissions from cows fed other diets ($P = 0.01$). With LIN+NIT, CH₄ emissions were close to those of animals fed NIT showing the absence of additive effect between nitrate and linseed.

Diets affected the daily pattern of CH₄ emissions in different ways (Figure 3). For CON, 2 peaks of CH₄ production were observed at around 2 h after feeding, with the largest peak after the morning feeding that represented 66% of the total daily ration. The CH₄ emissions pattern of LIN was similar to CON but emissions of LIN were consistently lesser throughout the day. In contrast to CON, with NIT and LIN+NIT, the peaks were not observed, and CH₄ emissions increased at 3 h post-feeding. Contrary to CH₄, CO₂ emissions (g/d or g/kg DMI) were not affected by dietary treatments.

Table 2 Methane and carbon dioxide emissions of non-lactating cows fed diets containing linseed oil and calcium nitrate alone or in association (n = 4)

Item ¹	Diet ²				SEM	P-value ³		
	CON	NIT	LIN	LIN+NIT		Nitrate	Linseed	Linseed \times nitrate
DM intake, kg/d	12.4	12.3	12.3	12.2	0.59	0.22	0.35	0.86
Methane emissions								
g CH ₄ /d	308.6	238.1	252.7	206.8	9.61	<0.01	<0.01	0.08
g CH ₄ /kg DM intake	25.0	19.4	20.7	17.0	0.70	<0.01	<0.01	0.18
g CH ₄ /kg digested DM	39.3	30.3	32.4	27.0	1.18	<0.01	<0.01	0.14
g CH ₄ /kg digested OM	36.8	28.3	30.3	25.1	1.06	<0.01	<0.01	0.12
g CH ₄ /kg digested NDF	55.9	43.1	47.1	43.1	2.42	0.01	0.06	0.07
% of GE intake	7.2	5.8	5.6	4.8	0.20	<0.01	<0.01	0.24
Carbon dioxide emissions								
g CO ₂ /d	9191	9323	8988	8789	562.1	0.84	0.06	0.35
g CO ₂ /kg DM intake	745	757	732	721	28.1	0.98	0.19	0.49

¹ Data were collected during 4 consecutive days in wk 5.

² CON = control; NIT = diet CON containing 3% calcium nitrate; LIN = diet CON containing 4% linseed oil; LIN+NIT = diet CON containing 4% linseed oil and 3% calcium nitrate.

³ Linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); Nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); Linseed \times nitrate = interaction between main effects of linseed and nitrate.

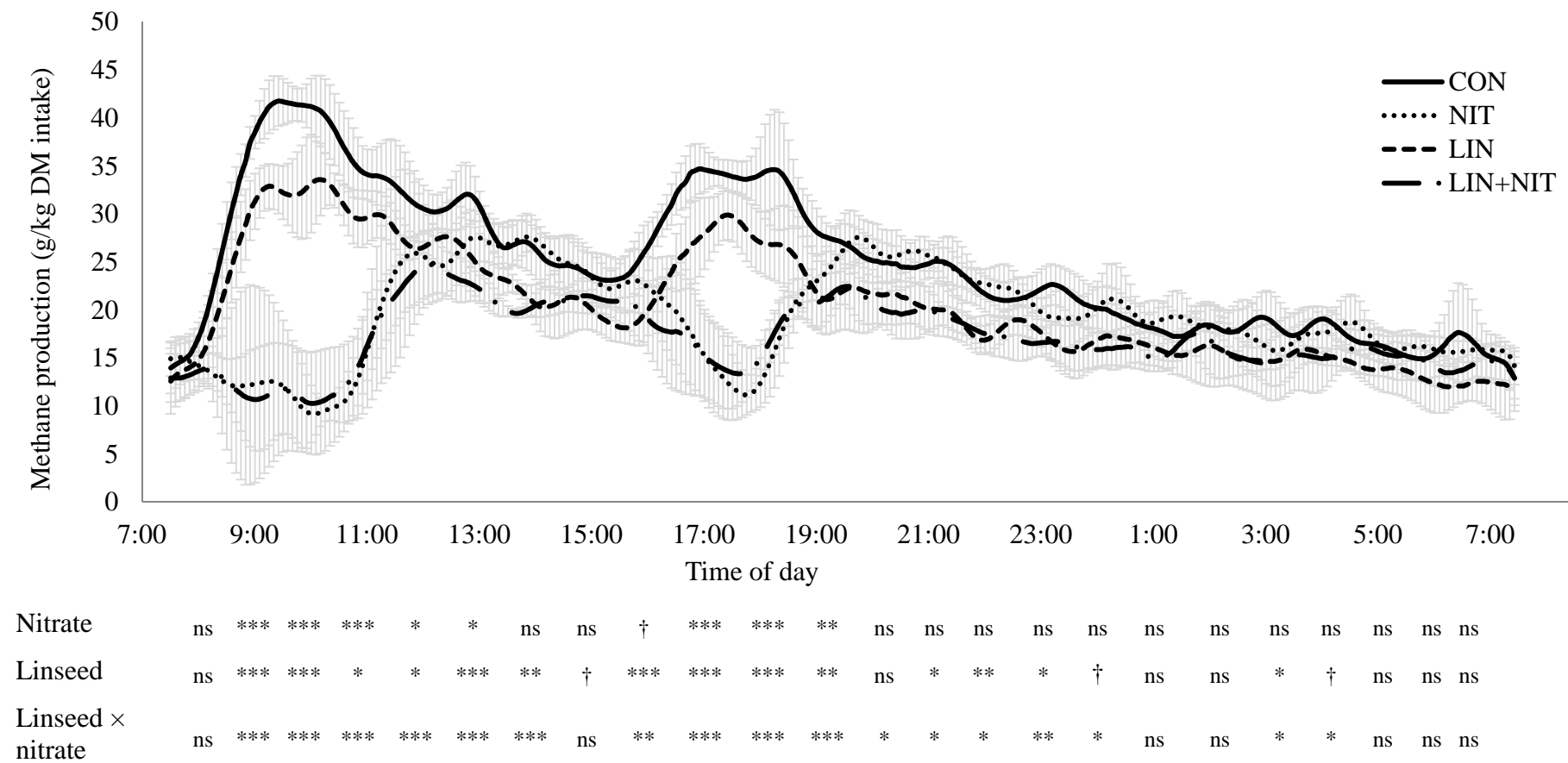


Figure 3 Daily methane production pattern of non-lactating cows fed diets containing linseed oil and calcium nitrate alone or in association (n = 4). Errors bars indicate SD. Treatments consisted of control diet (CON), CON containing 3% calcium nitrate (NIT), CON containing 4% linseed oil (LIN) and CON containing 4% linseed oil and 3% calcium nitrate (LIN+NIT). The arrows indicate time of feeding. Symbols indicate hourly statistical comparison (†*P*<0.10; **P*<0.05; ***P*<0.01; ****P*<0.001) between treatments: linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); linseed × nitrate = interaction between main effects of linseed and nitrate.

Rumen fermentation parameters

Mean rumen pH was greater for NIT and LIN+NIT compared with CON and LIN (Table 3; +0.23 units on average; $P = 0.03$). Diet LIN+NIT showed significantly greater pH values compared with CON during daytime, starting 3 h after the morning feeding (Figure 4). Mean dissolved H_2 concentrations in the rumen tended ($P = 0.07$) to be greater for diets including nitrate compared with other diets (+89%). The H_2 concentration was constantly low up to 6 h post-feeding for CON and LIN (3.8 μM ; Figure 5) but showed a significant jump as early as 1 h post-feeding nitrate (NIT and LIN+NIT). Hydrogen concentrations started to decrease 2 h post-feeding for LIN+NIT and at 3 h post-feeding for NIT. Compared with CON, H_2 concentrations were on average 5.9 and 12.6 times greater for LIN+NIT and NIT treatments, respectively.

Concentrations of total VFA were similar among diets before and after feeding. Linseed-containing diets increased propionate proportions before and after feeding ($P = 0.02$), leading to lesser acetate: propionate and (acetate + butyrate): propionate ratios compared with other diets. Nitrate-containing diets modified VFA profiles after feeding only ($P = 0.01$), with greater acetate and lesser propionate proportions, inducing greater acetate: propionate and (acetate + butyrate): propionate ratios compared with other diets. At least, nitrate-containing diets increased NH_3 -N (+20%; $P = 0.04$) concentrations before feeding. Nitrate concentrations in the rumen were lesser than the limit of quantification (13.3 mg/L or 0.22 mM).

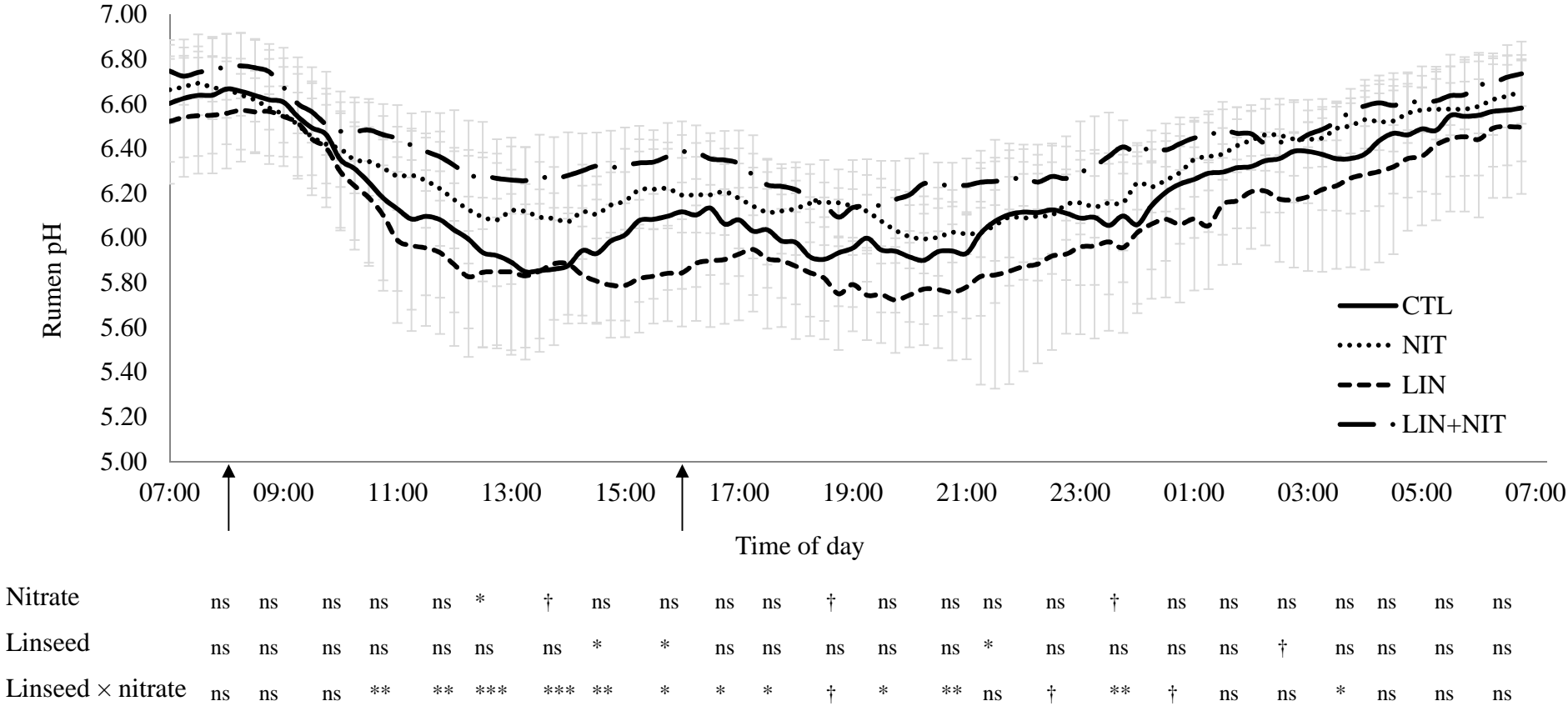


Figure 4 Daily pattern of rumen pH of non-lactating cows fed diets containing linseed oil and calcium nitrate alone or in association (n= 4). Errors bars indicate SD. Treatments consisted in control diet (CON), CON containing 3% calcium nitrate (NIT), CON containing 4% linseed oil (LIN) and CON containing 4% linseed oil and 3% calcium nitrate (LIN+NIT). The arrows indicate time of feeding. Symbols indicate hourly statistical comparison († $P<0.10$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$) between treatments: linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); linseed × nitrate = interaction between main effects of linseed and nitrate.

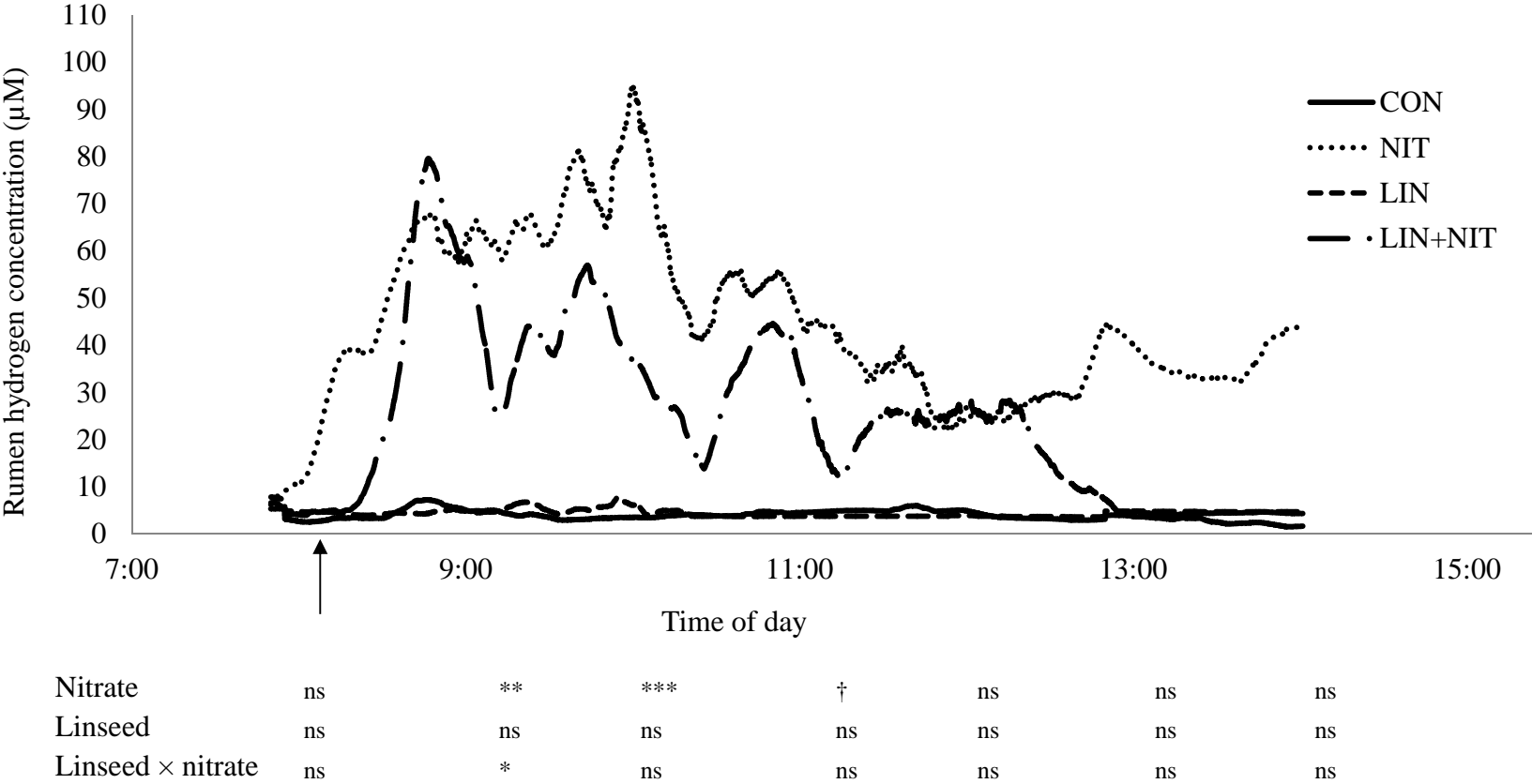


Figure 5 Rumen dissolved hydrogen concentrations up to 6 h after feeding non-lactating cows with diets containing linseed oil and calcium nitrate alone or in association (n = 4). Treatments consisted in control diet (CON), CON containing 3% calcium nitrate (NIT), CON containing 4% linseed oil (LIN) and CON containing 4% linseed oil and 3% calcium nitrate (LIN+NIT). The arrow indicates time of morning feeding. Symbols indicate hourly statistical comparison († $P<0.10$; * $P<0.05$; ** $P<0.01$; *** $P<0.001$) between treatments: linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); linseed × nitrate = interaction between main effects of linseed and nitrate.

Table 3 Rumen fermentation characteristics of non-lactating cows fed diets containing linseed oil and calcium nitrate alone or in association (n = 4)

Item ¹	Time after feeding (h)	Diet ²				SEM	P-value ³		
		CON	NIT	LIN	LIN+NIT		Nitrate	Linseed	Linseed × nitrate
Total VFA, mM	0	73.8	72.7	69.4	71.4	6.42	0.93	0.56	0.75
	3	111.9	102.6	102.6	107.7	6.52	0.74	0.74	0.28
VFA composition, mol/100 mol									
Acetate (A)	0	70.9	69.5	69.5	69.6	1.00	0.53	0.53	0.43
	3	70.2	73.4	67.0	73.1	1.07	0.01	0.15	0.23
Propionate (P)	0	15.0	15.0	17.6	16.0	0.59	0.20	0.02	0.23
	3	15.8	14.8	19.4	15.4	0.95	0.01	0.02	0.06
Butyrate (B)	0	10.3	11.4	9.0	10.4	0.71	0.08	0.11	0.81
	3	10.4	8.7	10.1	8.4	1.20	0.19	0.82	0.98
Minor VFA ⁴	0	3.79	4.15	3.58	3.94	0.321	0.31	0.54	1.00
	3	3.77	3.08	3.54	3.10	0.197	0.01	0.46	0.37
A:P	0	4.74	4.68	3.97	4.41	0.221	0.39	0.04	0.26
	3	4.48	5.03	3.52	4.79	0.233	<0.01	0.01	0.09
(A+B):P	0	5.43	5.44	4.48	5.06	0.230	0.20	0.02	0.22
	3	5.14	5.62	4.07	5.34	0.278	<0.01	0.01	0.08
NH ₃ -N, mM	0	5.84	6.79	4.87	6.68	0.555	0.04	0.34	0.44
	3	15.11	14.34	16.15	14.35	0.932	0.22	0.59	0.60
Total lactate, mM	0	0.56	0.65	0.57	0.65	0.039	0.06	0.81	0.97
	3	0.83	0.71	0.78	0.68	0.107	0.24	0.69	0.91
Nitrate, mg/L ⁵	0	<LoQ	<LoQ	<LoQ	<LoQ	--	--	--	--
	3	<LoQ	<LoQ	<LoQ	<LoQ	--	--	--	--
Nitrite, mg/L	0	0.12	0.58	0.12	0.83	0.246	0.07	0.66	0.66
	3	0.24	0.45	0.24	0.37	0.168	0.32	0.79	0.79
pH	Mean	6.20	6.30	6.07	6.42	0.101	0.03	0.94	0.15
Hydrogen, µM	Mean	3.58	45.28	4.03	21.00	14.097	0.07	0.41	0.39

¹ Data were collected during 2 non-consecutive days in wk 4.² CON = control; NIT = diet CON containing 3% calcium nitrate; LIN = diet CON containing 4% linseed oil; LIN+NIT = diet CON containing 4% linseed oil and 3% calcium nitrate.³ Linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); Nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); Linseed × nitrate = interaction between main effects of linseed and nitrate.⁴ Minor VFA = sum of isobutyrate, isovalerate, valerate and caproate.⁵ LoQ = Limit of Quantification = 13.3 mg/L or 0.22 mM.

The diet LIN decreased ($P = 0.03$) total protozoa concentration in the rumen before feeding whereas NIT did not affect this population. The toxic effect of linseed towards protozoa was not observed when associated with nitrate ($P = 0.02$; Table 4). Compared with CON, diet LIN reduced total protozoa concentration by specifically acting on entodiniomorphs (-52%).

Inversely, diet NIT tended to increase ($P = 0.09$) large entodiniomorphs and increased ($P = 0.02$) *Isotricha* before feeding.

Table 4 Rumen protozoa populations of non-lactating cows fed diets containing linseed oil and calcium nitrate alone or in association (n = 4)

Item ¹	Time after feeding (h)	Diet ²				SEM	P-value ³		
		CON	NIT	LIN	LIN+NIT		Nitrate	Linseed	Linseed × nitrate
Total protozoa, log ₁₀ /mL	0	5.87	5.71	5.55	5.73	0.060	0.91	0.03	0.02
	3	5.71	5.49	5.37	5.58	0.080	0.95	0.14	0.03
Entodiniomorphs, log ₁₀ /mL									
Small (<100 µm)	0	5.86	5.68	5.54	5.71	0.057	0.95	0.03	0.02
	3	5.69	5.46	5.36	5.56	0.080	0.86	0.16	0.03
Large (>100 µm)	0	4.09	4.18	3.66	4.01	0.110	0.09	0.03	0.29
	3	3.97	4.00	3.62	3.97	0.109	0.14	0.13	0.18
Holotrichs, log ₁₀ /mL									
<i>Dasytricha</i> (<100 µm)	0	3.51	3.65	2.67	3.58	0.497	0.29	0.35	0.42
	3	3.49	3.78	2.75	3.69	0.521	0.23	0.40	0.51
<i>Isotricha</i> (>100 µm)	0	1.90	3.19	2.29	3.11	0.484	0.02	0.63	0.47
	3	2.88	3.25	2.53	2.89	0.494	0.42	0.42	1.00

¹ Data were collected during 2 non-consecutive days in wk 4.

² CON = control; NIT = diet CON containing 3% calcium nitrate; LIN = diet CON containing 4% linseed oil; LIN+NIT = diet CON containing 4% linseed oil and 3% calcium nitrate.

³ Linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); Nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); Linseed × nitrate = interaction between main effects of linseed and nitrate.

Diet digestibility and nitrogen balance

Daily DM and OM intake were not affected by treatments and averaged 12.4 kg DMI/d (Table 5). Fiber intake was reduced with linseed-containing diets ($P < 0.01$) compared with other diets. Linseed associated with nitrate had a similar reducing effect towards fiber intake. Total tract digestibility of DM and OM was not affected by diets and linseed supplemented alone or in association with nitrate tended to reduce ($P < 0.10$) fiber digestibility.

Total N losses (% of N intake) were greater for diets including linseed compared with other diets ($P = 0.03$) leading to lesser N retention for LIN and LIN+NIT ($P = 0.03$; Table 6). This was not related to differences in daily fecal N losses between diets, but to numerically greater urinary N losses with linseed-containing diets ($P = 0.08$).

Table 5 Daily nutrient intake and total tract digestibility of non-lactating cows fed diets containing linseed oil and calcium nitrate alone or in association (n = 4)

Item ¹	Diet ²				SEM	P-value ³		
	CON	NIT	LIN	LIN+NIT		Nitrate	Linseed	Linseed × nitrate
Daily nutrient intake, kg/d								
DM	12.4	12.3	12.5	12.3	0.55	0.09	0.73	0.51
OM	11.4	11.3	11.4	11.3	0.51	0.14	0.74	0.45
NDF	5.0	5.0	4.8	4.7	0.22	0.08	<0.01	0.41
ADF	2.9	2.9	2.8	2.7	0.13	0.05	<0.01	0.76
GE intake, MJ/d	216.8	205.1	228.5	217.2	9.67	<0.01	<0.01	0.88
Total tract digestibility, %								
DM	63.7	64.1	64.0	63.3	0.77	0.85	0.65	0.43
OM	68.1	68.5	68.3	67.9	0.64	0.98	0.76	0.50
NDF	44.8	45.2	44.2	40.1	1.58	0.22	0.07	0.14
ADF	44.5	45.1	42.9	38.4	2.11	0.31	0.06	0.20

¹ Data were collected during 6 consecutive days in wk 4.

² CON = control; NIT = diet CON containing 3% calcium nitrate; LIN = diet CON containing 4% linseed oil; LIN+NIT = diet CON containing 4% linseed oil and 3% calcium nitrate.

³ Linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); Nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); Linseed × nitrate = interaction between main effects of linseed and nitrate.

Table 6 Nitrogen balance of non-lactating cows fed diets containing linseed oil and calcium nitrate alone or in association (n = 4)

Item ¹	Diet ²				SEM	P-value ³		
	CON	NIT	LIN	LIN+NIT		Nitrate	Linseed	Linseed × nitrate
N intake, g/d	252.5	242.5	242.5	227.5	11.59	<0.01	<0.01	0.13
Fecal N losses								
g/d	101.6	95.4	94.8	96.8	4.28	0.47	0.37	0.18
As % of N intake	40.1	39.4	39.5	42.5	1.18	0.27	0.25	0.10
Urinary N losses								
g/d	133.1	117.7	135.8	120.2	6.13	0.02	0.61	0.99
As % of N intake	52.5	48.8	56.5	52.7	1.82	0.09	0.08	0.97
Total N losses								
g/d	234.7	213.0	230.6	217.0	9.12	0.01	0.99	0.45
As % of N intake	92.6	88.3	96.0	95.2	2.25	0.20	0.03	0.35
N retained								
g/d	18.5	28.3	10.7	11.8	5.82	0.26	0.03	0.36
As % of N intake	7.4	11.8	4.0	4.8	2.25	0.20	0.03	0.35

¹ Data were collected during 6 consecutive days in wk 4.

² CON = control; NIT = diet CON containing 3% calcium nitrate; LIN = diet CON containing 4% linseed oil; LIN+NIT = diet CON containing 4% linseed oil and 3% calcium nitrate.

³ Linseed = main effect of linseed (CON and NIT versus LIN and LIN+NIT); Nitrate = main effect of nitrate (CON and LIN versus NIT and LIN+NIT); Linseed × nitrate = interaction between main effects of linseed and nitrate.

Discussion

Effect of nitrate on cows' health

In the rumen, nitrate is converted to nitrite and then ammonia. While nitrate is non-toxic, nitrite can be poisonous for the animal. If nitrite accumulates in the rumen, it can pass through the rumen wall into the blood and convert Hb to metHb, which cannot then transport oxygen to the tissues (Lewis, 1951). The level of blood metHb determines the severity of symptoms, which are brown mucous membrane discoloration, depressed feed intake and animal performances, and even coma and death in extreme cases (Bruning-Fann and Kaneene, 1993). Throughout this experiment, animals were unaffected by nitrate supplementation, as shown by the BW gain, the constant intake, and the low rumen concentrations of nitrate and nitrite and blood metHb. Nitrate feeding requires precise management of its distribution and careful control of animal health status. To deal with these issues, the use of slow-release encapsulated nitrate was shown to be effective at mitigating CH₄ emissions of lambs (3.4% nitrate in DM, inducing a 9.7% CH₄ reduction per percent added nitrate; El-Zaiat et al., 2014) or beef heifers

(2.3% nitrate in DM, inducing a 8.0% CH₄ reduction per percent added nitrate; Lee et al., 2014a, b) without raising blood metHb levels.

Methane emissions

We observed that supplying 2.6% added fat from linseed oil reduced CH₄ (g/kg DMI) by 17%, corresponding to a 6.5% reduction in CH₄ per percentage unit of added lipids from linseed. This result is in the range of previous meta-analysis data reporting that CH₄ (g/kg DMI) is reduced by 4.4% per percentage unit of fat (irrespective of lipid source) added to diet (Grainger and Beauchemin, 2011) or by 5.6% per percentage unit of linolenic acid from linseed (Doreau et al., 2011). Conversely, Veneman et al. (2013) did not explain the absence of any CH₄-mitigative effect (g/kg DMI, g/kg milk) of a similar level of linseed oil in lactating cows.

Nitrate fed alone reduced CH₄ (g/kg DMI) by 22%, corresponding to a 9.8% reduction per percentage unit of nitrate fed. This result is in the range of previous experimental data reporting a CH₄ (g/kg DMI) reduction of between 7.9 and 12.2% per percentage unit of added nitrate in the diet of sheep (Nolan et al., 2010; Van Zijderveld et al., 2010) or cattle (Van Zijderveld et al., 2011; Hulshof et al., 2012; Veneman et al., 2013). The CH₄-mitigating effect of nitrate is consequently greatly repeatable whatever the diet and the ruminant species.

The association of nitrate and linseed oil reduced CH₄ (g/kg DMI) by 32%. This result showed for the first time that there is a positive and additive effect between nitrate and linseed oil on methanogenesis. Theoretically, as these dietary strategies have different mechanisms of action, CH₄ reduction should reach 39% for a fully additive effect. Several reasons may explain the difference between theoretical and observed CH₄ reduction. First, we suggest that linseed reduced H₂ production and that nitrate only acted on this reduced H₂ pool. Then, according to stoichiometry and considering that control CH₄ emissions is equal to 100, CH₄ emissions corrected for the CH₄-mitigating effect of LIN (17%) would be $100 - 100 \times 0.17 = 83$. These CH₄ emissions corrected for the CH₄-mitigating effect of NIT (22%) would be $83 - 83 \times 0.22 = 65$. In total, this corresponds to an expected CH₄ reduction of 35% with LIN+NIT, which is close to the observed level of CH₄ reduction. In addition, LIN+NIT had lesser FA content compared with LIN, which may be linked to unnoticed pellets manufacturing issues. Knowing that 1% added fat from linseed reduced CH₄ by 6.5%, the difference in FA content between LIN+NIT (1.0% added fat) and LIN (2.6% added fat) corresponded to a CH₄ mitigation potential of 10.4%, suggesting a fully additive effect

between linseed oil and nitrate. At least, the formation of calcium salts via the reaction between lipids and soluble calcium from calcium nitrate may reduce the additive effects of LIN+NIT (Keyser et al., 1985).

The association of nitrate and linseed oil appears interesting: this same level of CH₄ reduction with linseed oil or nitrate fed individually could not be achieved without greater risks of metHb for nitrate or lesser diet digestibility for linseed oil. Other kinds of antimethanogenic combinations have shown various interactions. Tea saponin and soybean oil reduced CH₄ (g/kg DMI) from lambs by 27% and 14%, respectively, when distributed alone and by 19% when fed in association (Mao et al., 2010). Again in lambs, CH₄ (g/kg DMI) was reduced by 25% by chestnut tannin, 14% by coconut oil and 33% by the association chestnut tannin plus coconut oil (Liu et al., 2011). A fully additive effect was observed with two H₂-sink products fed to lambs, with a CH₄ reduction of 32% with nitrate, 16% with sulfate and 47% with nitrate plus sulfate (Van Zijderveld et al., 2010).

Mechanisms of CH₄ reduction: focus on rumen fermentation parameters

The reduction in CH₄ emissions observed in this trial did not cause a rumen dysfunction, as VFA concentration was not affected by diet and pH was only marginally modified. Two factors may explain the CH₄-mitigating effect of linseed oil. On the one hand, lipids from linseed oil half-reduced the rumen concentration of protozoa, although not as strongly as in previous experiments testing similar levels of lipids (-82% in a silage-based diet, Chung et al., 2011; -84% in a concentrate-rich hay-based diet, Ueda et al., 2003). The anti-protozoal effect of linseed combined with nitrate was less evident, probably because of the lesser fat content in LIN+NIT compared with LIN. Protozoa are known to be important H₂ producers via their hydrogenosomes (Morgavi et al., 2012) and their reduction is often associated with a decrease in methanogenesis (Guyader et al., 2014). Consequently, in this study, linseed supplementation reduced H₂ production, but as dissolved H₂ concentrations in the rumen were not affected by lipids, we assume that methanogens also used less H₂. On the other hand, linseed oil increased propionate proportion which is a H₂-consuming pathway competing with methanogenesis (Newbold et al., 2005). Most literature reports do not show an effect of linseed on rumen VFA composition (Chung et al., 2011; Doreau et al., 2009; Martin et al., 2011). To a minor extent, H₂ may have been consumed during PUFA biohydrogenation, but this pathway would deviate only 1 to 2.6% of ruminal H₂ (Czerkawski, 1986). The lesser CH₄

emissions throughout the day from LIN cows compared with CON cows indicated that linseed oil continuously modified rumen fermentation and microbial parameters.

Nitrate is an electron acceptor in several anaerobic environments. Its CH₄-mitigating effect is assumed to be related to a reduction of H₂ availability for methanogens due to its reduction to nitrite and ammonia (Ungerfeld and Kohn, 2006). To our knowledge, ours is the first study to report a post-feeding pattern of dissolved H₂ concentrations in the rumen. The CON and LIN diets presented stable and low rumen H₂ concentrations (3.8 μ M on average), which are in the range of concentrations (0.1 to 50 μ M) given by a literature review (Janssen, 2010). However, adding nitrate to the diet with or without linseed oil induced a peak in rumen dissolved H₂ concentrations up to 2 h post-feeding (up to 88 μ M on average), coinciding with a drop in CH₄ emissions and a rise of gaseous H₂ (measured in wk 5 of the last two experimental periods; data not shown) as already reported by Van Zijderveld et al. (2011). In presence of nitrate, the excess of dissolved H₂ further released in belched gas means that H₂ was produced at a greater rate than it was utilized. This may result from a toxic effect of nitrate (Van Zijderveld et al., 2010) or nitrite (Iwamoto et al., 2001) on H₂-users such as methanogens. This putative action is transient, lasting for 3 h post-feeding, as shown by the increase in CH₄ emissions from nitrate-fed cows up to levels similar to control-diet cows.

Diet digestibility and nitrogen balance

Supplying diets with linseed oil (2.6% added fat) did not affect total tract digestibility of DM and OM but tended to reduce total tract fiber digestibility to a same extent when fed alone or in association with nitrate. This result is not consistent with a previous study on lambs supplemented with crude linseed (2.4% added fat; Machmüller et al., 2000). These different results may be explained by the forms of linseed which affect availability of lipids supply: linseed oil would have a more negative effect on total tract digestibility than extruded and crude linseed (Martin et al., 2008). Adding 3% calcium nitrate as a substitute for urea did not reduce total tract digestibility confirming previous experiments on sheep fed hay and 4% potassium nitrate (Nolan et al., 2010) and on dairy cows fed maize silage and 2.8% calcium nitrate (Van Zijderveld et al., 2011). Nitrate neither affected N retention nor the distribution of N losses between urine and feces. Similar results were obtained with dairy cows (2.6% nitrate; Van Zijderveld et al., 2011), steers (2.3% nitrate; Lee et al., 2014a) and lambs (2.3% nitrate; Li et al., 2012) fed isonitrogenous diets, showing that nitrate can substitute urea as a source of non-protein N.

The association of nitrate and linseed oil is an efficient strategy to decrease CH₄ yields in non-lactating cows without altering diet digestibility. Linseed oil supplementation reduced CH₄ emissions throughout the day, while nitrate had a transient but marked action from when fed up to 3 h post-feeding. Methane production was further reduced when both linseed and nitrate were fed in association. Linseed oil reduced H₂-producers like protozoa, whereas nitrate acted as a H₂-sink and may have inhibited rumen H₂-users, as suggested by the rise of dissolved H₂ concentrations with this dietary treatment. Further work to characterize the quantity, activity and diversity of rumen microbiota should clarify the mechanisms behind the effects of these dietary treatments. In addition, it will be necessary to assess the long-term CH₄-mitigative effect of linseed oil associated with nitrate on farmed ruminants. Finally, the effect of nitrate on animal performances and the absence of residues in ruminant end-products still need further research.

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Literature cited

- AOAC. 2005. Official Methods of Analysis. 18th ed. Assoc. Off. Anal. Chem., Arlington, VA.
- Bruning-Fann, C. S., and J. B. Kaneene. 1993. The effects of nitrate, nitrite, and N-nitroso compounds on animal health. *Vet. Hum. Toxicol.* 35: 237-253.
- Chilliard, Y., C. Martin, J. Rouel, and M. Doreau. 2009. Milk fatty acids in dairy cows fed whole crude linseed, extruded linseed, or linseed oil, and their relationship with methane output. *J. Dairy Sci.* 92: 5199-5211.
- Chung, Y. H., M. L. He, S. M. McGinn, T. A. McAllister, and K. A. Beauchemin. 2011. Linseed suppresses enteric methane emissions from cattle fed barley silage, but not from those fed grass hay. *Anim. Feed Sci. Technol.* 166-167: 321-329.
- Czerkawski, J. W. 1986. An introduction to rumen studies. Pergamon Press, Oxfordshire and New York.

- Doreau, M., E. Aurousseau, and C. Martin. 2009. Effects of linseed lipids fed as rolled seeds, extruded seeds or oil on organic matter and crude protein digestion in cows. *Anim. Feed Sci. Technol.* 150: 187-196.
- Doreau, M., L. Bamière, C. Pellerin, M. Lherm, and M. Benoit. 2014a. Mitigation of enteric methane for French cattle: Potential extent and cost of selected actions. *Anim. Prod. Sci.* 54: 1417-1422.
- Doreau, M., A. Ferlay, Y. Rochette, and C. Martin. 2014b. Effects of dehydrated lucerne and soya bean meal on milk production and composition, nutrient digestion, and methane and nitrogen losses in dairy cows receiving two different forages. *Animal* 8: 420-430.
- Doreau, M., C. Martin, M. Eugène, M. Popova, and D. P. Morgavi. 2011. Leviers d'action pour réduire la production de méthane entérique par les ruminants. *INRA Prod. Anim.* 24: 461-474.
- El-Zaiat, H. M., R. C. Araujo, Y. A. Soltan, A. S. Morsy, H. Louvandini, A. V. Pires, H. O. Patino, P. S. Correa, and A. L. Abdalla. 2014. Encapsulated nitrate and cashew nut shell liquid on blood and rumen constituents, methane emission, and growth performance of lambs. *J. Anim. Sci.* 92: 2214-2224.
- Faisant, N., V. Planchot, F. Kozlowski, M. P. Pacouret, P. Colonna, and M. Champ. 1995. Resistant starch determination adapted to products containing high level of resistant starch. *Sci. Aliments* 15: 83-89.
- Gerber, P. J., H. Steinfeld, B. Henderson, A. Mottet, C. Opio, J. Dijkman, A. Falcucci, and G. Tempio. 2013. Tackling climate change through livestock – A global assessment of emissions and mitigation opportunities. Food and Agriculture Organization of the United Nations, Rome, Italy.
- Grainger, C., and K. A. Beauchemin. 2011. Can enteric methane emissions from ruminants be lowered without lowering their production ? *Anim. Feed Sci. Technol.* 166-167: 308-320.
- Guyader, J., M. Eugène, P. Nozière, D. P. Morgavi, M. Doreau, and C. Martin. 2014. Influence of rumen protozoa on methane emissions in ruminants: A meta-analysis approach. *Animal* 8: 1816-1825.
- Hegarty, R. S., and R. Gerdes. 1999. Hydrogen production and transfer in the rumen. *Recent Advances in Animal Nutrition* 12: 37-44.

- Hulshof, R. B. A., A. Berndt, W. J. J. Gerrits, J. Dijkstra, S. M. Van Zijderveld, J. R. Newbold, and H. B. Perdok. 2012. Dietary nitrate supplementation reduces methane emission in beef cattle fed sugarcane based diets. *J. Anim. Sci.* 90: 2317-2323.
- INRA. 2010. Alimentation des bovins, ovins et caprins. INRA Editions, Paris, France.
- Iwamoto, M., N. Asanuma, and T. Hino. 2001. Effects of energy substrates on nitrate reduction and nitrate reductase activity in a ruminal bacterium, *Selenomonas ruminantium*. *Anaerobe* 7: 315-321.
- Janssen, P. H. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Anim. Feed Sci. Technol.* 160: 1-22.
- Johnson, K. A., and D. E. Johnson. 1995. Methane emissions from cattle. *J. Anim. Sci.* 73: 2483-2492.
- Jouany, J. P. 1989. Effects of diet on populations of rumen protozoa in relation to fibre digestion. In: J. V. Nolan, R. A. Leng and D. I. Demeyer (eds.) *The roles of protozoa and fungi in ruminant digestion*, Proceedings of an International seminar sponsored by the OECD Co-operative research project on food production and preservation, and the University of New England, held 26-29 September, 1988. Penambul Books, Armidale, NSW 2351, Australia. p 59-74.
- Kaplan, J. C. 1965. Méthode de mesure rapide du taux de la méthémoglobine dans les globules rouges. *Rev. Fr. Etud. Clin. Bio.* 10: 856-859.
- Keyser, R. B., C. H. Noller, L. J. Wheeler, and D. M. Schaefer. 1985. Characterization of limestones and their effects in vitro and in vivo in dairy cattle. *J. Dairy Sci.* 68: 1376-1389.
- Krause, K. M., and G. R. Oetzel. 2006. Understanding and preventing subacute ruminal acidosis in dairy herds: A review. *Anim. Feed Sci. Technol.* 126: 215-236.
- Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2014a. Effects of encapsulated nitrate on nitrogen utilization and enteric methane emissions in beef cattle. In: *Linking animal science and animal agriculture: Meeting the global demands of 2050*, Kansas city, USA. p 323.
- Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2014b. Effects of encapsulated nitrate on toxicity, feed intake and feed consumption rates in beef cattle. In: *Linking animal science and animal agriculture: Meeting the global demands of 2050*, Kansas city, USA. p 846.

- Lee, C., and K. A. Beauchemin. 2014. A meta-analysis of effects of feeding nitrate on toxicity, production, and enteric methane emissions in ruminants. In: *Linking animal science and animal agriculture: Meeting the global demands of 2050*, Kansas City, USA. p 845-846.
- Lewis, D. 1951. The metabolism of nitrate and nitrite in the sheep. 1 The reduction of nitrate in the rumen of the sheep. *Biochem. J.* 48: 175-180.
- Li, L., J. Davis, J. V. Nolan, and R. S. Hegarty. 2012. An initial investigation on rumen fermentation pattern and methane emission of sheep offered diets containing urea or nitrate as the nitrogen source. *Anim. Prod. Sci.* 52: 653-658.
- Liu, H., V. Vaddella, and D. Zhou. 2011. Effects of chestnut tannins and coconut oil on growth performance, methane emission, ruminal fermentation, and microbial populations in sheep. *J. Dairy Sci.* 94: 6069-6077.
- Machmüller, A., D. A. Ossowski, and M. Kreuzer. 2000. Comparative evaluation of the effects of coconut oil, oilseeds and crystalline fat on methane release, digestion and energy balance in lambs. *Anim. Feed Sci. Technol.* 85: 41-60.
- Mao, H. L., J. K. Wang, Y. Y. Zhou, and J. X. Liu. 2010. Effects of addition of tea saponins and soybean oil on methane production, fermentation and microbial population in the rumen of growing lambs. *Livest. Sci.* 129: 56-62.
- Martin, C., D. Pomiès, A. Ferlay, Y. Rochette, B. Martin, Y. Chilliard, D. P. Morgavi, and M. Doreau. 2011. Methane output and rumen microbiota in dairy cows in response to long-term supplementation with linseed or rapeseed of grass silage- or pasture-based diets. *Proc. N.Z. Soc. Anim. Prod.* 71: 243-247.
- Martin, C., J. Rouel, J. P. Jouany, M. Doreau, and Y. Chilliard. 2008. Methane output and diet digestibility in response to feeding dairy cows crude linseed, extruded linseed, or linseed oil. *J. Anim. Sci.* 86: 2642-2650.
- McGinn, S. M., K. A. Beauchemin, T. Coates, and D. Colombatto. 2004. Methane emissions from beef cattle: Effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. *J. Anim. Sci.* 82: 3346-3356.
- Morgavi, D. P., E. Forano, C. Martin, and C. J. Newbold. 2010. Microbial ecosystem and methanogenesis in ruminants. *Animal* 4: 1024-1036.
- Morgavi, D. P., J. P. Jouany, and C. Martin. 2008. Changes in methane emission and rumen fermentation parameters induced by refaunation in sheep. *Anim. Prod. Sci.* 48: 69-72.

- Morgavi, D. P., C. Martin, J. P. Jouany, and M. J. Ranilla. 2012. Rumen protozoa and methanogenesis: not a simple cause-effect relationship. *Br. J. Nutr.* 107: 388-397.
- Morris, M. P., B. Cancel, and A. Gonzalez-Mas. 1958. Toxicity of nitrates and nitrites to dairy cattle. *J. Dairy Sci.* 41: 694-696.
- Newbold, C. J., S. Lopez, N. Nelson, J. O. Ouda, R. J. Wallace, and A. R. Moss. 2005. Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation in vitro. *Br. J. Nutr.* 94: 27-35.
- Nolan, J. V., R. S. Hegarty, J. Hegarty, I. R. Godwin, and R. Woodgate. 2010. Effects of dietary nitrate on fermentation, methane production and digesta kinetics in sheep. *Anim. Prod. Sci.* 50: 801-806.
- Pinares-Patiño, C. S., C. Hunt, R. Martin, J. West, P. Lovejoy, and G. Waghorn. 2012. Chapter 1: New Zealand ruminant methane measurement centre, AgResearch, Palmerston North. In: C. S. Pinares Patino and G. Waghorn (eds.) Technical manual on respiration chamber designs Ministry of Agriculture and Forestry, Wellington, New Zealand. p.
- Sar, C., B. Santoso, B. Mwenya, Y. Gamoa, T. Kobayashi, R. Morikawa, K. Kimura, H. Mizukoshi, and J. Takahashi. 2004. Manipulation of rumen methanogenesis by the combination of nitrate with b1-4 galacto-oligosaccharides or nisin in sheep. *Anim. Feed Sci. Technol.* 115: 129-142.
- Scollan, N. D., N. J. Choi, E. Kurt, A. V. Fisher, M. Enser, and J. D. Wood. 2001. Manipulating the fatty acid composition of muscle and adipose tissue in beef cattle. *Br. J. Nutr.* 85: 115-124.
- The United States Pharmacopeial Convention. 2008. Methylene blue (Veterinary-Systemic). <http://vetmed.tamu.edu/common/docs/public/aavpt/methyleneBlue.pdf>. Accessed 23 May 2014.
- Ueda, K., A. Ferlay, J. Chabrot, J. J. Looor, Y. Chilliard, and M. Doreau. 2003. Effect of linseed oil supplementation on ruminal digestion in dairy cows fed diets with different forage:concentrate ratios. *J. Dairy Sci.* 86: 3999-4007.
- Ungerfeld, E. M., and R. A. Kohn. 2006. The role of thermodynamics in the control of ruminal fermentation. In: K. Sejrsen, T. Hvelplund and M. O. Nielsen (eds.) Ruminant physiology: Digestion, metabolism and impact of nutrition on gene expression,

- immunology and stress Wageningen Academic Publishers, Wageningen, The Netherlands. p 55-85.
- Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and non starch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74: 3583-3597.
- Van Zijderveld, S. M., W. J. J. Gerrits, J. A. Apajalahti, J. R. Newbold, J. Dijkstra, R. A. Leng, and H. B. Perdok. 2010. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. *J. Dairy Sci.* 93: 5856-5866.
- Van Zijderveld, S. M., W. J. J. Gerrits, J. Dijkstra, J. R. Newbold, R. B. A. Hulshof, and H. B. Perdok. 2011. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. *J. Dairy Sci.* 94: 4028-4038.
- Veneman, J. B., S. Muetzel, K. J. Hart, C. L. Faulkner, J. M. Moorby, G. Molano, H. B. Perdok, J. R. Newbold, and C. J. Newbold. 2014. Dietary nitrate but not linseed oil decreases methane emissions in two studies with lactating dairy cows. In: *Livestock, Climate Change and Food Security Conference*, Madrid, Spain.
- Veneman, J. B., S. Muetzel, H. B. Perdok, and C. J. Newbold. 2013. Dietary nitrate but not linseed oil decreases methane emissions in lactating dairy cows fed a maize silage based diet. In: *Proceedings of the 5th Greenhouse Gases and Animal Agriculture Conference*, 23-26 June 2013, Dublin, Ireland
- Williams, A. G., and G. S. Coleman. 1992. *The rumen protozoa*. Springer-Verlag, New York.


STEP 2: Long-term methane mitigating effect of linseed plus nitrate supplemented to dairy cows

Objective

1/ To evaluate the long-term effect of association of feeding strategies acting on H₂ production (lipids from linseed, toxic effect towards protozoa) and H₂ utilization (nitrate from calcium nitrate, H₂-sink through nitrate reduction to nitrite and ammonia) on CH₄ emissions, lactating performances of dairy cows and animal health (blood metHb, nitrate and nitrite residues in milk and processed milk products).

2/ To check the effect of linseed plus nitrate on total tract digestibility, N balance and rumen fermentation after long-term supplementation.

Experimental approach

16 lactating cows  → 8 animals → **CON:** 54% corn silage + 6% hay + 40% pelleted concentrate
 → 8 animals → **LIN+NIT:** CON + 1.8% nitrate (from calcium nitrate)
 + 3.5% added lipids (from extruded linseed)

17 weeks of experiment (wk 1 to 3 = Adaptation; wk 4 to 17 = Measurement)

WEEK	4	5	6	7	8	9	10	11	12	13	14	15	16	17
Daily intake and milk yield														
Milk composition (once a week)														
Nitrate and nitrite residues in milk and milk products (once a week)														
Blood metHb (3.5 h after morning feeding, once a week)														
Daily kinetics of CH ₄ emissions (2 days)														
Total tract digestibility, N balance (5 days)														
Rumen fermentation (3.5 h after morning feeding, once a week)														



Main results

- Throughout the experiment, intake and milk production tended to be lower for dairy cows supplemented with LIN+NIT, but feed efficiency was similar between diets.
- From wk 4 to 17, average metHb level was 1.2%. No additional nitrate and nitrite residues were detected in milk and processed milk products from cows fed LIN+NIT.
- Diet LIN+NIT reduced CH₄ emissions by 29%, with a persistent effect throughout the 4 months of the experiment.
- Digestibility of nutrients and N balance were similar between diets. Diet LIN+NIT reduced total VFA concentration and increased C2/C3 ratio and protozoa concentration postfeeding.

Conclusion

The association of linseed plus nitrate is an efficient and long-term CH₄-mitigating strategy, which does not alter diet digestibility, N efficiency or animal health. However, the energetic benefits of the decreased CH₄ emissions did not appear beneficial for the animal.

Long-term methane-mitigating effect of linseed plus nitrate supplemented to dairy cows

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Abstract

The objective of this experiment was to study the long-term effect of linseed plus nitrate on CH₄ emission and performance in dairy cows. We also assessed the effect of this feeding strategy on the presence of nitrate and nitrite residues in milk products, total tract apparent digestibility, N balance and rumen fermentation. Sixteen lactating Holstein cows were allocated to 2 groups in a randomized design conducted in parallel for 17 weeks. Diets were (dry matter basis): 1) control (54% corn silage, 6% hay, and 40% concentrate) or 2) control plus 3.5% added fat from linseed and 1.8% nitrate (LIN+NIT). Diets were equivalent in terms of crude protein (16%), starch (28%), and neutral detergent fiber (33%), and were offered twice daily. Cows were fed ad libitum, except during wk 5, 16, and 17 in which feed was restricted to 95% of dry matter intake (DMI) to ensure complete consumption of meals. Milk production and DMI were measured weekly. Nitrate and nitrite concentration in milk and milk products was determined monthly. Daily methane emissions were quantified in open chambers (wk 5 and 16). Total tract apparent digestibility, N balance, and rumen fermentation parameters were determined at the end of the experiment (wk 17). Daily DMI tended to be lower with LIN+NIT from wk 4 to 16 (-5.1 kg/d on average). The LIN+NIT diet decreased milk production during 6 non-consecutive weeks (-2.5 kg/d on average). Nitrate or nitrite residues were not detected in milk and associated products of cows fed either diet. The LIN+NIT diet reduced CH₄ emissions to a similar extent at the beginning (wk 5) and end (wk 16) of the trial: CH₄ reduction averaged 46% (g/d), 29% (g/kg DMI), and 35% (g/kg milk). Both diets did not affect N efficiency and nutrients apparent digestibility. In the rumen, LIN+NIT did not affect protozoa number but reduced total volatile fatty acid concentration by 12% and propionate concentration by 31%. We concluded that linseed plus nitrate has a long-term methane-reducing effect in dairy cows. We also found a concomitant negative effect on milk production, despite a similar feed efficiency between diets. Further work is required to optimize the doses of linseed plus nitrate to avoid reduced cows performance. The consumption of milk products from animals fed nitrate is safe for human consumption in terms of nitrate and nitrite residues.

Keywords: linseed plus nitrate, long-term, methane, milk product, ruminant

Introduction

Linseed and nitrate are both proven dietary strategies for reducing CH₄ emissions from ruminants (Gerber et al., 2013). If used extensively, they could significantly abate enteric CH₄ emissions at a national scale (Doreau et al., 2014). However, the combination of these two feeding strategies on CH₄ production has not been studied before. In a short-term experiment on non-lactating cows, we reported that the combination of linseed oil (4% of DM) plus nitrate (2.25% of DM) reduced methanogenesis by 32% without affecting apparent diet digestibility. Compared to linseed oil and nitrate fed individually, the effect of this combination on CH₄ production was additive (Guyader et al., 2014a), because these two dietary strategies share different modes of action in the rumen. Polyunsaturated lipids from linseed are thought to act as inhibitors of H₂-producers such as protozoa (Guyader et al., 2014a), whereas nitrate is thought to act as a H₂-sink, competing with methanogenesis. Nitrate and nitrite are also toxic to methanogens (Guyader et al., 2014c).

In-practice, the use of these strategies at farm scale requires further investigation into their potential long-term effects. Linseed (3% added lipids) had a persistent CH₄-mitigating effect on dairy cows for up to 1 yr (Martin et al., 2011). The long-term CH₄-mitigating effect of nitrate (2.1% of DM) fed over 3 mo has been demonstrated in dairy cows (Van Zijderveld et al., 2011). However, the long-term CH₄-reducing effect of dietary linseed plus nitrate has not been tested.

Another issue to assess before practical application of linseed plus nitrate as an animal nutrition strategy is the potential for adverse effects of nitrate supplementation on human and animal health. To our knowledge, the effect of dietary nitrate on milk quality, including the absence of nitrate and nitrite residues in milk, has not been tested, whereas excess nitrite from nitrate reduction in the mouth may promote gastric irritation in humans (Weitzberg and Lundberg, 2013). One study did show an absence of additional nitrate and nitrite residues in meat when lambs were fed 3.4% encapsulated nitrate (El-Zaiat et al., 2013). Nitrate may also alter animal health by increasing the concentration of blood methemoglobin (metHb; Lewis, 1951). Without adaptation (Lee and Beauchemin, 2014), nitrite from nitrate reduction can accumulate in the rumen, passing through the blood and leading to subclinical methemoglobinemia (30-40% of metHb; Bruning-Fann and Kaneene, 1993).

The main objective of this experiment was to investigate the long-term effect of linseed plus nitrate on CH₄ emissions and lactation performance in dairy cows. As a secondary objective, nitrate metabolism was assessed by measuring metHb levels in blood and nitrate and nitrite

levels in milk and processed milk products. We also evaluated the effect of linseed plus nitrate on total tract apparent digestibility, N balance, and rumen fermentation parameters at the end of the experiment.

Materials and methods

The experiment was conducted at the UERT experimental dairy cow facilities at the INRA's Saint-Genès-Champanelle-based research centre in France from January to May 2014. All procedures involving animals were performed in accordance with French Ministry of Agriculture guidelines for animal research, and all applicable European guidelines and regulations on animal experimentation (http://www2.vet-lyon.fr/ens/expa/acc_regl.html).

Animals, Diets and Feeding

Sixteen lactating (including 7 primiparous) Holstein cows were used. At the start of the experiment, cows had an average milk yield of 33.4 ± 7.1 kg/d at 61 ± 23 DIM, and an average BW of 706 ± 67 kg. The experiment was conducted for 17 wk as a randomized block design where cows were separated into 2 groups balanced for calving date and milk production. Cows were housed in a freestall barn except during the 2 measurement periods (wk 5 and wk 16-17 for CH₄ and digestibility measurements) in which they were housed individually.

The first group of cows ($n = 8$ of which 4 primiparous) was fed the control diet (CON), and the second group of cows ($n = 8$ of which 3 primiparous) was fed CON with 9.8% extruded linseed and 2.4% calcium ammonium nitrate (75% NO₃ in DM) on a DM basis (LIN+NIT). The doses of extruded linseed and nitrate were estimated to reduce CH₄ emission by 10 to 15% when fed alone (Doreau et al., 2014) and by 20 to 30% when fed together. Diets were formulated to meet the requirements of lactating dairy cows (30 kg daily milk production without BW change) and to be equivalent in terms of CP, gross energy (GE) and starch content (INRA, 2010; Table 1). On a DM basis, diets were composed of 54% corn silage, 6% natural grassland hay, and 40% concentrate given as pellets (InVivo NSA, Longué Jumelles, France).

Table 1 Ingredients and chemical composition of the experimental diets

Item	Diet ¹	
	CON	LIN+NIT
Ingredients, % of DM		
Corn silage ²	54.00	54.00
Hay	6.00	6.00
Pelleted concentrate		
Corn	11.88	12.00
Barley	3.36	2.52
Soybean meal	5.24	1.28
Rapeseed meal	2.00	3.12
Sunflower meal	0.00	0.80
Extruded linseed ³	0.00	9.80
Soybean hulls	6.60	2.00
Wheat bran	6.00	4.20
Dehydrated beet pulp	0.94	0.00
Calcium ammonium nitrate ⁴	0.00	2.40
Urea	0.80	0.00
Calcium carbonate	1.13	0.00
Dicalcium phosphate	0.44	0.26
Beet molasses	1.20	1.20
Mineral-vitamin premix	0.20	0.20
Sodium chloride	0.17	0.18
Fungicide	0.02	0.02
Flavoring ⁵	0.02	0.02
Chemical composition ⁶ , % of DM		
OM	93.06	93.50
CP	15.81	15.59
NDF	34.74	31.91
ADF	18.20	16.58
Starch	27.98	28.78
Ether extract, % of DM	3.23	6.75
Total fatty acid, % of DM	2.54	5.86
Gross energy, MJ/kg of DM	17.64	18.37
FA profile, % of total FA		
C16:0	16.87	13.89
C18:0	2.40	2.74
C18:1 n-9	25.06	23.34
C18:2 n-6	43.24	31.59
C18:3 n-3	9.06	25.05

¹ CON = diet control; LIN+NIT = diet CON containing 10% extruded linseed and 1.8% nitrate on a DM basis.

² Fermentation characteristics of fresh silage juice: pH = 3.57; Acetic acid = 0.74 g/100g; Lactic acid = 3.01 g/100g; N-NH₃ = 0.02 g/100g.

³ Extruded linseed, InVivo NSA, Longué Jumelles, France

⁴ Calcium ammonium nitrate (5Ca(NO₃)₂.NH₄NO₃.10H₂O; Phytosem, Pont-du-Château, France) contained 75% NO₃ on a DM basis.

⁵ Gusti, Nutriad, Chester, England.

⁶ Average of chemical composition from samples (n = 3) taken in wk 5, 16 and 17.

Two weeks before starting the experiment, all cows were fed CON diet ad libitum. Then, LIN+NIT-group animals were diet-adapted by progressively replacing CON concentrate with LIN+NIT concentrate over a 2-wk adaptation period to achieve the dose of 2.4% calcium ammonium nitrate at the beginning of wk 3. Hay was offered once daily (0800 h) and corn silage mixed with concentrates was offered twice daily (66% at 0930 h and 34% at 1600 h). All cows were fed ad libitum except during measurement weeks in which offered feed was restricted to 95% of individual voluntary feed intake to ensure complete consumption of the diet. Forage-to-concentrate ratio was kept as close as possible to the target ratio by adjusting the amounts of offered feed every week based on quantity and composition of the refusals of the previous week. Cows had free access to water throughout the experiment.

Measurements and Analyses

Liveweight and Blood Methemoglobin. Animals were weighed the week before starting the experiment (wk 0) then in wk 5, 10, 14, and 20. Blood metHb levels were measured 3.5 h after morning feeding on cows fed LIN+NIT and compared with levels of control samples taken on these same animals in wk 0. Blood was then sampled twice a week from wk 1 to wk 3 (adaptation to nitrate) and once a week from wk 4 to the end of the experiment (wk 17). Blood (10 mL) was sampled from the tail vein into K2-EDTA collection tubes (Venosafe, Terumo, Guyancourt, France) then carried on ice to the nearest hospital (CHU Gabriel Montpied, Clermont-Ferrand, France) to determine metHb concentrations by spectrophotometry within 1 h (UV-160, Shimadzu, Marne-La-Vallée, France; Kaplan, 1965).

Intake. Offered feed and refusals were weighed and recorded daily throughout the experiment. During the 2 measurement periods (wk 5 and wk 16-17), samples (200 g) of hay and concentrates were taken once a week, and samples (200 g) of corn silage were taken twice a week. For each feed sample, one aliquot was used to determine DM content (103°C for 24 h) and the other aliquot was stored at 4°C (hay and concentrates) or -20°C (corn silage) until analysis of chemical composition. Refusals were measured for DM when they exceeded 2 kg/d per animal during measurement weeks. Composition of refusals was identified as forage (hay, corn silage) or concentrate, and their chemical composition was considered similar to that of feed.

Chemical composition analyses were carried out on fresh (hay, concentrates) or freeze-dried (corn silage) feedstuff samples after grinding (1 mm) (InVivo Labs, Chierry, France). Organic

matter was determined by ashing at 550°C for 6 h (method 942.05; AOAC, 2005). Total N was analyzed by combustion according to the Dumas method (method 968.06; AOAC, 2005), and CP content was calculated as $N \times 6.25$. Fiber (NDF and ADF) was determined by sequential procedures (Van Soest et al., 1991) after pretreatment with amylase and sulfuric acid, and was expressed exclusive of residual ash. Starch was analyzed using an enzymatic method (Faisant et al., 1995), and gross energy was analyzed by adiabatic bomb calorimetry (C200 model, IKA, Staufen, Germany). Ether extract was determined after acid hydrolysis (method 954.02; AOAC, 2005), and FA composition was determined by gas chromatography of methyl esters (method 969.33; AOAC, 2005). Juice from fresh corn silage was obtained by maceration to analyze pH, N-NH₃ (Kjeldahl method, method 2001.11; AOAC, 2005), acetic and lactic acid (gas chromatography with a flame ionization detector) concentrations (InVivo Labs, Chierry, France).

Methane Emissions. Daily total CH₄ emissions were measured continuously using 4 open chambers (1 animal per chamber) in wk 5 and 16. Each animal spent 2 consecutive days (48 h) in a chamber to measure the CH₄ emissions of the 8 animals from a same group within the week. Animals were allocated to the same chamber for both measurement periods.

The chambers (16.6 m³) were made of steel uprights with clear polycarbonate walls allowing sight contact between animals and with the farm personnel. They operated at a slight negative pressure, with an airflow oscillating between 700 and 800 m³/h (approximately 45 air renewals per hour). Airflow entered the chamber through an aperture at the bottom of the rear door (0.42 m²), and exited through an exhaust duct situated at the top of the chamber, over the head of the animal. Airflow in the exhaust duct of each chamber was continuously measured (CP300, KIMO, Montpon-Ménestérol, France), and recorded once every 5 min (KT-210-AO, KIMO, Montpon-Ménestérol, France). Concentration of gases in the barn and in the 4 chambers was alternatively analyzed at a 0.1 Hz sample frequency for 5 min every 25 min using an infrared detector (Ultramat 6, Siemens, Karlsruhe, Germany) and recorded (Nanodac Invensys, Eurotherm Automation SAS, Dardilly, France). Gas concentrations between 2 measurement intervals in the barn and in the chambers were estimated by linear regression. The detector was manually calibrated the day before each measurement week using pure N₂ and a mixture of CH₄ (650 ppm) and CO₂ (700 ppm) in N₂.

Chamber rear doors were opened twice daily: in the morning for milking and to remove feces and urine, and in the afternoon for milking. Chamber front doors were opened 3 times a day

for feeding. Front and rear doors were not simultaneously opened in order to avoid an air stream into the chamber. In total, the doors of each chamber were opened for 30 min per 24 h. Data collected while doors were open were deleted and a proportional calculation was applied to recover 24-h CH₄ emissions.

Diet Apparent Digestibility and Nitrogen Balance. Total tract apparent digestibility and N balance were determined from total and separate collection of feces and urine for 5 d during wk 17. To separate urine from feces, cows were fitted with flexible tubes connected to a 30-L flask containing 500 mL of 3 M sulfuric acid to achieve a urine pH lower than 3 and thereby avoid N volatilization. Feces and urine were removed once daily.

Every day, after weighing and mixing of feces, a 1% fresh aliquot was used to determine DM (103°C for 24 h), and another 1% fresh aliquot was pooled across days for each animal and frozen (-20°C). At the end of the experiment, pooled samples were thawed, freeze-dried, and ground (1 mm) to determine OM, N, NDF, and ADF content as previously described for feed (InVivo Labs, Chierry, France).

For urine, every day after weighing, a 1% fresh aliquot was pooled across days for each animal and frozen (-20°C). At the end of the experiment, after thawing, the N content of urine was determined by the Kjeldahl method (method 2001.11; AOAC, 2005 ; InVivo Labs, Chierry, France).

Milk Yield and Composition. Throughout the experiment, milk yield was determined daily. For determination of milk composition (fat, protein, lactose, and urea concentration), individual milk samples (30 mL) mixed with potassium bichromate (Merck, Fontenay-Sous-Bois, France) were taken and stored at 4°C before analysis within 2 d (Galilait, Theix, France). Samples were taken at morning and afternoon milking 2 d per week when animals were in the CH₄ chambers (wk 5 and 16). Milk fat, protein, and lactose content were analyzed by infrared spectrometry with a 3-channel spectrophotometer (MilkoScan, Foss Electric, Hillerød, Denmark; method 972.16; AOAC, 1990). Milk urea concentration was analyzed by the dimethylamino-4-benzaldehyde colorimetric method (Potts, 1967).

For analysis of nitrate and nitrite residues in individual milk, samples (300 mL) from the morning milking were taken once a week in wk 5, 9, 13, and 17. For analysis of nitrate and nitrite residues in pooled milk and milk products, the morning milk of all animals was pooled by diet in wk 9 and 17. Pooled milk was sampled (100 mL) and local farmhouse-style

products were made (yoghurts, whey, curd and 6-wk ripened Saint-Nectaire cheese). All samples were stored at 4°C before analysis within 2 d (Eurofins Analytics, Nantes, France). Nitrate and nitrite residues in individual milk samples were analyzed by ion chromatography (method 993.30; AOAC, 1990) with a limit of quantification (LoQ) of 10 mg/kg for nitrate and 5 mg/kg for nitrite. In pooled milk samples and processed milk products, nitrate and nitrite residues were analyzed by spectrometry after nitrate reduction with cadmium (ISO 14673; ISO, 2004) with a LoQ of 5 mg/kg for nitrate and 0.5 mg/kg for nitrite.

Rumen Fermentation Parameters. On the last day of wk 17, rumen samples were collected 3.5 h after the morning feeding by stomach tubing (Shen et al., 2012). Samples were strained through a polyester monofilament fabric (250 µm pore size) and the filtrate was subsampled for VFA and NH₃ concentration analyses and protozoa counting. For VFA analysis, 0.8 mL of filtrate was mixed with 0.5 mL of a 0.5 M HCl solution containing 2% (w/v) metaphosphoric acid and 0.4% (w/v) crotonic acid. For NH₃ analysis, 1 mL of filtrate was mixed with 0.1 mL of 5% orthophosphoric acid. These samples were stored at -20°C until analysis. For protozoa counting, 2 mL of filtrate was mixed with 2 mL of methyl green-formalin saline solution, and stored at room temperature in the dark until counting.

Concentrations of VFA and NH₃ were analyzed by gas chromatography with a flame ionization detector and by colorimetry, respectively (Morgavi et al., 2008). Protozoa were counted by microscopy, 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 log₁₀-transformed before statistical analysis.

Statistical Analyses

Data were analyzed using the MIXED procedure of SAS (Version 9.4; SAS Institute, 2009). All statistical models included the animal nested within diet as random effect.

Data collected throughout the experiment (intake, milk production and composition) or on two occasions (CH₄ emissions) were averaged per week as there was no statistical difference between days within a week. The statistical model included diet (n = 2), week (n = 17 for intake and milk and n = 2 for CH₄), and diet × week interaction as fixed effects. Week was treated as a repeated measurement. For intake, milk production and composition (except for urea), data collected the week before starting the experiment (wk 0) were used as covariates. For continuous measures of CH₄ emissions, the model included diet (n = 2), week (n = 2),

hour ($n = 24$), diet \times week and diet \times hour interactions as fixed effects. Hour was treated as a repeated measurement. As the interaction diet \times week was not statistically significant, averaged data of the two weeks are presented in Figure 4. For the repeated measurements, several covariance structures were tested (variance component, autoregressive, compound symmetry, unstructured, and toeplitz) and structure with the lowest Akaike's information criteria was chosen. Then, variance component was always used as covariance structure, except for daily CH₄ emissions where compound symmetry was used.

Data collected at the end of the experiment (apparent digestibility, N balance, rumen fermentation and microbial parameters) were analyzed with diet ($n = 2$) as fixed factor.

Differences between diets were considered significant at $P \leq 0.05$, and trends were discussed at $0.05 < P \leq 0.1$. Least squares means are reported throughout.

Results

Liveweight and Blood Methemoglobin

During the 17-wk experiment, cows fed CON or LIN+NIT lost on average 32 and 22 kg to reach a final BW of 697 ± 62 kg and 662 ± 67 kg, respectively. During the 3-wk period of adaptation to nitrate, the maximum metHb level was 13.0% (Figure 1). From wk 4 to wk 17, average metHb level was 1.2%. Maximum metHb level peaked at 30.8% for one cow in wk 17, whereas average metHb level for all other cows on that week averaged 4.4%.

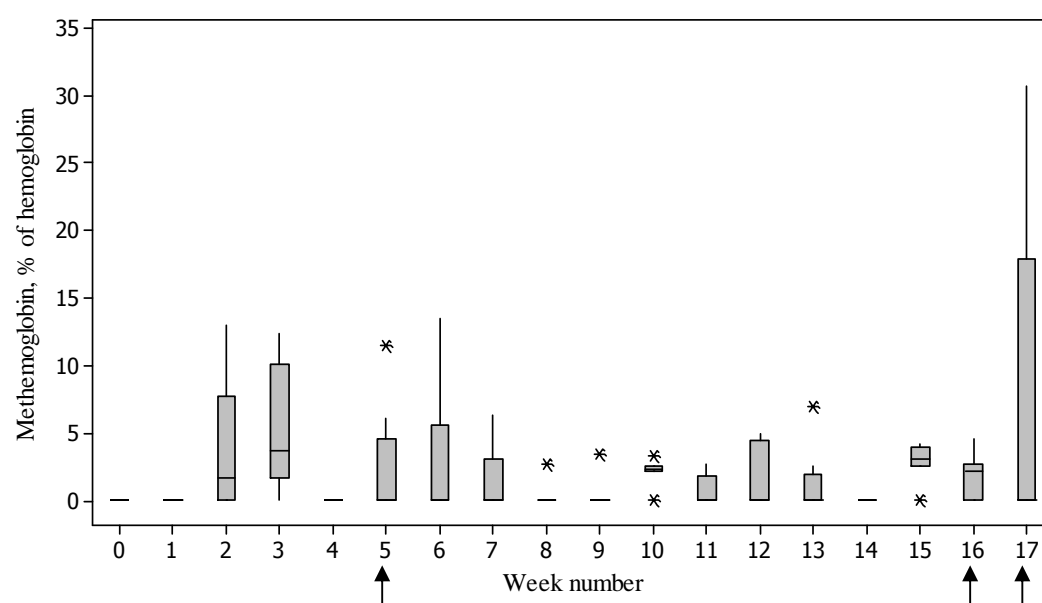


Figure 1 Boxplot of blood methHb levels of lactating cows fed 10% extruded linseed plus 1.8% nitrate ($n = 8$) during 17 weeks. In wk 0, animals were fed a control diet. Linseed and nitrate were firstly incorporated in wk 1. Blood was analyzed in wk 0 and then twice a week during wk 1, 2 and 3 and once a week from wk 4 to 17. The box represents the quartiles with the median within the box, and the vertical lines represent the maximum and minimum value within 1.5 interquartile range of the higher and lower quartile, respectively. Values greater than 1.5 interquartile range are considered as outliers and are identified with a star. The arrows indicate the measurement weeks.

Intake and Milk Yield

Daily DMI was similar between diets in wk 1, 2, 3, and 17 (Figure 2) and tended to be lower with LIN+NIT from wk 4 to 16 (-5.1 kg/d on average; $P \leq 0.10$). This tendency between diets was also observed for DM and OM intake ($P = 0.070$ and $P = 0.078$, respectively) when cows were in chambers for 2 d for CH_4 measurements (wk 5 and 16; Table 2). Fiber intakes were lower with LIN+NIT ($P = 0.008$ for NDF and $P = 0.007$ for ADF) whereas dietary treatments did not affect gross energy intake (Table 2).

We found no between-diet difference in milk production over two thirds of the experiment (11 wk out of 17; Figure 3), whereas in wk 4, 5, 7, 9, 10, and 17, milk production was lower with LIN+NIT (-2.5 kg/d on average; $P \leq 0.05$). During the 2 d in chambers (wk 5 and 16), cows fed LIN+NIT also tended to produce less milk (-2.8 kg/d on average; $P = 0.078$; Table 2). Feed efficiency was similar between diets in wk 5 and tended to be higher for LIN+NIT in wk 16 (diet \times week, $P = 0.079$; Table 2).

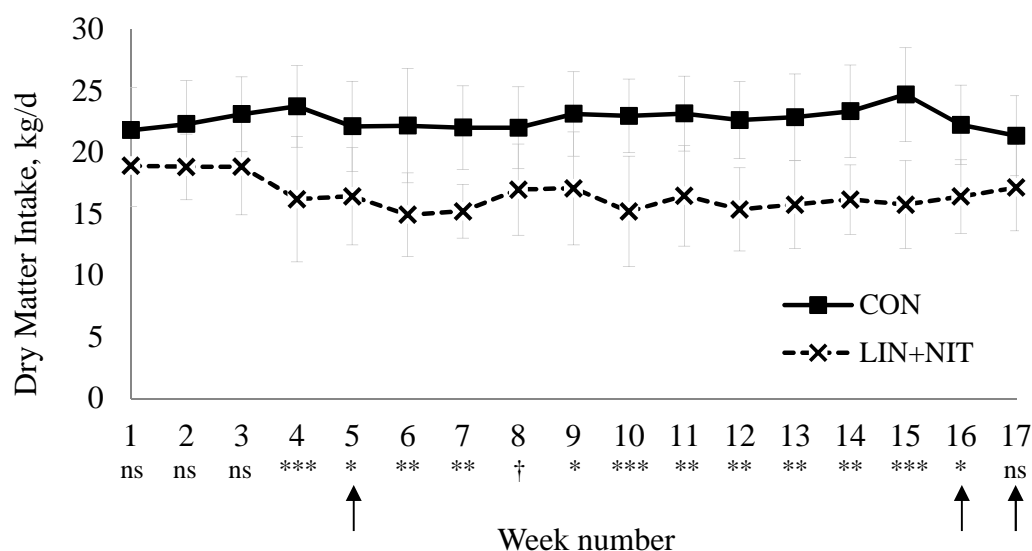


Figure 2 Dry matter intake of lactating cows fed a control diet (CON; $n = 8$) or CON supplemented with 10% extruded linseed plus 1.8% nitrate (LIN+NIT; $n = 8$) during 17 weeks (averages of 4 days per week). Errors bars indicate SD. Symbols indicate weekly statistical comparison between CON and LIN+NIT ($†P \leq 0.10$; $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$). Arrows indicate measurement weeks.

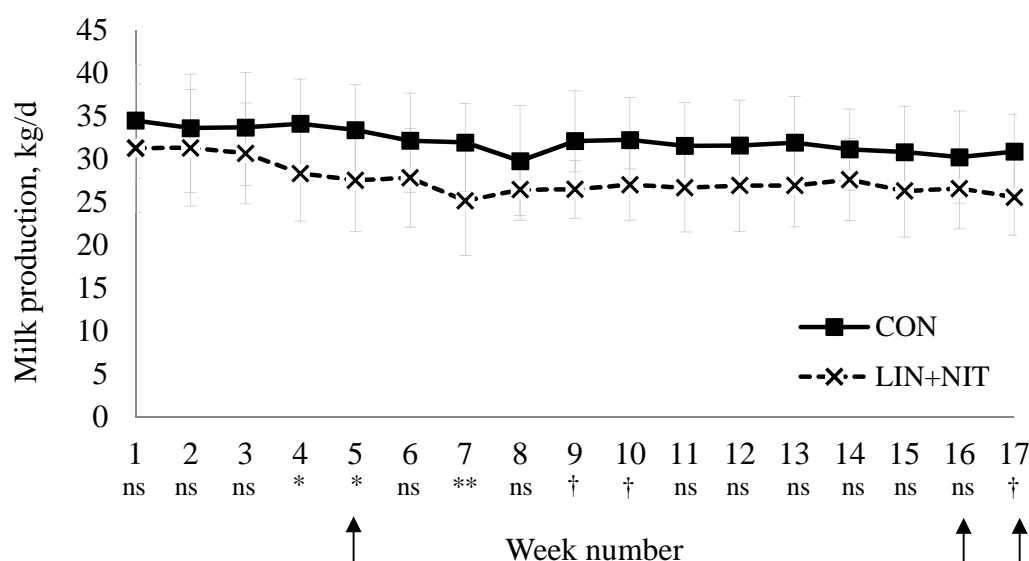


Figure 3 Milk yield of lactating cows fed a control diet (CON; $n = 8$) or CON supplemented with 10% extruded linseed plus 1.8% nitrate (LIN+NIT; $n = 8$) during 17 weeks (averages of 4 days per week). Errors bars indicate SD. Symbols indicate weekly statistical comparison between CON and LIN+NIT ($†P \leq 0.10$; $*P \leq 0.05$; $**P \leq 0.01$; $***P \leq 0.001$). Arrows indicate measurement weeks.

Table 2 Daily nutrient intake, milk yield and composition, and methane emissions of lactating cows fed a control diet (n = 8) or a diet supplemented with a combination of linseed and nitrate (n = 8)

Item ²	Diet ¹				SEM	P-value		
	CON		LIN+NIT			Diet	Week	Diet × Week
Week number ³	5	16	5	16				
Nutrient intake								
DM, kg/d	20.8	20.7	18.8	17.3	1.00	0.070	0.182	0.293
OM, kg/d	19.4	19.2	17.6	16.2	0.93	0.078	0.183	0.292
NDF, kg/d	7.25	7.19	6.03	5.55	0.338	0.008	0.204	0.326
ADF, kg/d	3.80	3.80	3.13	2.88	0.177	0.007	0.205	0.319
Gross energy, MJ/d	367.5	364.3	345.3	318.0	17.82	0.183	0.172	0.276
Milk yield and composition								
Milk yield, kg/d	32.6	29.9	28.9	28.1	1.05	0.078	0.001	0.052
Feed efficiency ⁴ , kg milk/kg DMI	1.57	1.46	1.58	1.67	0.064	0.148	0.888	0.079
Fat, g/d	1393.1	1205.7	1030.3	1075.1	91.67	0.060	0.198	0.045
Protein, g/d	1031.0	996.9	851.4	865.3	45.24	0.026	0.615	0.243
Lactose, g/d	1654.6	1501.8	1489.3	1365.5	54.60	0.060	<0.001	0.608
Urea, g/d	7.5	6.1	2.4	2.0	0.55	<0.001	0.061	0.223
Fat, g/kg	41.9	39.1	36.5	39.1	2.23	0.298	0.961	0.185
Protein, g/kg	31.5	33.2	29.4	30.9	0.78	0.045	0.009	0.902
Lactose, g/kg	50.7	50.1	51.9	48.8	0.72	0.948	0.002	0.027
Urea, mg/dL	22.2	19.4	8.7	7.7	1.51	<0.001	0.216	0.524
Methane emission								
g CH ₄ /d	470.6	459.1	254.0	247.6	34.13	<0.001	0.640	0.895
g CH ₄ /kg DM intake	21.5	20.8	14.6	15.3	1.30	0.003	1.000	0.310
g CH ₄ /kg milk	14.0	14.8	9.4	9.3	1.02	0.002	0.560	0.516
% of gross energy intake	6.1	5.9	4.0	4.2	0.36	0.001	0.988	0.307

¹ CON = diet control; LIN+NIT = diet CON containing 10% extruded linseed and 1.8% nitrate on a DM basis. ² Average of 2 d in chambers in wk 5 and 16. For intake, milk yield and composition, a covariate (data obtained in wk 0) was included in the statistical model. ³ Number of weeks of distribution of dietary treatment. ⁴ Feed efficiency = milk yield/DMI.

In chambers, milk fat and lactose concentrations were similar between diets, whereas LIN+NIT reduced milk protein ($P = 0.045$) and urea ($P < 0.001$) contents by 6.8% and 60.6%, respectively. For both diets, nitrate and nitrite concentrations in individual milk samples, pooled milk samples, and milk products were lower than the LoQ, except for curd from CON in wk 17 and cheese from CON and LIN+NIT in wk 9 in which low nitrite concentrations were detected (1.5 mg/kg).

Methane Emissions

Diet LIN+NIT reduced CH₄ emissions by 29.3% when expressed in grams per kilogram of DMI ($P = 0.003$), and by 35.1% when expressed in grams per kilogram of milk ($P = 0.002$). Whatever the mode of expression of CH₄ emission, there was no significant effect of week or diet \times week interaction (Table 2). This shows that CH₄ emissions of CON and LIN+NIT were similar between the 2 wk of measurements, and that the difference between diets was repeatable, even after 11 wk of dietary treatments.

Methane emissions for a 24-h period, averaged for the 2 wk of measurements, are presented in Figure 4. Methane emissions were similar between diets during the 4 h preceding the morning feeding, then LIN+NIT reduced CH₄ emissions for the first 12 h after the morning meal ($P \leq 0.05$).

Diet Apparent Digestibility and Nitrogen Balance

Apparent digestibility of DM, OM, and NDF was similar between diets, and averaged 67.5, 69.4, and 50.6%, respectively (Table 3). The LIN+NIT diet tended to reduce ADF (-3.8%; $P = 0.070$) and CP (-2.9%; $P = 0.074$) apparent digestibility.

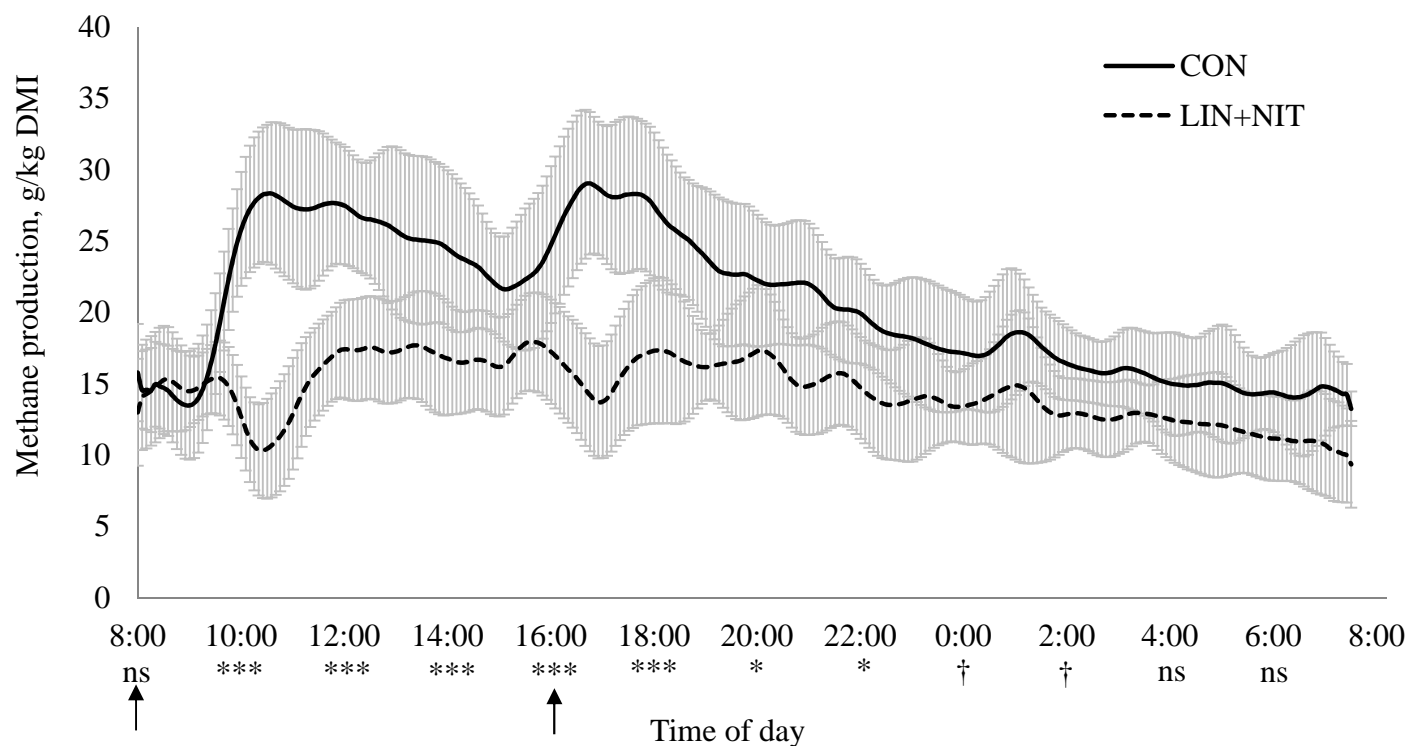


Figure 4 Daily CH₄ production pattern of lactating cows fed a control diet (CON; n = 8) or CON supplemented with 10% extruded linseed plus 1.8% nitrate (LIN+NIT; n = 8) during 17 weeks (raw data; averages of 2 days and 2 weeks of CH₄ measurement; wk 5 and 16). Errors bars indicate SD. Symbols indicate hourly statistical comparison between CON and LIN+NIT († $P \leq 0.10$; * $P \leq 0.05$; ** $P \leq 0.01$; *** $P \leq 0.001$). Arrows indicate time of feeding.

Nitrogen intake was 22% lower with LIN+NIT ($P = 0.001$). Consequently, LIN+NIT led to lower fecal N losses, urinary N losses, and N retained in milk ($P = 0.016$, $P < 0.001$, and $P = 0.003$, respectively). However, N distribution was unaffected by diet. On average for both diets, 35.7% ($P = 0.074$), 24.1% ($P = 0.071$), and 29.9% ($P = 0.937$) of N intake was directed towards feces, urine, and milk, respectively. Finally, N balance was positive and similar between diets and averaged 52.6 g/d or 10.5% of N intake.

Table 3 Total tract apparent digestibility and nitrogen balance of lactating cows after 17 weeks feeding a control diet (n = 8) or a diet supplemented with a combination of linseed and nitrate (n = 8)

Item ²	Diet ¹		SEM	P-value
	CON	LIN+NIT		
Total tract apparent digestibility, %				
DM	67.8	67.2	0.74	0.531
OM	69.8	69.0	0.73	0.458
NDF	51.3	49.9	1.11	0.393
ADF	47.5	43.7	1.35	0.070
CP	65.8	62.9	1.05	0.074
Starch	98.5	97.9	0.24	0.109
N intake, g/d	548.1	425.1	21.56	0.001
Fecal N losses				
g/d	187.3	156.8	7.88	0.016
% of N intake	34.2	37.1	1.05	0.074
Urinary N losses				
g/d	138.2	96.1	5.74	<0.001
% of N intake	25.4	22.7	0.98	0.071
Total fecal and urinary N losses				
g/d	325.5	253.0	11.16	<0.001
% of N intake	59.6	59.7	1.36	0.939
Milk N output				
g/d ³	163.5	126.1	7.24	0.003
% of N intake	29.9	29.8	1.05	0.937
N Balance ⁴				
g/d	59.1	46.1	9.87	0.365
% of N intake	10.5	10.5	1.73	0.990

¹ CON = diet control; LIN+NIT = diet CON containing 10% extruded linseed and 1.8% nitrate on a DM basis.

² Average of 5 d of total tract apparent digestibility and N balance measurement in wk 17. No covariate was included in the statistical model.

³ Milk N output = (milk yield × milk protein concentration)/average N content in milk (6.38 g N/g milk protein).

⁴ N balance = N intake - total fecal and urinary N losses - milk N output.

Rumen Fermentation and Microbial Parameters

Concentration of NH_3 in the rumen did not change with diets (Table 4). Diet LIN+NIT reduced total VFA (-12 mM; $P = 0.020$) and propionate concentrations (-8 mM; $P = 0.003$) without affecting acetate and butyrate concentrations. These differences in VFA profile induced an increase in C2/C3 and C2+C4/C3 ratios ($P = 0.003$) with LIN+NIT.

Total concentration of protozoa in the rumen tended to increase with LIN+NIT (+53%; $P = 0.052$). This was linked to a higher concentration of small entodiniomorphs and *Dasytricha* ($P = 0.047$ and $P = 0.014$, respectively). Concentrations of large entodiniomorphs and *Isotricha* were unaffected by diets.

Table 4 Fermentation parameters and protozoal concentration in the rumen of lactating cows after 17 weeks feeding a control diet (n = 8) or a diet supplemented with a combination of linseed and nitrate (n = 8)

Item ²	Diet ¹		SEM	P-value
	CON	LIN+NIT		
NH_3 , mM	10.14	10.97	1.648	0.736
VFA concentration, mM				
Total VFA	104.1	91.7	3.35	0.020
Acetate (C2)	58.6	56.9	1.95	0.561
Propionate (C3)	25.6	17.6	1.65	0.003
Butyrate (C4)	15.2	14.1	1.61	0.635
Minor VFA ³	4.71	3.08	0.577	0.055
C2/C3	2.36	3.27	0.170	0.003
(C2+C4)/C3	2.99	4.08	0.213	0.003
Total protozoa, log ₁₀ /mL	5.03	5.32	0.095	0.052
Entodiniomorphs, log ₁₀ /mL				
Small (< 100 μm)	5.01	5.31	0.095	0.047
Large (> 100 μm)	3.39	3.11	0.217	0.387
Holotrichs, log ₁₀ /mL				
<i>Dasytricha</i> (< 100 μm)	2.22	3.02	0.191	0.014
<i>Isotricha</i> (> 100 μm)	3.24	2.48	0.115	0.140

¹ CON = diet control; LIN+NIT = diet CON containing 10% extruded linseed and 1.8% nitrate on a DM basis.

² Data from rumen samples taken the last day of wk 17. No covariate was included in the statistical model.

³ Minor VFA = sum of isobutyrate, isovalerate, valerate and caproate.

Discussion

Intake, Milk Production, and Nitrogen Balance

Throughout the experiment, intake and milk production tended to be lower for dairy cows supplemented with LIN+NIT. As feed efficiency (kg of milk per kg of feed) was similar between diets, the lower intake may explain the lower milk production. The lower intake with LIN+NIT is difficult to explain because diets had similar net energy content. In addition, in a short-term experiment, intake was similar between non-lactating cows fed with or without linseed plus nitrate (Guyader et al., 2014a). Individual nitrate supplementation at higher doses than here (1.8%) did not reduce intake of restricted-fed dairy cows (2.1%, Van Zijderveld et al., 2011; 2.0%, Veneman et al., 2014) and sheep (2.5%, Nolan et al., 2010; 2.6%, Van Zijderveld et al., 2010) but tended to reduce DMI of dairy cows (2.0%, Veneman et al., 2014) and steers (2.3%, Hulshof et al., 2012) fed ad libitum. Linseed applied at doses higher than here (3.5% added fat) did not have a negative effect on the intake or milk production of dairy cows (5.1% added fat, Ferlay et al., 2013; 4% added fat, Veneman et al., 2014) fed ad libitum or restricted. One study reported a lower DMI (-7%) by lactating cows fed a grass silage-based diet supplemented with linseed (3% added fat; Martin et al., 2011). The only study that simultaneously used linseed plus nitrate (4% added fat plus 2.3% nitrate) on cows did not result in intake changes, but the cows were non-lactating and not fed ad libitum (Guyader et al., 2014a). Consequently, we hypothesize that LIN+NIT fed together ad libitum may have an inhibitory effect on voluntary intake linked to a tendency for lower ADF apparent digestibility. Earlier reviews have highlighted the negative correlation between fiber digestibility and voluntary intake through a lower passage rate of particles from the rumen and greater rumen filling (Allen, 1996). Further work would help determine the optimal quantity of dietary LIN+NIT that can be provided without reducing intake, which is also an essential step towards making this feeding strategy acceptable at the farm scale.

The LIN+NIT diet had no effect on concentration and production of fat and lactose. This result confirms previous experiments on dairy cows supplemented with nitrate (2.1% nitrate in a corn silage based diet; Van Zijderveld et al., 2011) or with incremental amounts of extruded linseed (up to 5.1% added fat in hay- or corn silage-based diets; Ferlay et al., 2013). The LIN+NIT diet reduced milk protein concentration by 7% (-2.2 g/kg milk) and milk protein production by 15% (-155.6 g/d). In dairy cows fed 2.1% nitrate, Van Zijderveld et al. (2011) also reported reduced milk protein concentrations (-5% or -1.4 g/kg of milk) but no

effect on milk protein production whereas milk yield was stable. The reduced milk protein content may not be linked to linseed supplementation, as milk protein content of dairy cows was not affected by 3.5% added fat from extruded linseed in hay- or corn silage-based diets (Ferlay et al., 2013).

Nitrogen balance was positive for both diets, even if it may be overestimated because volatile N losses from faeces and urine, dermal and scurf N losses were not taken into account (Spanghero and Kowalski, 1997). Nevertheless, N balance was similar between diets with the same N distribution between milk, feces and urine. In addition, average N efficiency (N in milk/N intake) was similar between CON and LIN+NIT (30%) and close to the data given in the literature (25%, with a range between 15 and 40%; Calsamiglia et al., 2010). This result shows that dairy cows use nitrate in the same way as they use other N sources. With LIN+NIT, milk urea concentration and production were 12.6 mg/dL and 4.6 g/d less, respectively, than CON. This marked decrease was surprising and in contradiction with previous experiments on dairy cows showing no effect of extruded linseed (1.1% added fat; Pezzi et al., 2007) or nitrate (2.1% nitrate; Van Zijderveld et al., 2011) on milk urea content. We assumed that the between-diet difference in milk urea comes from the lower N intake of animals fed LIN+NIT, as N intake is known to correlate positively with milk urea (Spek et al., 2013).

The main concern when using nitrate in animal nutrition is its potential negative effect on animal and human health. To avoid increase of blood metHb in animals (Lewis, 1951), progressive adaptation to nitrate is essential (Lee and Beauchemin, 2014). In addition, it is important that nitrate is homogeneously incorporated in the ration, not top dressed, to avoid swift ingestion of the daily dose. By applying these recommendations, we did not observe rises in metHb levels in animals fed LIN+NIT, similarly to a previous experiment on dairy cows fed 2.1% nitrate (Van Zijderveld et al., 2011). However, we cannot explain the greater metHb level observed in the last week of the experiment. In terms of human health, nitrate and nitrite are common food additives used for their anti-bacterial properties against lethal pathogens (European Food Safety Authority, 2009). However, an excess of nitrite from nitrate reduction in the mouth may promote gastric inflammation (Weitzberg and Lundberg, 2013). Regulations have been adopted to keep concentrations of nitrate and nitrite residues within recommended daily allowances for nitrate and nitrite intake (3.75 and 0.13 mg/kg BW per day, respectively; European Food Safety Authority, 2009), and Europe has limited nitrate concentration in drinking water to 50 mg/L (Benjamin, 2000). Nitrate intake mainly comes

from vegetables (60 to 80%), water (15 to 20%) and animal-based products (10 to 15%), while 80 to 85% of nitrite exposure comes from conversion of nitrate in the mouth. Vegetables such as spinach can contain up to 1,614 mg nitrate per kg. Here, nitrate and nitrite residues in milk or milk products were lower than the LoQ of the technique (5 mg/kg for nitrate and 0.5 mg/kg for nitrite), except in cheese from CON and LIN+NIT (1.5 mg/kg nitrite). These novel data confirm previous work on lamb meat (El-Zaiat et al., 2013), and show that animals can metabolize nitrate and nitrite without transferring residues into animal products. Consequently, long-term supplementation with nitrate (4 months) can be safely proposed in ruminant nutrition without risks for human health, as a CH₄-mitigating strategy and a source of non-protein nitrogen to replace urea.

Methane Emissions and Associated Digestive Mechanisms

In our experiment using open chambers, CH₄ emissions of dairy cows fed CON averaged 21.2 g/kg DMI. This value is close to the estimate calculated by an equation based on OM content of the diet and OM digestibility (21.4 g/kg DMI; Sauvant et al., 2011), and is also in accordance with the average CH₄ emission of cattle fed diets without supplementation of CH₄-mitigating treatments (20.7 g/kg DMI, number of treatments = 33) as compiled from a database used for a previous meta-analysis (Guyader et al., 2014b).

The reduction in CH₄ emission (g/kg of DMI) averaged 29% when dairy cows were supplemented with 1.8% nitrate plus 3.5% added fat from extruded linseed, corresponding to our expected theoretical CH₄ reduction. This confirms our previous results obtained on non-lactating cows supplemented with 2.2% nitrate plus 4% added fat from linseed oil (Guyader et al., 2014a) and shows that LIN+NIT can efficiently reduce CH₄ emissions regardless of the physiological stage of cows. We also observed a severe CH₄-mitigating effect of LIN+NIT just after feeding, which was most probably linked to the effect of nitrate quickly metabolized in the rumen. This result agrees with previous studies (Van Zijderveld et al., 2010; Guyader et al., 2014a). Methane reduction with LIN+NIT corresponds to a saving of 2% of gross energy intake, without positive responses on apparent digestibility, weight gain or body condition score (data not shown) of the animals. The absence of relationship between CH₄ reductions and dairy cow performance has also been reported previously (Van Zijderveld et al., 2011).

The CH₄-mitigating effect of LIN+NIT was maintained throughout the 4 months of the experiment, indicating that this dietary strategy could be applied on farms. The long-term CH₄-mitigating effect of nitrate (2.1%) and extruded linseed (2.5% added fat) fed individually

to dairy cows was also maintained during 3 mo (Van Zijderveld et al., 2011) and 1 yr (Martin et al., 2011), respectively.

The LIN+NIT diet did not change rumen protozoa concentration as previously observed with non-lactating cows supplemented with 2.2% nitrate plus 4% added fat from linseed (Guyader et al., 2014a). Diet LIN+NIT increased the acetate/propionate and (acetate+butyrate)/propionate ratios due to a decrease in ruminal propionate which is normally a competitive pathway of methanogenesis (Martin et al., 2010). This contrasts with our previous work in which LIN+NIT did not change rumen fermentation parameters (Guyader et al., 2014a). However, in the present work, the relationship between CH₄ production and rumen fermentation and microbial parameters should be interpreted with caution given the large differences in time scale between CH₄ measurement periods and rumen samplings through stomach tubing. Consequently, the CH₄-mitigating effect of LIN+NIT would not be explained by a reduction in acetate and butyrate synthesis, nor by a reduction in protozoa which are important H₂-producers. Other mechanisms must be involved in the CH₄-mitigating effect of LIN+NIT. Both supplements may act as H₂ sinks. Based on stoichiometric calculation and assuming complete reduction of nitrate to nitrite and ammonia, and complete biohydrogenation of polyunsaturated fatty acids, the reduction of 1 mol nitrate reduces CH₄ by 1 mol, and the biohydrogenation of 1 mol C18:1, C18:2, and C18:3 reduces CH₄ by 0.25, 0.50 and 0.75 mol, respectively. Extending this calculation here, 325.8 g/d of nitrate ingested by dairy cows would have reduced CH₄ by 5.25 mol/d (or 90.1 g/d) and 600.9 g of fatty acid ingested by dairy cows (23, 32, and 25% of C18:1, C18:2, and C18:3, respectively) would have reduced CH₄ by 0.87 mol/d (or 14.9 g/d). In total, H₂ consumption by LIN+NIT would have reduced CH₄ emissions by 105.0 g/d, explaining 49% of the observed CH₄ reduction. The remaining decrease can thus be explained by non-stoichiometric processes. The LIN+NIT diet may also act on rumen microbiota. Previous work showed that nitrate reduced both quantity (2.6% nitrate to sheep, Van Zijderveld et al., 2010) and activity (2.3% nitrate to non-lactating cows, Guyader et al., 2014c) of methanogens. The anti-methanogenic effect of polyunsaturated fatty acid has also been demonstrated in pure culture of methanogens (Prins et al., 1972) and in previous experiments with cattle (4% added fat, Guyader et al., 2014c; 3.5% added fat, C. Martin, unpublished data). In addition, H₂ production must have been lowered with LIN+NIT owing to a lower quantity of fermentable substrates in the rumen (lower DMI, quantity of carbohydrates due to lipids substitution and fiber digestibility) which directly reduced CH₄ emissions.

Conclusions

The association of linseed plus nitrate is an efficient feeding strategy to reduce CH₄ emissions in the long-term without altering diet apparent digestibility, N efficiency or animal health. However, the energetic benefits of the decreased CH₄ emissions to the animals were not observed. Additional data is needed on changes in rumen microbiota in order to fully understand the CH₄-mitigating effect of the association of linseed plus nitrate. Moreover, to make this dietary strategy acceptable by farmers, further work is required to optimize the doses of linseed plus nitrate in an effort to avoid concomitant reduction in intake and milk production. A life cycle assessment will also be needed to evaluate the environmental benefit and economic cost of this dietary strategy in order to raise the prospects of using this strategy at farm level.

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References

- Allen, M. S. 1996. Physical constraints on voluntary intake of forages by ruminants. *J. Anim. Sci.* 74:3063-3075.
- AOAC. 1990. Official Methods of Analysis. Assoc. Off. Anal. Chem. Vol. 77. Arlington, VA.
- AOAC. 2005. Official Methods of Analysis. 18th ed. Assoc. Off. Anal. Chem., Arlington, VA.
- Benjamin, N. 2000. Nitrates in the human diet – good or bad? *Ann Zootech (Paris)* 49:207-216.
- Bruning-Fann, C. S. and J. B. Kaneene. 1993. The effects of nitrate, nitrite, and N-nitroso compounds on animal health. *Vet. Hum. Toxicol.* 35:237-253.
- Calsamiglia, S., A. Ferret, C. K. Reynolds, N. B. Kristensen, and A. M. van Vuuren. 2010. Strategies for optimizing nitrogen use by ruminants. *Animal* 4:1184-1196.
- Doreau, M., L. Bamière, C. Pellerin, M. Lherm, and M. Benoit. 2014. Mitigation of enteric methane for French cattle: Potential extent and cost of selected actions. *Anim. Prod. Sci.* 54:1417-1422.

- El-Zaiat, H. M., H. O. Patiño, Y. A. Soltan, A. S. Morsy, R. C. Araujo, H. Louvandini, and A. L. Abdalla. 2013. Additive effect of nitrate and cashew nut shell liquid in an encapsulated product fed to lambs on enteric methane emission and growth performance. Page 346 in Proc. 5th Greenhouse Gases and Animal Agriculture conference, Dublin, Ireland.
- European Food Safety Authority. 2009. Scientific opinion of the panel on contaminants in the food chain on a request from the European Commission on nitrite as undesirable substances in animal feed. The EFSA J. 1017:1-47.
- Faisant, N., V. Planchot, F. Kozlowski, M. P. Pacouret, P. Colonna, and M. Champ. 1995. Resistant starch determination adapted to products containing high level of resistant starch. Sci. Aliments 15:83-89.
- Ferlay, A., M. Doreau, C. Martin, and Y. Chilliard. 2013. Effects of incremental amounts of extruded linseed on the milk fatty acid composition of dairy cows receiving hay or corn silage. J. Dairy Sci. 96:6577-6595.
- Gerber, P. J., H. Steinfeld, B. Henderson, A. Mottet, C. Opio, J. Dijkman, A. Falcucci, and G. Tempio. 2013. Tackling climate change through livestock – A global assessment of emissions and mitigation opportunities. Food and Agriculture Organization of the United Nations, Rome, Italy.
- Guyader, J., M. Eugène, M. Doreau, Y. Rochette, D. P. Morgavi, and C. Martin. 2014a. Association of nitrate and linseed oil effectively reduces methane emission in ruminants. Proc. Aust. Soc. Anim. Prod. 30:87.
- Guyader, J., M. Eugène, P. Nozière, D. P. Morgavi, M. Doreau, and C. Martin. 2014b. Influence of rumen protozoa on methane emissions in ruminants: A meta-analysis approach. Animal 8:1816-1825.
- Guyader, J., M. Silberberg, M. Popova, A. R. Seradj, D. P. Morgavi, and C. Martin. 2014c. Dietary nitrates decrease methane emission by inhibiting rumen methanogenic archaea without influencing nitrate reducing bacteria. in Proc. Gut microbiology: From sequence to function, Aberdeen, UK.
- Hulshof, R. B. A., A. Berndt, W. J. J. Gerrits, J. Dijkstra, S. M. Van Zijderveld, J. R. Newbold, and H. B. Perdok. 2012. Dietary nitrate supplementation reduces methane emission in beef cattle fed sugarcane based diets. J. Anim. Sci. 90:2317-2323.
- INRA. 2010. Alimentation des bovins, ovins et caprins. INRA Editions, Paris, France.

- ISO. 2004. ISO 14673-1:2004 (IDF 189-1: 2004) : Lait et produits laitiers -- Détermination des teneurs en nitrates et en nitrites -- Partie 1: Méthode par réduction au cadmium et spectrométrie. Accessed June 19, 2014. http://www.iso.org/iso/fr/home/store/catalogue_tc/catalogue_detail.htm?csnumber=38702.
- Kaplan, J. C. 1965. Méthode de mesure rapide du taux de la méthémoglobine dans les globules rouges. *Rev. Fr. Etud. Clin. Bio.* 10:856-859.
- Lee, C. and K. A. Beauchemin. 2014. A meta-analysis of effects of feeding nitrate on toxicity, production, and enteric methane emissions in ruminants. Pages 845-846 in *Proc. Linking animal science and animal agriculture: Meeting the global demands of 2050*, Kansas City, USA.
- Lewis, D. 1951. The metabolism of nitrate and nitrite in the sheep. 1 The reduction of nitrate in the rumen of the sheep. *Biochem. J.* 48:175-180.
- Martin, C., D. P. Morgavi, and M. Doreau. 2010. Methane mitigation in ruminants: from microbe to the farm scale. *Animal* 4:351-365.
- Martin, C., D. Pomiès, A. Ferlay, Y. Rochette, B. Martin, Y. Chilliard, D. P. Morgavi, and M. Doreau. 2011. Methane output and rumen microbiota in dairy cows in response to long-term supplementation with linseed or rapeseed of grass silage- or pasture-based diets. *Proc. N.Z. Soc. Anim. Prod.* 71:243-247.
- Morgavi, D. P., J. P. Jouany, and C. Martin. 2008. Changes in methane emission and rumen fermentation parameters induced by refaunation in sheep. *Anim. Prod. Sci.* 48:69-72.
- Nolan, J. V., R. S. Hegarty, J. Hegarty, I. R. Godwin, and R. Woodgate. 2010. Effects of dietary nitrate on fermentation, methane production and digesta kinetics in sheep. *Anim. Prod. Sci.* 50:801-806.
- Pezzi, P., M. Giammarco, G. Vignola, and N. Brogna. 2007. Effects of extruded linseed dietary supplementation on milk yield, milk quality and lipid metabolism of dairy cows. *Ital. J. Anim. Sci* 6:333-335.
- Potts, T. J. 1967. Colorimetric determination of urea in feeds (report of AOAC Committee). *Journal of the AOAC* 50:56-58.
- Prins, R. A., C. J. Van Nevel, and D. I. Demeyer. 1972. Pure culture studies of inhibitors for methanogenic bacteria. *Antonie Van Leeuwenhoek* 38:281-287.

- Sauvant, D., S. Giger-Reverdin, A. Serment, and L. Broudiscou. 2011. Influences des régimes et de leur fermentation dans le rumen sur la production de méthane par les ruminants. *INRA Prod. Anim.* 24:433-446.
- Shen, J. S., Z. Chai, L. J. Song, J. X. Liu, and Y. M. Wu. 2012. Insertion depth of oral stomach tubes may affect the fermentation parameters of ruminal fluid collected in dairy cows. *J. Dairy Sci.* 95:5978-5984.
- Spanghero, M. and Z. M. Kowalski. 1997. Critical analysis of N balance experiments with lactating cows. *Livest. Prod. Sci.* 52:113-122.
- Spek, J. W., J. Dijkstra, G. van Duinkerken, and A. Bannink. 2013. A review of factors influencing milk urea concentration and its relationship with urinary urea excretion in lactating dairy cattle. *J. Agric. Sci.* 151:407-423.
- Van Soest, P. J., J. B. Robertson, and B. A. Lewis. 1991. Methods for dietary fiber, neutral detergent fiber, and non starch polysaccharides in relation to animal nutrition. *J. Dairy Sci.* 74:3583-3597.
- Van Zijderveld, S. M., W. J. J. Gerrits, J. A. Apajalahti, J. R. Newbold, J. Dijkstra, R. A. Leng, and H. B. Perdok. 2010. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. *J. Dairy Sci.* 93:5856-5866.
- Van Zijderveld, S. M., W. J. J. Gerrits, J. Dijkstra, J. R. Newbold, R. B. A. Hulshof, and H. B. Perdok. 2011. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. *J. Dairy Sci.* 94:4028-4038.
- Veneman, J. B., S. Muetzel, K. J. Hart, C. L. Faulkner, J. M. Moorby, G. Molano, H. B. Perdok, J. R. Newbold, and C. J. Newbold. 2014. Dietary nitrate but not linseed oil decreases methane emissions in two studies with lactating dairy cows. in *Proc. Livestock, Climate Change and Food Security Conference*, Madrid, Spain.
- Weitzberg, E. and J. O. Lundberg. 2013. Novel aspects of dietary nitrate and human health. *Annu. Rev. Nutr.* 33:129-159.
- Williams, A. G. and G. S. Coleman. 1992. *The rumen protozoa*. Springer-Verlag, New York.

STEP 3: Absence of methane mitigating effect of tea saponin fed to non-lactating and lactating cows

Objective

- 1/ To test the effect of a different feeding strategy acting on H_2 production (saponin from tea, toxic effect towards protozoa) on CH_4 emissions and associated ruminal mechanisms of non-lactating and lactating cows.
- 2/ To assess its effect on diet digestibility, N balance and lactating performances.

Experimental approach

Trial 1

4 non-lactating cows \rightarrow 2×2 Factorial design \rightarrow **CON-1:** 50% hay + 50% pelleted concentrate



NIT-1: CON + 2.3% nitrate (from calcium nitrate)

TEA-1: CON + 0.5% saponin (from tea)

TEA+NIT-1: CON + 0.5% saponin + 2.3% nitrate



4 experimental periods of 5 weeks (wk 1 to 3 = Adaptation; wk 4 to 5 = Measurement)

WEEK	1	2	3	4	5
Daily intake					
Blood metHb (3 h after morning feeding, once a week)					
Total tract digestibility, N balance (6 days)					
Rumen fermentation (3 h after morning feeding, twice a week)					
Daily kinetics of CH_4 emissions (4 days)					

Experimental approach

Trial 2

8 lactating cows \rightarrow 2×2 Crossover design \rightarrow **CON-2:** 54% corn silage + 6% hay + 40% pelleted concentrate



TEA-2: CON + 0.5% saponin (from tea)

4 experimental periods of 5 weeks (wk 1 to 3 = Adaptation; wk 4 to 5 = Measurement)

WEEK	1	2	3	4	5
Daily intake, milk yield					
Total tract digestibility, N balance					
Rumen fermentation (3.5 h after morning feeding, once a week)					
Daily kinetics of CH_4 emissions (2 days)					
Milk composition (once a week)					

Main resultsTrial 1Trial 2

- Intake tended to be reduced by tea saponin (-12% in trial 2). Milk yield was reduced by 18% without modification in its composition.
- Methane emissions from non-lactating and lactating cows were unaffected by tea saponin. This plant extract also poorly modified rumen fermentation parameters.
- Tea saponin did not affect N balance but tended to improve fiber digestibility in both experiments.

Conclusion

Tea saponin tended to reduce zootechnical performances of cattle, without reducing their CH₄ emissions whatever the physiological stage. We assume that the active compound of the plant was degraded during the pelleting process. This plant extract tended to increase fiber digestibility of lactating cows, without affecting N balance. Further work is required to improve tea saponin palatability and to confirm its positive effect on fiber digestibility.

Absence of methane-mitigating effect of tea saponin fed to non-lactating and lactating cows

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In progress

Abstract

Two *in vivo* trials were conducted to study the effect of tea saponin alone (or in association with nitrate) on methane emissions and digestive processes in cows. Trial 1 was designed as a 2×2 factorial design on four rumen cannulated non-lactating cows fed four diets: 1/ control (CON-1) consisting of hay and concentrate (50:50 on a DM basis), 2/ control with 0.5% tea saponin (TEA-1), 3/ control with 2.3% nitrate (NIT-1) and 4/ control with 0.5% tea saponin and 2.3% nitrate (TEA+NIT-1). Trial 2 was carried out on eight lactating cows fed two diets in a 2×2 crossover design: 1/ control (CON-2) consisting of maize silage, hay and concentrate (54:6:40 on a DM basis) and 2/ control with 0.5% tea saponin (TEA-2). In both trials, each experimental period lasted five weeks including two last weeks of measurement during which animals were restricted fed between 90-95% of *ad libitum* intake. Intake and milk production were daily measured all along trials. Daily methane emissions were quantified using open chambers, total tract digestibility and nitrogen balance were determined from total feces and urine collected separately, rumen fermentation parameters and protozoal concentration were analyzed from samples taken after morning feeding. In both trials, tea saponin tended to reduce DM intake (-12% in trial 2). Milk production was reduced (-18%) with TEA-2, most likely because of the tendency for lower intake as feed efficiency was similar between diets. Methane emissions (g/kg dry matter intake) were similar between CON-1 and TEA-1, and were reduced to the same extent with NIT-1 and TEA+NIT-1 (-28% on average). On dairy cows, methane emissions (g/kg dry matter intake) were increased by 14% with TEA-2. Total tract digestibility and nitrogen balance were similar among diets in the two trials, except for ADF digestibility which tended to be improved with TEA-2 (+8%). Ruminal fermentative parameters (ammonia, lactate, and volatile fatty acids ratios) were poorly changed by diets: we observed an increase of acetate and a decrease of butyrate with nitrate-containing diets in trial 1, and an increase of acetate with tea saponin in trial 2. Whatever trial, protozoa concentrations were similar among diets. We conclude that tea saponin was not efficient to reduce methane emissions from cattle in our experimental conditions. Further work is required to confirm positive effect of this plant extract on fiber digestibility.

Keywords: cattle, digestibility, methane, nitrate, tea saponin

Implications

The use of plant extracts as saponins may be a natural method to mitigate methane emissions from ruminants. Diets supplemented with tea saponin included into pelleted concentrates failed to reduce methane emissions in non-lactating and lactating cows. The tendency of this plant extract to improve fiber digestibility in lactating cows needs to be confirmed. Milk production was reduced, most likely because of the tendency for lower intake, but feed efficiency was similar between diets. We suspect that the plant active compound in tea saponin was denatured during the pelleting process.

Introduction

Saponins have been considered as promising natural substances for methane (CH₄) mitigation in ruminants. This plant extract would have a toxic effect on protozoa through the formation of complex with sterols present in their membrane, inducing cell lysis (Goel and Makkar, 2012). However, the *in vivo* effect of saponins on methanogenesis and protozoa in the rumen presents contradictory results according to the source and supplemented dose. The decrease of protozoa (between 58 and 88%) with saponins supplementation either involved a reduction (-13% with 1% sarsaponin from *Yucca schidigera*, YS; Lila et al., 2005), an increase (+14% with 4% saponin from *Medicago sativa*; Klita et al., 1996), or no variation (up to 0.13% saponins from YS and *Quillaja saponaria*, QS; Pen et al., 2007; Holtshausen et al., 2009) in CH₄ emissions.

Recent reviews highlighted a high anti-methanogenic potential for tea saponin (Wang et al., 2012; Gerber et al., 2013). This novel saponin is extracted from the seeds, leaves and roots of the tea tree from Japan (*Camellia sinensis*) or Sri Lanka (*Camellia assamica*). Reduction of CH₄ emissions (g/kg dry matter intake, DMI) with tea saponin (0.25 to 0.5% of DM) supplemented to sheep (Yuan et al., 2007; Mao et al., 2010; Zhou et al., 2011) or steers (Li and Powers, 2012) averaged 26% per percentage added tea saponin. Mao et al. (2010) related this CH₄-mitigating effect with a significant reduction of ruminal protozoa concentration (-41%). In addition, the association of dietary strategies acting on both protozoa (linseed, saponin) and methanogens (nitrate) additively lowered methanogenesis *in vitro* (saponin from QS plus nitrate; Patra and Zhongtang, 2013), and *in vivo* (linseed plus nitrate fed to cows; Guyader et al., 2014b). Tea saponin would also improve *in vitro* organic matter (OM) digestibility (+21%; Wei et al., 2012) but this effect has never been tested *in vivo*.

The objective of this study was to test the effect of tea saponin alone or in association with nitrate fed to lactating and non-lactating cows on CH₄ emissions, diet digestibility, fermentation parameters and protozoa concentration in the rumen.

Material and methods

Two experiments were conducted at the animal facilities of the Experimental Unit UERT at the INRA's Theix Research Centre (Saint-Genès-Champanelle, France). Trial 1 was led from January to June 2013 and trial 2 was led from January to April 2014. Procedures involving animals were performed in accordance with the French Ministry of Agriculture guidelines for animal research and with the applicable EU guidelines and regulations on experiments with animals (http://www2.vet-lyon.fr/ens/expa/acc_regl.html).

Experimental design and animal feeding in trial 1

Four multiparous non-lactating Holstein cows fitted with rumen cannulas (initial average BW of 658 ± 26 kg, mean \pm s.d.) were randomly assigned to four dietary treatments in a 2×2 factorial design, using either calcium nitrate or tea saponin at two different doses (0 and 3% for calcium nitrate; 0 and 0.5% for tea saponin). Each experimental period lasted five weeks, with measures performed in the final two weeks (weeks 4 & 5). All along the experiment, animals were housed in individual stalls. On a dry matter (DM) basis, diets were: 1) control (CON-1), 2) CON-1 with 0.5 % tea saponin (TEA-1), 3) CON-1 with 2.3% nitrate (NIT-1), 4) CON-1 with 0.5% tea saponin and 2.3% nitrate (TEA+NIT-1). The doses of tea saponin and nitrate were calculated to achieve a theoretical CH₄ reduction of 20% (Mao et al., 2010; Van Zijderveld et al., 2011) and 40% when distributed alone or in association, respectively.

Diet CON-1 consisted of 50% natural grass hay and 50% concentrate (DM basis; Table 1) and met the maintenance requirements of non-lactating cows (INRA, 2010). Diets were formulated to get similar levels of starch (26.0%), protein (12.2%), NDF (40.1%) and calcium (Ca, 0.67%). Diets were adjusted to have the same nitrogen (N) and Ca concentrations by including urea and calcium carbonate in CON-1 and TEA-1. Forage was distributed without further processing and all other ingredients including tea saponin or nitrate or both were pelleted in concentrates (InVivo NSA, Chierry, France).

Two weeks before starting the experiment, cows were fed CON-1 *ad libitum*. Then, all along the trial, feed was restricted to 90% of individual voluntary feed intakes to ensure complete consumption of the diet. At the beginning of each experimental period, TEA-1, NIT-1 and TEA+NIT-1 concentrates were progressively supplied by replacing CON-1 concentrate. The

TEA-1 concentrate was distributed at maximal dose after a 5-day transition period, whereas the NIT-1 and TEA+NIT-1 concentrates were distributed at their maximal dose after a 10-day transition period.

Feeds were offered twice daily (66% at 0800 h and 34% at 1600 h for hay; 60% between 0800 and 0930 h in three equal portions and 40% between 1600 and 1630 h in two equal portions for concentrates). Forage-to-concentrate ratio was kept as close as possible to the target ratio by adjusting the amounts of feed offered daily based on the composition of the refusals of the previous day. Cows had free access to water throughout the experiment.

Table 1 Ingredients and chemical composition of the experimental diets (trial 1)

	Diet ¹			
	CON-1	NIT-1	TEA-1	TEA+NIT-1
Ingredients (% of DM)				
Hay	50.00	50.00	50.00	50.00
Pelleted concentrates				
Wheat	25.23	25.23	25.23	25.23
Maize	15.00	15.00	15.00	15.00
Calcium nitrate ²	0	3.00	0	3.00
Tea saponin extract ³	0	0	0.77	0.77
Calcium carbonate	1.70	0	1.70	0
Urea	1.22	0	1.22	0
Dehydrated beet pulp	4.08	4.00	3.31	3.23
Molasses beet	1.00	1.00	1.00	1.00
Binder	1.00	1.00	1.00	1.00
Mineral-vitamin mix	0.75	0.75	0.75	0.75
Aroma	0.02	0.02	0.02	0.02
Chemical composition (% of DM)				
OM	91.3	91.5	91.4	91.4
CP	12.7	12.2	12.4	11.6
NDF	40.1	40.2	40.2	40.0
ADF	23.3	23.1	23.1	23.1
Starch	25.4	25.7	26.3	26.4
GE (MJ/kg of DM)	17.4	16.6	17.5	16.5

¹ CON-1 = diet control; NIT-1 = diet CON-1 containing 2.3% nitrate; TEA-1 = diet CON-1 containing 0.5% tea saponin; TEA+NIT-1 = diet CON-1 containing 0.5% tea saponin and 2.3% nitrate.

² 5Ca(NO₃)₂.NH₄NO₃.10H₂O; 75% NO₃ in DM (Phytosem, Pont-du-Château, France).

³ 688 g saponins/kg of DM according to supplier (Choisun Tea Sci-Tech Co. Ltd., Hangzhou, Zhejiang, China) indications.

Experimental design and animal feeding in trial 2

Eight lactating Holstein cows (four primiparous and four multiparous) were used. At the beginning of the experiment, the average BW was 629 ± 53 kg, milk production was 29 ± 7

kg and number of days in milk was 106 ± 21 days. Cows were separated into two groups balanced for number of primiparous, calving date, and milk production. The two groups were conducted in a 2×2 crossover design. Each experimental period lasted five weeks with the two last weeks for measurement (weeks 4 & 5). Cows were housed in a freestall barn except during the measurement weeks in which they were tied individually.

Table 2 Ingredients and chemical composition of the experimental diets (trial 2)

	Diet ¹	
	CON-2	TEA-2
Ingredients (% of DM)		
Maize silage	54.00	54.00
Hay	6.00	6.00
Pelleted concentrates		
Maize	11.88	11.88
Barley	3.36	2.96
Soybean meal	5.24	5.24
Rapeseed meal	2.00	2.00
Soybean hulls	6.60	6.60
Wheat bran	6.00	5.24
Dehydrated beet pulp	0.94	0.94
Urea	0.80	0.80
Calcium carbonate	1.13	1.13
Dicalcium phosphate	0.44	0.44
Molasses beet	1.20	1.60
Mineral-vitamin mix	0.20	0.20
Salt	0.17	0.17
Fungicide	0.02	0.02
Aroma	0.02	0.02
Tea saponin extract ²	0.00	0.76
Chemical composition (% of DM)		
OM	93.0	93.1
CP	16.1	16.1
NDF	35.1	35.6
ADF	18.4	18.7
Starch	28.2	27.8
GE (MJ/kg of DM)	17.7	17.9

¹ CON-2= diet control; TEA-2 = diet CON-2 containing 0.5% tea saponin.

² 689 g saponins/kg of DM according to supplier (Choisun Tea Sci-Tech Co. Ltd., Hangzhou, Zhejiang, China) indications.

Each group of cows received two dietary treatments consisting in (on a DM basis): 1) control (CON-2), 2) CON-2 with 0.5% tea saponin (TEA-2). Dosage of tea saponin and manufacturer were similar to trial 1 but the extract came from different batches as purchased separately. Diet CON-2 was made of 54% maize silage, 6% hay and 40% pelleted concentrates (InVivo

NSA, Longué-Jumelles, France; Table 2) and met the requirements of lactating dairy cows (INRA, 2010). Diets were equivalent in terms of starch (28.0%), crude protein (16.1%) and fiber (35.4%).

Two weeks before starting the experiment, cows were fed *ad libitum* with CON-2. Then, all along the experiment, cows were fed *ad libitum*, except during measurement weeks in which offered feed was restricted to 95% of individual voluntary feed intake. At the beginning of each experimental period, TEA-2 concentrate was progressively supplied by replacing CON-2 concentrate, to achieve the maximal dose after a one week transition period. During the experiment, hay was offered once daily (0800 h) and maize silage mixed with concentrates was distributed two times per day (66% at 0930 h and 34% at 1600 h). Forage-to-concentrate ratio was kept as close as possible to the target ratio by adjusting the amounts of feed offered weekly based on the composition of the refusals of the previous week. Cows had free access to water throughout the experiment.

Measurements and analyses for trials 1 & 2

Intake. During the 2 trials, offered feed and refusals were weighed and recorded daily to estimate DMI. Feed (hay and concentrate for trial 1; silage, hay and concentrate for trial 2) were sampled as described previously (Guyader et al., 2014b). Briefly, one sample of each feed was taken on two days during weeks 4 and 5. For each sample, one aliquote was used to determine DM (103°C for 24h) and another aliquote was stored at 4°C (hay and concentrate) or -20°C before freeze drying (maize silage). Refusals DM content was determined if they exceeded 1 kg/day and per animal in weeks 4 and 5. At the end of the experiment, each feed samples were pooled per treatment and ground (1 mm screen) before chemical analyses (InVivo Labs, Saint-Nolff, France for trial 1; InVivo Labs, Chierry, France for trial 2).

Organic matter was determined by ashing at 550°C for 6h (method 942.05; AOAC, 2005). Fiber (NDF and ADF) was determined by sequential procedures (Van Soest et al., 1991) after pretreatment with amylase, and expressed exclusive of residual ash. Total N was analyzed by combustion according to the Dumas method (method 968.06; AOAC, 2005), and CP content was calculated as $N \times 6.25$. Starch was analyzed using an enzymatic method (Faisant et al., 1995) and gross energy (GE) was analyzed by isoperibolic calorimetry (C200 model, IKA, Staufen, Germany).

Liveweight and blood methemoglobin. Animals were weighed at the end of each experimental period. In trial 1, levels of blood methemoglobin (metHb) were controlled 3h after morning meal for animals fed nitrate (NIT-1 and TEA+NIT-1). One control sample was taken from all animals the week preceding the start of the experiment. Then, blood was sampled at days 3, 5, 10, 12, 17, 19 and 29 of each experimental period. Blood was sampled from jugular vein and packed onto ice before metHb content analysis (Kaplan, 1965) within 1h at the nearest hospital (CHU Gabriel Montpied, Clermont-Ferrand, France).

Methane emissions. Kinetics of CH₄ emissions were determined using open chambers in week 4 as described in Guyader et al. (2014b), during four (trial 1) and two (trial 2) consecutive days. Chambers rear doors were opened twice daily for cleaning and milking, and front doors were opened for each feed distribution (five times per day for trial 1 and three times per day for trial 2). In total, doors were opened on average 15 min/day (trial 1) and 30 min/day (trial 2). As far as possible, doors were not opened during gases concentration analysis or deleted if it was the case. Air fluxes were not corrected for environmental data, as trial 1 showed that this correction did not influence final values.

Digestibility and nitrogen balance. Total tract digestibility of nutrients and N balance were determined via daily total and separate collection of feces and urine in week 5. Collection lasted six days in trial 1 and five days in trial 2. Each day, after weighing and mixing of feces, one aliquote (1%) was used to determine DM (103°C for 24 h) and another aliquote (1%) was pooled per week and per animal before freezing (-20°C). At the end of trials, samples were defrosted and dried (trial 1) or freeze-dried (trial 2) before grinding (1 mm screen). Chemical composition (OM, NDF, ADF, CP) was analyzed similarly to feed.

Urine was collected in vessels containing 500 mL sulfuric acid 3 M to maintain a urine pH lower than 3 to avoid N volatilization. Each day, after weighing, one aliquote (1%) was pooled per week and per animal before freezing (-20°C). At the end of each trial, samples were defrosted and N content was determined according to Kjeldahl method (InVivo Labs, Chierry, France; method 2001.11; AOAC, 2005) as it was not possible to apply the Dumas method on fresh urine. In trial 1, a second aliquote (0.25%) was diluted (1:4) with distilled water and pooled per week and per animal before freezing (-20°C). At the end of the trial, samples were defrosted and concentration in derivatives of puric bases (DPB; xanthin, hypoxanthin, allantoiné, uric acid) was determined by high pressure liquid chromatography (Shingfield and Offer, 1999) to assess microbial synthesis within the rumen.

Rumen fermentation parameters. Rumen content was sampled in the ventral sac of each cow through the cannula, 3h after morning meal, during two non-consecutive days in week 5 (trial 1) or by stomach tubing, 3.5h after the morning meal, on the last day of week 5 (trial 2). All rumen samples were strained through a polyester monofilament fabric (250 μm pore size) and filtrate was subsampled for volatile fatty acids (VFA, 0.8 mL filtrate in 0.5 mL of a 0.5 M HCl solution containing 2% (w/v) metaphosphoric acid and 0.4% (w/v) crotonic acid), ammonia (NH_3 , 1 mL filtrate in 0.1 mL of 5% orthophosphoric acid), and protozoa (2 mL filtrate in 2 mL of methyl green-formalin solution) concentrations analyses. In trial 1, lactate (3 mL filtrate without preservative), nitrate and nitrite (20 mL filtrate without preservative) concentrations were also determined as well as dynamics of rumen pH which was followed during six consecutive days in week 4 with boluses (eBolus, eCow, Exeter, United Kingdom; Guyader et al., 2014b).

Samples were stored at -20°C before analysis, except for protozoa samples which were stored at room temperature and away from direct light until counting. Concentrations of VFA and NH_3 were analyzed by gas chromatography with a flame ionization detector and by colorimetry, respectively (Morgavi et al., 2008). Lactate concentrations were determined by colorimetry (D/L-lactic acid, BioSentec, Auzeville-Tolosane, France). Nitrate and nitrite concentrations were analyzed by spectrometry (Laboratoire Vétérinaire et Biologique, Lempdes, France). Protozoa were counted by microscopy and categorized as either small ($<100\ \mu\text{m}$) or large ($>100\ \mu\text{m}$) entodiniomorphs, or as holotrichs (Dasytricha or Isotricha) (Williams and Coleman, 1992). Protozoa concentrations were \log_{10} -transformed before statistical analysis.

Milk yield and composition in trial 2. Milk production was daily quantified. Milk composition was determined at each milking on samples (30 mL) taken one day in week 4, mixed with potassium bichromate (Merck, Fontenay Sous Bois, France), and stored at 4°C . Milk fat, protein and lactose content were analyzed by infrared spectrometry with a 3-channel spectrophotometer (Galilait, Theix, France; method 972.16; AOAC, 1990) and urea concentration was determined by colorimetry (Galilait, Theix, France; Potts, 1967).

Statistical analyses

All statistical analyses were carried out with the mixed procedure of SAS (Version 9.2; SAS Institute, 2009). As sampling day effect ($n = 2$ for rumen fermentation parameters in trial 1; n

= 4 or 2 for CH₄ emissions in trials 1 and 2, respectively) was never significant, this factor was not considered in subsequent analyses, and all data were averaged per period.

In trial 1, the statistical model included the random effect of cow ($n = 4$) and fixed effects of period ($n = 4$), nitrate (CON-1 and TEA-1 versus NIT-1 and TEA+NIT-1), tea saponin (CON-1 and NIT-1 versus TEA-1 and TEA+NIT-1) and the interaction nitrate \times tea saponin. In trial 2, one animal passed away during the second period after a fall not linked with the trial. All data for this animal were deleted in further statistical analyses. The statistical model included the animal ($n = 7$) as random effect, and period ($n = 2$) and diet ($n = 2$) as fixed effects.

Daily kinetics of ruminal pH (trial 1) and CH₄ emissions (trials 1 and 2) were analyzed by repeated time. Hour ($n = 24$ for both trials) was treated as a repeated measurement with compound symmetry as covariance structure. In trial 1, the model included the fixed effects of period, hour, nitrate, tea saponin, nitrate \times tea saponin and the interactions between hour and dietary treatments (tea saponin \times hour, nitrate \times hour, tea saponin \times nitrate \times hour). In trial 2, the model included period, diet, hour and diet \times hour interactions as fixed effects.

Differences between diets were considered significant at $P < 0.05$, and trends were discussed at $0.05 \leq P \leq 0.1$. Least squares means are reported throughout.

Results

Trial 1 on non-lactating cows

Animals weight and metHb levels. At the end of the trial, animals weighed 699 ± 42 kg, which corresponded to an average weight gain of 10 kg per animal and per period. Levels of blood metHb progressively increased until the end of the second week of adaptation, before going down and remaining at stable and low levels at the beginning of measurement weeks (Supplementary material Figure S1). The maximal level reached by one animal fed NIT-1 was 25.9% on day 12.

Intake, diet digestibility and nitrogen balance (Table 3). Both nitrate and tea saponin reduced daily intake (DM, OM, NDF, ADF and GE; $P < 0.05$), with an additive effect between tea saponin and nitrate (tea saponin \times nitrate, $P > 0.05$). Nutrient digestibility was similar between diets ($P > 0.05$), with an average DM digestibility of 63.7%. Saponin-containing diets (TEA-1 and TEA+NIT-1) tended to improve NDF digestibility ($P = 0.126$) and nitrate-containing diets

(NIT-1 and TEA+NIT-1) tended to increase ADF digestibility ($P=0.073$). Nitrogen balance was positive (+17.3 g/day) and similar between diets.

Table 3 Daily nutrient intake, total tract digestibility and N balance of non-lactating cows fed diets containing tea saponin and calcium nitrate alone or in association (n = 4; trial 1)

	Diet ¹				SEM	<i>P</i> -value ²		
	CON-1	NIT-1	TEA-1	TEA+NIT-1		Saponin	Nitrate	Saponin × nitrate
Daily nutrient intake								
DM (kg/day)	12.3	12.0	12.0	11.8	0.40	0.032	0.040	0.914
OM (kg/day)	11.2	11.0	11.0	10.7	0.37	0.032	0.048	0.756
NDF (kg/day)	4.93	4.84	4.83	4.71	0.164	0.032	0.047	0.758
ADF (kg/day)	2.86	2.79	2.77	2.71	0.095	0.018	0.044	0.962
GE (MJ/day)	214	200	210	194	6.9	0.021	<0.001	0.548
Total tract digestibility (%)								
DM	62.8	63.8	64.5	63.7	1.15	0.270	0.845	0.220
OM	66.9	67.9	68.1	67.7	1.11	0.336	0.451	0.180
NDF	42.3	43.7	45.2	44.6	2.60	0.126	0.697	0.369
ADF	41.9	44.8	44.3	45.3	2.83	0.160	0.073	0.331
CP	59.1	54.4	58.6	55.1	3.35	0.972	0.241	0.852
N balance (g/day)								
N intake	247.5	232.5	242.5	217.5	8.54	0.003	<0.001	0.050
N in feces	102.1	108.4	99.2	97.9	10.22	0.458	0.778	0.671
N in urine	123.1	106.6	133.4	103.2	9.47	0.638	0.016	0.363
N in feces + urine	225.2	215.0	232.6	201.1	14.56	0.763	0.089	0.339
N balance	25.0	20.4	6.8	16.9	10.79	0.286	0.778	0.459

¹ CON-1 = diet control; NIT-1 = diet CON-1 containing 2.3% nitrate; TEA-1 = diet CON-1 containing 0.5% tea saponin; TEA+NIT-1 = diet CON-1 containing 0.5% tea saponin and 2.3% nitrate.

² Saponin = main effect of tea saponin (CON-1 and NIT-1 *versus* TEA-1 and TEA+NIT-1); Nitrate = main effect of nitrate (CON-1 and TEA-1 *versus* NIT-1 and TEA+NIT-1); Saponin × nitrate = interaction between main effects of tea saponin and nitrate.

Methane emissions (Table 4). Animals fed TEA-1 produced the same quantities of CH₄ (expressed as g/day, g/kg DMI, g/kg digested DM, g/kg digested OM, g/kg digested NDF, % of GE intake) than animals fed CON-1. Animals fed nitrate-containing diets (NIT-1 and TEA+NIT-1) produced the same quantities of CH₄ but in a lower amount than CON-1 (-28% on average; $P<0.05$). Kinetics of CH₄ emissions (Supplementary material Figure S2) confirmed the absence of CH₄-mitigating effect of tea saponin fed alone all along the day. Inversely, nitrate-containing diets induced lower emissions during 3h following meals before rising to similar levels than CON-1.

Table 4 Methane emissions of non-lactating cows fed diets containing tea saponin and calcium nitrate alone or in association (n = 4; trial 1)

	Diet ¹				SEM	P-value ²		
	CON-1	NIT-1	TEA-1	TEA+NIT-1		Saponin	Nitrate	Saponin × nitrate
g CH ₄ /day	312.3	219.2	294.0	206.3	13.37	0.248	<0.001	0.830
g CH ₄ /kg DMI	25.4	18.6	24.6	17.8	1.41	0.529	0.001	0.973
g CH ₄ /kg dDM	40.5	29.1	38.3	28.1	2.30	0.446	0.002	0.768
g CH ₄ /kg dOM	37.9	27.4	36.3	26.4	2.11	0.488	0.001	0.846
g CH ₄ /kg dNDF	60.2	42.5	55.2	41.4	4.29	0.395	0.003	0.571
% of GE intake	7.3	5.6	7.0	5.4	0.42	0.519	0.003	0.956

¹ CON-1 = diet control; NIT-1 = diet CON-1 containing 2.3% nitrate; TEA-1 = diet CON-1 containing 0.5% tea saponin; TEA+NIT-1 = diet CON-1 containing 0.5% tea saponin and 2.3% nitrate.

² Saponin = main effect of tea saponin (CON-1 and NIT-1 *versus* TEA-1 and TEA+NIT-1); Nitrate = main effect of nitrate (CON-1 and TEA-1 *versus* NIT-1 and TEA+NIT-1); Saponin × nitrate = interaction between main effects of tea saponin and nitrate.

Rumen fermentation parameters and protozoa concentrations (Table 5). Tea saponin fed alone increased total VFA concentrations after feeding compared to CON-1 (+19%; $P<0.05$) without modifying VFA profile. Diets supplemented with nitrate (NIT-1 and TEA+NIT-1) increased acetate proportion (+10% on average; $P<0.01$), reduced butyrate proportion (-39% on average; $P<0.01$) and reduced ammonia concentrations (-23.6% on average; $P<0.05$). No treatment affected nitrite concentrations and nitrate was never detected in the rumen. Average daily pH was similar between diets (6.20 on average), despite a reduction for TEA-1 between 3 and 5h after the morning meal and between 1 and 4h after the afternoon meal (Supplementary material Figure S3). Saponin-containing diets (TEA-1 and TEA+NIT-1) tended to increase protozoa concentration ($P<0.10$).

Table 5 Daily average pH, rumen fermentation characteristics and protozoa concentration 3 h after feeding non-lactating cows with diets containing tea saponin and calcium nitrate alone or in association (n = 4; trial 1)

	Diet ¹				SEM	P-value ²		
	CON-1	NIT-1	TEA-1	TEA+NIT-1		Saponin	Nitrate	Saponin × nitrate
Total VFA (mM)	101.50	98.43	120.58	98.13	5.515	0.013	0.003	0.011
VFA profile (%)								
Acetate (C2)	67.83	75.53	69.10	73.56	1.512	0.787	0.003	0.234
Propionate (C3)	16.57	14.68	16.39	16.38	1.911	0.489	0.397	0.401
Butyrate (C4)	11.64	7.01	11.08	7.16	0.854	0.813	0.002	0.678
Minor VFA ³	3.97	2.78	3.43	2.93	0.382	0.563	0.040	0.322
C2/C3	4.27	5.33	4.46	4.65	0.554	0.507	0.120	0.260
(C2+C4)/C3	5.00	5.82	5.18	5.11	0.628	0.501	0.347	0.279
NH ₃ -N (mM)	18.32	14.84	18.42	13.15	1.790	0.570	0.016	0.525
Total lactate (mM)	0.80	0.80	0.93	0.51	0.196	0.624	0.214	0.210
Nitrite (mg/L)	0.24	4.17	0.24	1.63	1.756	0.482	0.172	0.482
pH ⁴	6.24	6.31	6.01	6.22	0.104	0.137	0.187	0.480
Total protozoa (log ₁₀ /mL)	5.38	5.40	5.53	5.58	0.146	0.067	0.655	0.875

¹ CON-1 = diet control; NIT-1 = diet CON-1 containing 2.3% nitrate; TEA-1 = diet CON-1 containing 0.5% tea saponin; TEA+NIT-1 = diet CON-1 containing 0.5% tea saponin and 2.3% nitrate.

² Saponin = main effect of tea saponin (CON-1 and NIT-1 *versus* TEA-1 and TEA+NIT-1); Nitrate = main effect of nitrate (CON-1 and TEA-1 *versus* NIT-1 and TEA+NIT-1); Saponin × nitrate = interaction between main effects of tea saponin and nitrate.

³ Minor VFA = sum of isobutyrate, isovalerate, valerate and caproate.

⁴ Daily average.

Trial 2 on lactating cows

Animals lost 11 kg on average per period, to end with a final BW of 608 ± 33 kg.

Intake, diet digestibility and nitrogen balance (Table 6). Diet TEA-2 numerically reduced daily DMI (-2.3 kg/day), and did not affect intake of OM, NDF, ADF and GE. Nutrients digestibility (DM, OM, NDF, CP) was similar between diets with an average DM digestibility of 66.2%, but TEA-2 tended to improve ADF digestibility (+8%; P<0.10). N balance was positive and similar between CON-2 and TEA-2 (+54.6 g/day on average).

Table 6 Daily nutrient intake, total tract digestibility and N balance of lactating cows fed a diet containing tea saponin (n = 7; trial 2)

	Diet ¹		SEM	<i>P</i> -value
	CON-2	TEA-2		
Daily nutrient intake				
DM (kg/day)	20.0	17.7	1.23	0.109
OM (kg/day)	18.6	16.5	1.15	0.111
NDF (kg/day)	7.04	6.31	0.434	0.143
ADF (kg/day)	3.69	3.31	0.227	0.139
GE (MJ/day)	354	316	21.8	0.129
Total tract digestibility (%)				
DM	65.8	66.6	0.78	0.362
OM	67.5	68.4	0.77	0.359
NDF	48.3	52.1	1.55	0.147
ADF	43.9	47.9	1.38	0.086
CP	63.9	63.0	0.99	0.345
N balance (g/day)				
N intake	515.6	457.4	33.02	0.118
N in feces	186.4	169.2	14.07	0.254
N in urine	136.3	120.6	5.75	0.112
N in feces + urine	322.8	289.8	18.36	0.199
N in milk	143.9	123.0	14.54	0.486
N balance	52.2	56.9	12.74	0.878

¹ CON-2 = diet control; TEA-2 = diet CON-2 containing 0.5% tea saponin.

Milk production and methane emissions (Table 7). Diet TEA-2 reduced milk production by 18% (23.6 versus 28.9 kg/day; $P < 0.001$) without affecting milk content in fat (34.3 g/kg on average), protein (30.8 g/kg on average), lactose (50.5 g/kg on average) and urea (20.0 mg/dL on average). Feed efficiency was similar between CON-2 and TEA-2 (1.39 kg milk/kg DMI on average).

Expressed in g/day, CH₄ emissions were similar between CON-2 and TEA-2, and were higher for TEA-2 when expressed in g/kg DMI (+12.7%; $P < 0.001$), g/kg milk (+20.9%; $P < 0.05$), g/kg digested nutrients (+11.9% for OM; $P < 0.05$) or as a percentage of GE intake (+12.8%; $P < 0.001$). These differences between diets were maintained all along the day as observed on daily kinetics of CH₄ emissions (Supplementary material Figure S4).

Table 7 Milk production and CH₄ emission of lactating cows fed a diet containing tea saponin (n = 7; trial 2)

	Diet ¹		SEM	P-value
	CON-2	TEA-2		
Milk yield (kg/day)	28.9	23.6	1.97	<0.001
Feed efficiency ² (kg milk/kg DMI)	1.45	1.33	0.083	0.251
Fat concentration (g/kg)	30.3	38.2	3.48	0.321
Protein concentration (g/kg)	31.6	29.9	0.70	0.326
Lactose concentration (g/kg)	50.2	50.7	0.92	0.052
Urea concentration (mg/dL)	21.7	18.2	4.36	0.611
CH ₄ emissions				
g CH ₄ /day	435.2	442.2	38.69	0.840
g CH ₄ /kg DMI	21.3	24.7	1.10	0.004
g CH ₄ /kg milk	15.1	19.1	1.22	0.018
g CH ₄ /kg dDM	32.5	37.0	1.61	0.021
g CH ₄ /kg dOM	34.1	38.7	1.69	0.023
g CH ₄ /kg dNDF	126.2	133.2	5.99	0.454
% of GE intake	6.01	6.89	0.310	0.006

¹ CON-2 = diet control; TEA-2 = diet CON-2 containing 0.5% tea saponin.

² Feed efficiency = milk yield/DMI.

Rumen fermentation parameters and protozoa concentrations (Table 8). Concentrations in NH₃ and total VFA were similar between CON-2 and TEA-2 (15.1 and 105.2 mM, respectively). The VFA profile differed only in acetate proportion, which was higher for TEA-2 (+6.2%; $P < 0.05$) inducing a tendency for a higher C2/C3 ratio compared to CON-2 ($P < 0.10$). Protozoa concentrations were similar between diets (5.1 log₁₀/mL on average).

Table 8 Rumen fermentation characteristics and protozoa concentration 3 h after feeding lactating cows with a diet containing tea saponin (n = 7; trial 2)

	Diet ¹		SEM	P-value
	CON-2	TEA-2		
NH ₃ -N (mM)	16.08	14.15	2.763	0.643
Total VFA (mM)	107.07	103.32	10.720	0.806
VFA profile (%)				
Acetate (C2)	55.68	61.87	2.005	0.035
Propionate (C3)	23.25	20.47	1.650	0.185
Butyrate (C4)	16.57	13.72	1.429	0.199
Minor VFA ²	4.26	3.95	0.326	0.516
C2/C3	2.49	3.07	0.226	0.062
(C2+C4)/C3	3.26	3.75	0.272	0.176
Total protozoa (log ₁₀ /mL)	5.02	5.18	0.117	0.360

¹ CON-2 = diet control; TEA-2 = diet CON-2 containing 0.5% tea saponin.

² Minor VFA = sum of isobutyrate, isovalerate, valerate and caproate.

Discussion

Intake and reduction of lactating performances in cows fed tea saponin

In both trials, intake was reduced by tea saponin supplementation, even if the plant extract was included into pelleted concentrates which should have improved its palatability thanks to the presence of aroma. We also faced difficulties to feed tea saponin as a powder, as handling of the powder led to respiratory irritation problems for users and animals refused to eat it. This issue has never been highlighted in previous studies testing this plant extract (Mao et al., 2010; Zhou et al., 2011; Li and Powers, 2012).

Tea saponin clearly reduced milk production without affecting milk composition. The reduction of milk yield can be explained by the tendency for a lower DMI, as feed efficiency was similar between diets. To our knowledge, the negative effect of tea saponin on lactating performances of dairy cattle has never been observed. Instead, inconsistent results have been reported on beef cattle and lambs. Mao et al. (2010) did not observe differences in growth of lambs supplemented with 0.5% tea saponin. With steers, Li and Powers (2012) reported no effect of 0.05% tea saponin on the average daily weight gain, whereas a higher dose (0.11%) reduced the average daily weight gain by 80% linked to a drop of DMI (-27%). Overall results show that a dose response study on dairy cattle is required to complete this work.

Absence of positive methane mitigating effect of tea saponin

Tea saponin supplementation (0.5% DM) did not affect CH₄ emissions (g/kg DMI) of non-lactating cows and increased CH₄ emissions (g/kg DMI) of lactating cows, after 4 weeks of feeding saponin. This result is linked to the absence of the expected reduction of ruminal protozoa in both studies suggesting an adaptation of this population. Indeed, in sheep, a decrease of protozoa number after 4 days of feeding saponins (*Sesbania sesban*) was reported but this population recovered 10 days later (Newbold et al., 1997). The absence of CH₄-mitigating effect of tea saponin was reported previously on steers but animals were fed low tea saponin doses (0.11% maximum; Li and Powers, 2012). However, with similar doses than ours (0.5% tea saponin), CH₄ emissions (g/kg DMI) were reduced by 27% (Mao et al., 2010) and 11% (Zhou et al., 2011) in sheep, and were linked to a reduction of protozoa concentrations (-41% and -43% of total bacterial 16S rDNA, respectively) after 3-8 weeks saponin feeding.

Several reasons may explain the inefficiency of our tea saponin extract on methanogenesis and on associated rumen microbial and fermentative parameters. In our trials, tea saponin was

included into granulated concentrates whereas it was distributed as a powder in other studies. During the pelleting process, the saponin was heated (~40°C), which may have damaged its anti-methanogenic and -protozoal properties. Indeed, a modification of the miscellaneous structure of QS was already observed after heating between 20 and 60°C (Mitra and Dungan, 1997). An animal species effect (sheep *versus* cattle) may be also considered. Finally, we cannot exclude an effect of the batch production; plant maturity, geographical area of production and extraction methods are three parameters affecting the final concentration and quality of the saponin (Li and Powers, 2012).

The mode of action of nitrate to mitigate methanogenesis is different from saponins as it does not reduce protozoa. Nitrate may not only act as a hydrogen-sink but may also have a direct inhibiting effect towards rumen methanogens (Guyader et al., 2014a). Nitrate fed alone reduced CH₄ emissions related to DMI by 27%, corresponding to a 12% reduction per percentage unit of nitrate fed. This result confirms once more time the efficiency and repeatability of the nitrate CH₄-mitigating effect in cattle (Hulshof et al., 2012; Guyader et al., 2014b; Veneman et al., 2014). Moreover, a recent meta-analysis reported a linear dose-response effect of nitrate (0.3 to 1.2 g/kg BW/day) on enteric CH₄ emissions with a reduction of 12% of CH₄ yield (g/kg DMI) per 0.1 g added nitrate/kg BW/day (Lee and Beauchemin, 2014). Association of nitrate plus tea saponin did not accentuate the CH₄-mitigating effect of nitrate, suggesting that the CH₄ reduction with this association was linked to the nitrate effect. Nitrate fed alone or in association with tea saponin to non-lactating cows increased acetate without changing propionate concentrations in the rumen, which confirmed previous findings (Nolan et al., 2010; Hulshof et al., 2012; Veneman et al., 2014). Increased acetate concentration may compensate the hydrogen deficiency in the rumen (Janssen, 2010) linked to nitrate reduction.

Improvement of fiber digestibility with tea saponin

Tea saponin did not modify diet digestibility of non-lactating cows, whereas with lactating cows, it tended to improve ADF digestibility (+4 units). To our knowledge, our study is the first one to show a beneficial effect of tea saponin on nutrient digestibility of cattle. This effect was not reported on goats supplemented with low doses (0.04, 0.06 and 0.08%; Zhou et al., 2012). Generally, saponins have an undermined effect on diet digestibility, which seems to be linked to their source and dose. Only Pen et al. (2007) observed an increased NDF digestibility (+3.7 units) on ovine supplemented with 0.08% saponin from QS. Most authors reported no effect of saponins on diet digestibility in bovine (0.03% saponin from YS or QS,

Holtshausen et al., 2009) or in ovine (0.13% saponin from YS, Pen et al., 2007; 1-4% saponin from *Medicago sativa*, Klita et al., 1996). A depressive effect on fiber digestibility was even shown with 0.5 and 1% saponin from YS (-2.6 and -2.9 units, respectively; Lila et al., 2005) and with 1% saponin extracted from the tropical tree *Sapindus saponaria* (-3 units, Hess et al., 2004).

Nitrate supplementation did not affect diet digestibility and N balance in both trials, confirming previous studies on sheep (Nolan et al., 2010) and lactating cows (Van Zijderveld et al., 2011; Guyader et al., 2014b) supplemented up to 2.5% nitrate. Nitrate was well metabolized by the animals and can substitute urea as a non-protein N source in diets low in fermentescible N content (Leng, 2008). Moreover, the absence of animals' health issue in terms of methemoglobinemia supports the use of this chemical at the farm scale under controlled conditions. It is recommended to feed animals with maximum doses of 1% nitrate (Doreau et al., 2014) and to apply a long enough adaptation period (Lee and Beauchemin, 2014).

In conclusion, tea saponin supplementation did not reduce CH₄ emissions and rumen protozoa concentrations in cattle. The inefficiency may be explained by the denaturation of the active compound of the plant when heating during the pelleting process. To test this hypothesis, an *in vitro* experiment may be carried out to compare gas production and composition and protozoa number with pelleted or non-pelleted tea saponin supplementation. This plant extract tended to increase fiber digestibility of lactating cows, without improving animals' performances. Further work is required to improve tea saponin palatability and to confirm its positive effect on digestibility via a dose response study.

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References

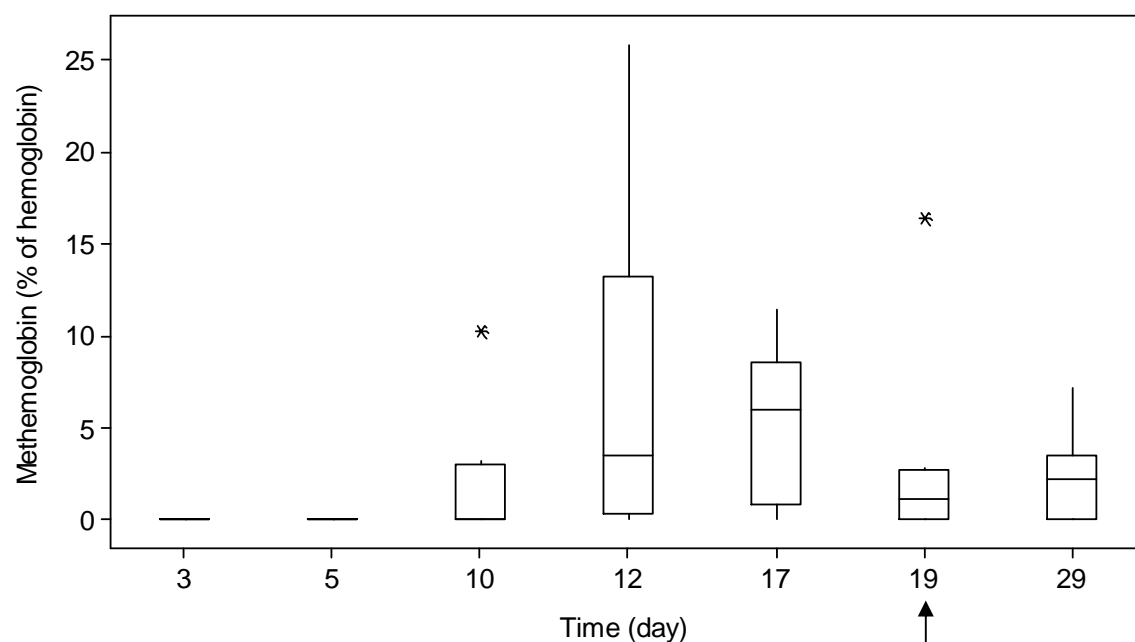
- AOAC 1990. Official Methods of Analysis. Assoc. Off. Anal. Chem., Arlington, VA.
- AOAC 2005. Official Methods of Analysis. 18th ed. Assoc. Off. Anal. Chem., Arlington, VA.
- Doreau M, Bamière L, Pellerin C, Lherm M and Benoit M 2014. Mitigation of enteric methane for French cattle: Potential extent and cost of selected actions. *Animal Production Science* 54, 1417-1422.

- Faisant N, Planchot V, Kozlowski F, Pacouret MP, Colonna P and Champ M 1995. Resistant starch determination adapted to products containing high level of resistant starch. *Sciences des Aliments* 15, 83-89.
- Gerber PJ, Henderson B and Makkar HPS 2013. Mitigation of greenhouse gas emissions in livestock production: a review of technical options for non-CO₂ emissions. Food and Agriculture Organization of the United Nations, Rome, Italy.
- Goel G and Makkar HPS 2012. Methane mitigation from ruminants using tannins and saponins. *Tropical Animal Health and Production* 44, 729-739.
- Guyader J, Silberberg M, Popova M, Morgavi DP, Seradj AR, Gerard C, Loncke C and Martin C 2014a. Dietary nitrate inhibits rumen methanogenic archaea without influencing genes coding for microbial nitrate or nitrite reductases. *Archaea* (Special issue: Methanogens of the gastrointestinal tract and methane mitigation from livestock) Submitted (November 2014).
- Guyader J, Eugène M, Meunier B, Doreau M, Morgavi DP, Silberberg M, Rochette Y, Gerard C, Loncke C and Martin C 2014b. Positive interaction between dietary linseed oil and nitrate as methane emissions-reducers in cattle. *Journal of Animal Science* Accepted.
- Hess HD, Beuret RA, Lötscher M, Hindrichsen IK, Machmüller A, Carulla JE, Lascano CE and Kreuzer M 2004. Ruminal fermentation, methanogenesis and nitrogen utilization of sheep receiving tropical grass hay-concentrate diets offered with *Sapindus saponaria* fruits and *Cratylia argentea* foliage. *Animal Science Journal* 79, 177-189.
- Holtshausen L, Chaves AV, Beauchemin KA, McGinn SM, McAllister TA, Odongo NE, Cheeke PR and Benchaar C 2009. Feeding saponin-containing *Yucca schidigera* and *Quillaja saponaria* to decrease enteric methane production in dairy cows. *Journal of Dairy Science* 92, 2809-2821.
- Hulshof RBA, Berndt A, Gerrits WJJ, Dijkstra J, Van Zijderveld SM, Newbold JR and Perdok HB 2012. Dietary nitrate supplementation reduces methane emission in beef cattle fed sugarcane based diets. *Journal of Animal Science* 90, 2317-2323.
- INRA 2010. Alimentation des bovins, ovins et caprins. INRA Editions, Paris, France.
- Janssen PH 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Animal Feed Science and Technology* 160, 1-22.
- Kaplan JC 1965. Méthode de mesure rapide du taux de la méthémoglobine dans les globules rouges. *Revue Française d'Etudes Cliniques et Biologiques* 10, 856-859.

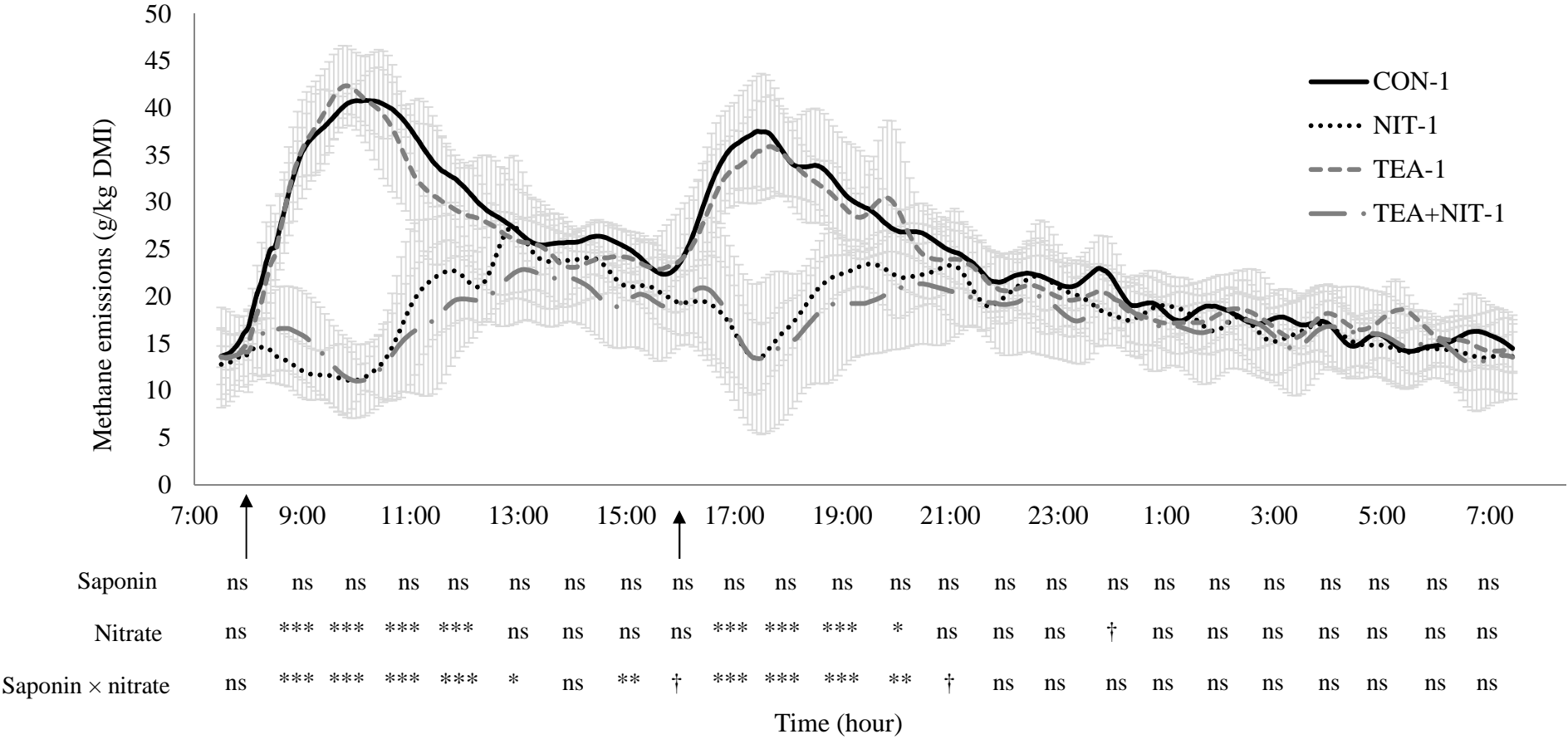
- Klita PT, Mathison GW, Fenton TW and Hardin RT 1996. Effects of alfalfa root saponins on digestive function in sheep. *Journal of Animal Science* 74, 1144-1156.
- Lee C and Beauchemin KA 2014. A review of feeding supplementary nitrate to ruminant animals: nitrate toxicity, methane emissions, and production performance. *Canadian Journal of Animal Science* 94.
- Leng RA 2008. The potential of feeding nitrate to reduce enteric methane production in ruminants. Department of Climate Change, Commonwealth Government of Australia, Canberra, ACT, Australia.
- Li W and Powers W 2012. Effects of saponin extracts on air emissions from steers. *Journal of Animal Science* 90, 4001-4013.
- Lila ZA, Mohammed N, Kanda S, Kurihara M and Itabashi H 2005. Sarsaponin effects on ruminal fermentation and microbes, methane production, digestibility and blood metabolites in steers. *Asian-Australasian Journal of Animal Sciences* 18, 1746-1751.
- Mao HL, Wang JK, Zhou YY and Liu JX 2010. Effects of addition of tea saponins and soybean oil on methane production, fermentation and microbial population in the rumen of growing lambs. *Livestock Science* 129, 56-62.
- Mitra S and Dungan SR 1997. Micellar properties of Quillaja saponin. 1. Effects of temperature, salt, and pH on solution properties. *Journal of Agricultural and Food Chemistry* 45, 1587-1595.
- Morgavi DP, Jouany JP and Martin C 2008. Changes in methane emission and rumen fermentation parameters induced by refaunation in sheep. *Animal Production Science* 48, 69-72.
- Newbold CJ, El Hassan SM, Wang J, Ortega ME and Wallace RJ 1997. Influence of foliage from African multipurpose trees on activity of rumen protozoa and bacteria. *British Journal of Nutrition* 78, 237-249.
- Nolan JV, Hegarty RS, Hegarty J, Godwin IR and Woodgate R 2010. Effects of dietary nitrate on fermentation, methane production and digesta kinetics in sheep. *Animal Production Science* 50, 801-806.
- Patra AK and Zhongtang Y 2013. Effective reduction of enteric methane production by a combination of nitrate and saponin without adverse effect on feed degradability, fermentation, or bacterial and archaeal communities of the rumen. *Bioresource Technology* 148, 352-360.
- Pen B, Takaura K, Yamaguchi S, Asa R and Takahashi J 2007. Effects of *Yucca schidigera* and *Quillaja saponaria* with or without β 1-4 galacto-oligosaccharides on ruminal

- fermentation, methane production and nitrogen utilization in sheep. *Animal Feed Science and Technology* 138, 75-88.
- Potts TJ 1967. Colorimetric determination of urea in feeds (report of AOAC Committee). *Journal of the AOAC* 50, 56-58.
- Shingfield KJ and Offer NW 1999. Simultaneous determination of purine metabolites, creatinine and pseudouridine in ruminant urine by reversed-phase high-performance liquid chromatography. *Journal of Chromatography B Biomedical Applications* 723, 81-94.
- Van Soest PJ, Robertson JB and Lewis BA 1991. Methods for dietary fiber, neutral detergent fiber, and non starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* 74, 3583-3597.
- Van Zijderveld SM, Gerrits WJJ, Dijkstra J, Newbold JR, Hulshof RBA and Perdok HB 2011. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. *Journal of Dairy Science* 94, 4028-4038.
- Veneman JB, Muetzel S, Hart KJ, Faulkner CL, Moorby JM, Molano G, Perdok HB, Newbold JR and Newbold CJ 2014. Dietary nitrate but not linseed oil decreases methane emissions in two studies with lactating dairy cows. In *Livestock, Climate Change and Food Security Conference*, Madrid, Spain.,
- Wang JK, Ye JA and Liu JX 2012. Effects of tea saponins on rumen microbiota, rumen fermentation, methane production and growth performance - a review. *Tropical Animal Health and Production* 44, 697-706.
- Wei ML, Ren LP, Zhou ZM and Meng QX 2012. Effect of addition of three plant extracts on gas production, ruminal fermentation, methane production and ruminal digestibility based on an in vitro technique. *Journal of Animal and Veterinary Advances* 11, 4304-4309.
- Yuan ZP, Zhang CM, Zhou L, Zou CX, Guo YQ, Li WT, Liu JX and Wu YM 2007. Inhibition of methanogenesis by tea saponin and tea saponin plus disodium fumarate in sheep. *Journal of Animal and Feed Sciences* 16, 560-565.
- Zhou CS, Xiao WJ, Tan ZL, Salem AZM, Geng MM, Tang SX, Wang M, Han XF and Kang JH 2012. Effects of dietary supplementation of tea saponins (*Ilex kudingcha* C.J. Tseng) on ruminal fermentation, digestibility and plasma antioxidant parameters in goats. *Animal Feed Science and Technology* 176, 163-169.

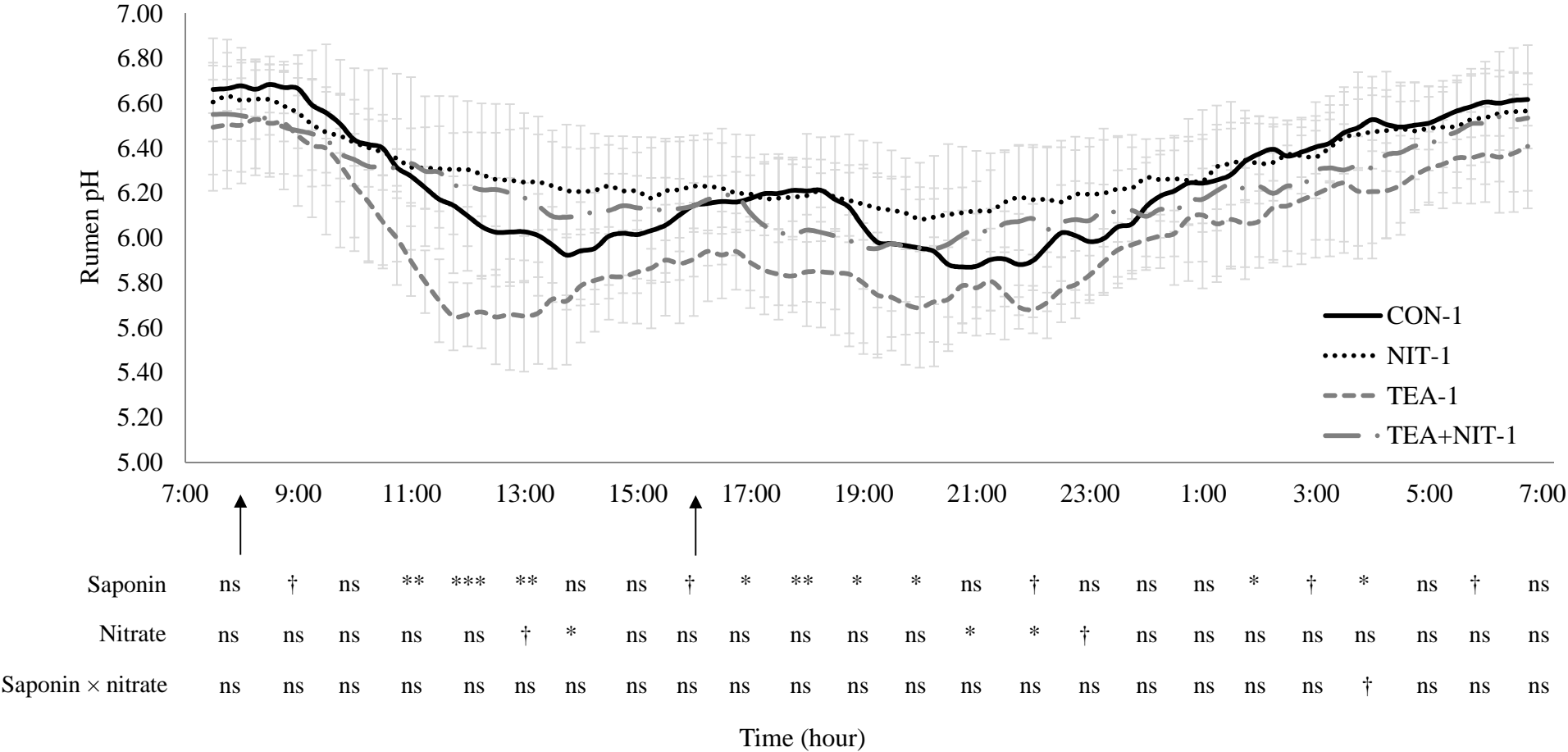
Zhou YY, Mao HL, Jiang F, Wang JK, Liu JX and McSweeney CS 2011. Inhibition of rumen methanogenesis by tea saponins with reference to fermentation pattern and microbial communities in Hu sheep. *Animal Feed Science and Technology* 166-167, 93-100.

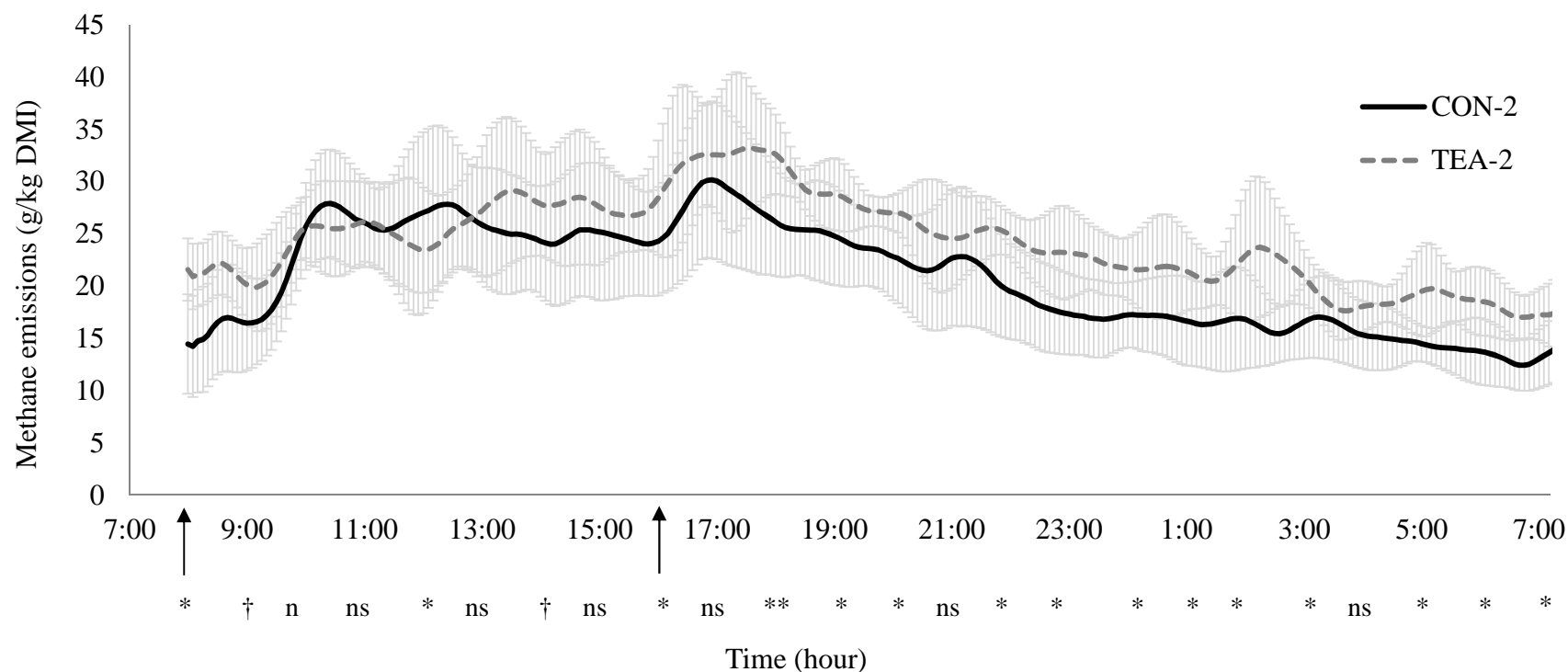
Supplementary File – for Online Publication Only

Supplementary Figure S1. Boxplot of blood methHb levels of non-lactating cows fed diets containing 2.3% nitrate with or without 0.5% tea saponin (n = 8; trial 1). The box represents the quartiles with the median at the center and the vertical lines represent the maximum and minimum value within 1.5 interquartile range of the higher and lower quartile, respectively. Values greater than 1.5 interquartile range are considered as outliers and are identified with a star. Blood was analyzed during the three weeks adaptation period, the arrow indicates the start of the measurement period.



Supplementary Figure S2. Daily CH₄ production pattern of non-lactating cows fed diets containing 2.3% nitrate with or without 0.5% tea saponin (n = 4; trial 1). Errors bars indicate s.d. Treatments consisted in diet control (CON-1), diet CON-1 containing 2.3% nitrate (NIT-1), diet CON-1 containing 0.5% tea saponin (TEA-1) and diet CON-1 containing 0.5% tea saponin and 2.3% nitrate (TEA+NIT-1). The arrows indicate time of feeding. Symbols indicate hourly statistical comparison († = $P < 0.10$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$) between treatments: saponin = main effect of tea saponin (CON-1 and NIT-1 *versus* TEA-1 and TEA+NIT-1); nitrate = main effect of nitrate (CON-1 and TEA-1 *versus* NIT-1 and TEA+NIT-1); saponin × nitrate = interaction between main effects of tea saponin and nitrate.





Supplementary Figure S4. Daily CH₄ production pattern of lactating cows fed a diet containing 0.5% tea saponin (n = 7; trial 2). Errors bars indicate s.d. Treatments consisted in diet control (CON-2) and diet CON-2 containing 0.5% tea saponin (TEA-2). The arrows indicate time of feeding. Symbols indicate hourly statistical comparison between CON-2 and TEA-2 († = $P < 0.10$; * = $P < 0.05$; ** = $P < 0.01$; *** = $P < 0.001$).

STEP 4: Dietary nitrate inhibits rumen methanogenic archaea without influencing genes coding for microbial nitrate or nitrite reductases

Objective

To study the effect of CH₄-mitigating strategies acting on H₂ production (lipids from linseed or saponin from tea, toxic effect towards protozoa) and H₂ utilization (nitrate from calcium nitrate, H₂-sink through nitrate reduction to nitrite and ammonia) on abundance, activity and diversity of rumen microbiota from non-lactating cows.

Experimental approach

4 non-lactating cows → 2 × 2 Factorial design → **FD 1**

CON: 50% hay + 50% pelleted concentrate
NIT: CON + 2.3% nitrate (from calcium nitrate)
LIN: CON + 2.6% added lipids (from linseed oil)
LIN+NIT: CON + 1.0% added lipids + 2.3% nitrate

4 non-lactating cows → 2 × 2 Factorial design → **FD 2**

CON: 50% hay + 50% pelleted concentrate
NIT: CON + 2.3% nitrate (from calcium nitrate)
TEA: CON + 0.5% saponin (from tea)
TEA+NIT: CON + 0.5% saponin + 2.3% nitrate

Rumen samples taken in wk 5 (FD1) or wk 4 (FD2) 3 h after morning feeding

Total nucleic acids extraction (DNA and RNA) and cDNA synthesis

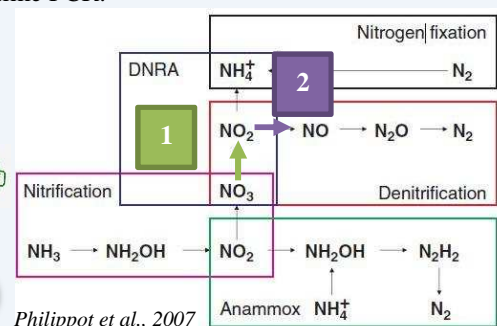
Abundance (DNA) and gene expression (cDNA) analysis by real-time PCR:

- Total bacteria (*rrs*) & methanogens (*mcrA*)
- Nitrate reducing bacteria (*napA* and *narG* /1)
- Nitrite reducing bacteria (*nirK* /2)

Diversity (DNA and cDNA) analysis with MiSeq, Illumina:

- Bacteria, protozoa, methanogens, fungi

In progress
(Annex 1)



Main resultsFD 1FD 2

- Total rumen bacteria abundance was similar between diets.
- Methanogens abundance was reduced by nitrate- and lipids- containing diets in FD1 but not in FD2. Methanogens activity was reduced by 2.3 folds on average in diets including nitrate (NIT and LIN+NIT in FD1; NIT and TEA+NIT in FD2).
- Relative abundance of *napA*, *narG* and *nirK* DNA copies were similar between diets in FD1 and FD2. Only *narG* activity was detected without difference between dietary treatments.

Conclusion

Lipids from linseed, saponin from tea, nitrate and their association (linseed plus nitrate and linseed plus tea saponin) act differently on rumen microbiota. Linseed reduced methanogens abundance, which may be explained by a toxic effect of fatty acids. Tea saponin did not affect targeted microbial population. Nitrate fed alone or in association with linseed or tea saponin did not affect nitrate and nitrite reducing bacteria, but had a toxic effect towards abundance and activity of methanogens, probably linked to nitrite toxicity. Further work is in progress to assess the effect of these three dietary treatments on diversity of rumen microbiota.

Dietary nitrate inhibits rumen methanogenic archaea without influencing genes coding for microbial nitrate or nitrite reductases

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In progress

Abstract

This work assessed the effect of nitrate fed alone or in association with linseed or tea saponin on the abundance and activity of rumen bacteria, methanogens and nitrate and nitrite reductases. Two 2×2 factorial design experiments (FD1 and FD2) were performed using four non-lactating cows each. Diets were: 1) control, 2) control with 2.3% nitrate, 3) control with 4% linseed oil (FD1) or 0.77% tea saponin (FD2), and 4) control with 2.3% nitrate and 4% linseed oil (FD1) or 0.77% tea saponin (FD2). Rumen content was sampled after morning feeding at the end of each experimental period. Extracted nucleic acids were used for microbial quantification and gene expression analysis by qPCR. Targeted genes were: *rrs* (total bacteria), *mcrA* (methanogens), *narG*, *napA* and *nirK* (nitrate and nitrite reductase). Total bacteria abundance was similar among diets. Nitrate fed alone or in association with linseed reduced methanogens abundance and *mcrA* expression (FD1). Nitrate fed alone or in association with tea saponin only reduced *mcrA* expression (FD2). Abundance and expression of *narG*, *napA* and *nirK* were unaffected by diets. Dietary nitrate inhibited rumen methanogens but did not affect microbial genes coding for nitrate or nitrite reductases.

Keywords: Methanogens; Nitrate; Nitrate reductase; Nitrite reductase; Rumen

Background

We found a methane (CH₄)-mitigating effect of nitrate (2.3% in dry matter, DM) fed alone or in association with linseed oil (2.6% added fat in DM, [8]) or tea saponin (0.5% saponin in DM; Guyader et al., personal communication) in non-lactating cows. The predominant pathway of nitrate metabolism in the rumen is the reduction of nitrate to nitrite and nitrite to ammonia which consumes four moles of hydrogen (H₂) [14] thus reducing H₂ availability for methanogens. Another pathway of nitrate reduction consists in denitrification to produce gaseous nitrous oxide (N₂O) [23]. These mechanisms require the presence of bacteria known to reduce nitrate or nitrite such as *Selenomonas ruminantium*, *Veillonella parvula* and *Wolinella succinogenes* [11]. However, the quantity of these rumen bacteria was not affected when nitrate was supplemented to goats (1% in DM; [2]) or steers (1.2% in DM; [15]). Nevertheless, the effect of nitrate supplementation on both abundance and expression of universal genes coding for nitrate reductases in the rumen has never been assessed. In addition, whereas N₂O has been detected in eructated gaseous emissions of dairy cattle supplemented with 2.1% nitrate in DM [22], the abundance and expression of genes targeting nitrite reductases in the rumen have never been studied.

Sheep fed a corn silage-based diet had reduced abundance of rumen methanogens when supplemented with 2.1% nitrate (in DM) [26]. We also observed that nitrate supplementation induced a rise of dissolved H₂ concentrations in the rumen of cows following ingestion [9]. These results suggest that nitrate may not only act as a H₂-sink but may also have a direct inhibiting effect on rumen methanogens. Nevertheless, the abundance and activity of methanogens in the rumen of cattle supplemented with nitrate is unknown.

The objective of this work was to assess the effect of nitrate fed alone or in association with linseed or tea saponin on i) the abundance and activity of methanogens, and ii) the abundance and expression of microbial genes targeting nitrate and nitrite reductases in the rumen of cows.

Materials and methods

The experiment was conducted at the animal experimental facilities of INRA's Herbivores Research Unit (UERT, Saint-Genès-Champagnelle, France) from January to June 2013. All procedures involving animals were conducted in accordance with the French Ministry of Agriculture guidelines for animal research, and all applicable European guidelines and regulations on animal experimentation. The experiment was approved by the Auvergne regional ethic committee for animal experimentation, approval number CE50-12.

Animals, experimental design and feeding management

Eight non-lactating Holstein cows were separated into two groups conducted in parallel according to 2×2 factorial designs. Within each experiment, four cows were randomly assigned to four dietary treatments during 5-week experimental periods. In factorial design 1 (FD1), diets were on a DM basis: 1) control diet (CON, 50% natural grassland hay and 50% concentrate), 2) control diet with 4% linseed oil (LIN; 2.6% added fat), 3) control diet with 3% calcium nitrate (NIT; 2.3% nitrate), and 4) control diet with 4% linseed oil plus 3% calcium nitrate (LIN+NIT; 2.6% added fat plus 2.3% nitrate) [8]. In factorial design 2 (FD2), diets were on a DM basis: 1) control diet (CON, 50% natural grassland hay and 50% concentrate), 2) control diet with 0.77% tea saponin (TEA; 0.5% saponin), 3) control diet with 3% calcium nitrate (NIT; 2.3% nitrate), and 4) control diet with 0.77% tea saponin plus 3% calcium nitrate (TEA+NIT; 0.5% saponin plus 2.3% nitrate). Chemical composition of diets CON and NIT were similar between the two experiments.

Rumen content sampling for microbial analysis

At the end of each experimental period, rumen contents of cows were sampled over two days. Whole rumen content samples (200 g) were taken, through the cannula, from multiple sites within the rumen. Sampling was done 3 h after the morning feeding when CH₄ emissions differences between diets measured on the same animals were maximal [8]. A part of each sample (~30 g) was mixed with 30 mL ice cold PBS pH 6.8 and homogenized using a Polytron grinding mill (Kinematica GmbH, Steinhofhalde, Switzerland) for three cycles of 1 min with intervals of 1 min on ice. Then, approximately 0.5 g were transferred to a 2.5 mL Eppendorf tube and mixed with 1 mL of RNeasy® Stabilization Solution (Applied Biosystems, Austin, TX, USA). Tubes were immediately stored at -80°C until total nucleic extractions which were done within 3 months of storage. Remaining rumen samples were used to determine DM of rumen content (103°C for 24h).

Total nucleic acids extraction and cDNA synthesis

Total nucleic acids (DNA and RNA) were co-extracted from all samples by bead-beating and phenol-chloroform extraction followed by saline-alcohol precipitation [24]. The yield and purity of extracted DNA and RNA were assessed using a Nanodrop Lite Spectrophotometer (Thermo Fisher Scientific, Wilmington, USA), by measuring the absorbance intensity at 260 nm and the absorbance ratio 260/280, respectively. RNA integrity was estimated with an Agilent RNA 6000 Nano Kit on an Agilent 2100 bioanalyzer (Agilent Technologies, Santa

Clara, CA, USA) according to the manufacturer's instructions. RNA Integrity Number (RIN) and the ratio between ribosomal RNA (rRNA) 23S/16S were calculated using the Software 2100 Expert, version B 02.08. SI648 (SR2; Agilent Technologies, Waldbronn, Germany).

Following extraction and quality assessment, RNA was reverse transcribed using the Reverse Transcriptase Kit with random primers (Promega, Madison, USA) according to manufacturer's instructions, on a T-100 thermocycler (BioRad, Hercules, USA). Both DNA and cDNA were stored at -20°C before subsequent analyses within 2 months following DNA extraction and cDNA synthesis.

Quantification and gene expression of microbial communities

Samples from each cow from the two sampling days of each experimental period were pooled by mixing an equal quantity of DNA or cDNA reaction volume, respectively. Quantification of gene targets were performed on microbial DNA and cDNA by quantitative PCR (qPCR) using a Step One Plus apparatus (Applied Biosystems, Villebon sur Yvette, France). Reactions were run in triplicate in 96-well plates, using 15.5 µL of 1X Takara SYBR Premix Ex Taq (Lonza, France), 0.25 µmoles of each forward and reverse primer and 20 ng of DNA or 2 µL of cDNA in a final volume of 20 µL. In this study, we used universal primers targeting the bacterial *rrs* gene and methanogenic specific primers, which were both designed for the rumen ecosystem. We also used universal primers to target nitrate and nitrite reductase genes; however, these pairs of primers were designed based on sequences recovered from non-rumen ecosystems. Primers description, average amplification efficiency, slope and R^2 of qPCR are described in Table 1, as required by MIQE guidelines for PCR [4]. Negative controls without templates were run in each assay to assess overall specificity.

Abundance of total bacteria (based on *rrs* DNA copies) was assessed using absolute quantification. Standard curve [19], amplification and melting curve were carried out as previously described [7]. Abundance of methanogenic archaea (based on *mcrA* DNA copies) was also assessed using absolute quantification, with standard curve prepared as previously described [19]. Level of expression of the functional *mcrA* gene (based on *mcrA* cDNA copies) was assessed using relative quantification with *rrs* cDNA copies used as reference. For both *mcrA* gene quantification and expression analyses, amplification and melting curve programs were performed as previously described [5].

Copy number and level of expression of genes involved in nitrate and nitrite reduction were analyzed by targeting two genes coding for a membrane-bound (*narG*) and a periplasmic (*napA*) nitrate reductase commonly found in bacteria from anaerobic estuarine sediments [25]

and one gene coding for a nitrite reductase found in bacteria from soil (*nirK* [10]). The presence of these three genes in rumen metagenomes was checked using the metagenomics RAST server [18]: *narG* and *napA* were already described in the rumen, whereas *nirK* was not reported. The qPCR program was the same as for total bacteria. Abundance (based on DNA copies) and activity (based on cDNA copies) of these genes were assessed using relative quantification with *rrs* as the reference gene (DNA *rrs* or cDNA *rrs*).

Table 1 Description of primers (sequences, product size, average amplification efficiency, slope and R²) used for quantifying abundance and activity of total bacteria, methanogenic archaea and nitrate and nitrite reductases by qPCR

Organism or enzyme	Target gene	Primer set	Primer sequences 5'-3'	Product size (bp)	Efficiency	Slope	R ²
Total bacteria [7]	<i>rrs</i>	520 F 799 R2	AGCAGCCGCGGTAAT CAGGGTATCTAATCCTGTT	280	1.88	-3.64	0.999
Methanogenic archaea [5]	<i>mcrA</i>	<i>qmcrA</i> F <i>qmcrA</i> R	TTCGGTGGATCDCARAGRGC GBARGTCGWAWCCGTAGAAATCC	140	1.96	-3.43	0.995
Nitrate reductase [25]	<i>napA</i>	<i>napA</i> 1F <i>napA</i> 1R	GTYATGGARGAAAAATTCAA GARCCGAACATGCCAC	111	2.01	-3.29	0.999
	<i>narG</i>	<i>narG</i> 2F <i>narG</i> 2R	CTCGAYCTGGTGGTYGA TTYTCGTACCAGGTSGC	89	1.97	-3.39	1.000
Nitrite reductase [10]	<i>nirK</i>	<i>nirK</i> 876 F <i>nirK</i> 1040 R	ATYGGCGGVAYGGCGA GCCTCGATCAGRTRTGGTT	165	1.99	-3.34	0.999

Quantitative PCR calculations and statistical analysis

Technical triplicates were averaged while checking overlaying of amplification plots at threshold cycle (C_t) value. Absolute quantification of total bacteria and methanogenic archaea were expressed as log₁₀ *rrs* or *mcrA* copies/g DM rumen content, respectively. Relative quantification and expression of genes coding for nitrate (*narG* and *napA*) or nitrite (*nirK*) reductases, as well as gene expression of *mcrA* were assessed by the C_t of the qPCR and the 2^{-ΔC_t} method [16]:

$$2^{-\Delta C_t} = 2^{-(C_t \text{ target gene} - C_t \text{ rrs})}$$

Data were analyzed using the MIXED procedure of SAS (Version 9.2; SAS Institute, 2009) and for the two experiments separately. The statistical model included the random effect of cow (n = 4) and fixed effects of period (n = 4), nitrate (CON and LIN *versus* NIT and

LIN+NIT in FD1; CON and TEA *versus* NIT and TEA+NIT in FD2), linseed (CON and NIT *versus* LIN and LIN+NIT in FD1), tea saponin (CON and NIT *versus* TEA and TEA+NIT in FD2) and the interaction linseed \times nitrate (FD1) or tea saponin \times nitrate (FD2). Data were considered significant at $P \leq 0.05$. Trends were discussed at $0.05 < P \leq 0.1$. Least square means are reported throughout.

Results

Total nucleic acids were extracted with similar yields and purity for the 2 experiments. Electropherograms obtained for RNA integrity analysis presented two peaks corresponding to the 16S and 23S rRNA. Diets did not affect RIN which averaged 7.30 and 7.24 for FD1 and FD2, respectively.

Abundance and activity of total bacteria and methanogens

Diets did not change abundance of total bacteria that averaged 7.31 and 7.45 \log_{10} *rrs* copies/g DM rumen content for FD1 and FD2, respectively (Tables 2 and 3). For control diets, abundance of methanogens was similar between the two experiments. In FD1, abundance of methanogens was reduced by nitrate-containing diets (NIT and LIN+NIT; 5.01 \log_{10} *mcrA* copies/g DM rumen content on average) as compared to CON and LIN (5.18 \log_{10} *mcrA* copies/g DM rumen content on average; $P=0.01$). Linseed-containing diets (LIN and LIN+NIT) also tended to reduce abundance of methanogens ($P < 0.10$). Inversely, methanogens abundance was similar among diets in FD2.

Expression of *mcrA* was reduced by nitrate-containing diets for both experiments ($P < 0.05$; Tables 2 and 3). The level of *mcrA* expression with NIT and LIN+NIT compared to CON and LIN was reduced by 2.5 folds in FD1. Similarly, the level of *mcrA* expression was reduced by 2.1 folds with NIT and TEA+NIT compared to CON and TEA in FD2.

Table 2 Abundance of total bacteria, and abundance and activity of methanogenic archaea in the rumen of non-lactating cows supplemented with nitrate fed alone or in association with linseed oil (FD1, n = 4)

Item	Diet ¹				SEM	P-Value ²		
	CON	NIT	LIN	LIN+NIT		Nitrate	Linseed	Linseed × nitrate
Total bacteria (<i>rrs</i>)								
Concentration								
(log ₁₀ copies/g DM rumen content)	7.44	7.24	7.27	7.27	0.056	0.13	0.23	0.14
Methanogenic archaea (<i>mcrA</i>)								
Concentration								
(log ₁₀ copies/g DM rumen content)	5.30	4.97	5.05	5.05	0.056	0.01	0.06	0.01
Activity (2 ^{-ΔCt} × 10 ⁶)	23.91	10.49	21.54	8.19	3.384	0.01	0.51	0.99

¹CON = control; NIT = diet CON including 2.3% nitrate from calcium nitrate; LIN = diet CON including 2.6% added fat from linseed oil; LIN+NIT = diet CON including 2.6% added fat from linseed oil plus 2.3% nitrate from calcium nitrate.

²Linseed = main effect of linseed oil (CON and NIT *versus* LIN and LIN+NIT); Nitrate = main effect of nitrate (CON and LIN *versus* NIT and LIN+NIT); Linseed × nitrate = interaction between main effects of linseed oil and nitrate.

Table 3 Abundance of total bacteria, and abundance and activity of methanogenic archaea in the rumen of non-lactating cows supplemented with nitrate fed alone or in association with tea saponin (FD2, n = 4)

Item	Diet ¹				SEM	P-Value ²		
	CON	NIT	TEA	TEA+NIT		Nitrate	Saponin	Saponin × nitrate
Total bacteria (<i>rrs</i>)								
Concentration								
(log ₁₀ copies/g DM rumen content)	7.44	7.43	7.37	7.54	0.066	0.24	0.78	0.19
Methanogenic archaea (<i>mcrA</i>)								
Concentration								
(log ₁₀ copies/g DM rumen content)	5.37	5.38	5.24	5.47	0.090	0.24	0.80	0.29
Activity (2 ^{-ΔCt} × 10 ⁶)	18.67	7.40	16.08	8.28	4.463	0.004	0.70	0.44

¹CON = control; NIT = diet CON including 2.3% nitrate from calcium nitrate; TEA = diet CON including 0.5% saponin from tea; TEA+NIT = diet CON including 0.5% saponin from tea.

²Saponin = main effect of tea saponin (CON and NIT *versus* TEA and TEA+NIT); Nitrate = main effect of nitrate (CON and TEA *versus* NIT and TEA+NIT); Saponin × nitrate = interaction between main effects of tea saponin and nitrate.

Quantification and expression of genes coding for nitrate or nitrite reductases

Relative abundance of *napA*, *narG* and *nirK* DNA copies were similar between diets for both experiments (Tables 4 and 5). In FD1, the $2^{-\Delta Ct}$ values for DNA copies of *napA*, *narG* and *nirK* averaged 0.77, 10.06 and 13.40, respectively. These values averaged 1.61, 15.26 and 24.04, respectively in FD2. Expression of *napA* and *nirK* genes was below the detection limits. Expression of *narG* was detected at similar levels between all diets: the $2^{-\Delta Ct}$ values were equal to 1.85 and 1.31 in FD1 and FD2, respectively.

Table 4 Abundance and activity of nitrate (*napA* and *narG*) and nitrite (*nirK*) reductases in the rumen of non-lactating cows supplemented with nitrate fed alone or in association with linseed oil (FD1, n = 4)

Item ²	Diet ¹				SEM	P-Value ²		
	CON	NIT	LIN	LIN+NIT		Nitrate	Linseed	Linseed × nitrate
Nitrate reductase (<i>napA</i>)								
Concentration (2 ^{-ΔCt} × 10 ⁶)	0.79	0.75	0.82	0.73	0.123	0.61	0.96	0.84
Activity (2 ^{-ΔCt} × 10 ⁶)	<LD	<LD	<LD	<LD	--	--	--	--
Nitrate reductase (<i>narG</i>)								
Concentration (2 ^{-ΔCt} × 10 ⁶)	10.80	10.05	10.42	8.96	1.281	0.42	0.58	0.78
Activity (2 ^{-ΔCt} × 10 ⁶)	1.90	2.09	1.54	1.87	0.474	0.60	0.56	0.88
Nitrite reductase (<i>nirK</i>)								
Concentration (2 ^{-ΔCt} × 10 ⁶)	14.39	16.20	12.19	10.83	2.173	0.92	0.13	0.49
Activity (2 ^{-ΔCt} × 10 ⁶)	<LD	<LD	<LD	<LD	--	--	--	--

<LD = below limit of detection

¹CON = control; NIT = diet CON including 2.3% nitrate from calcium nitrate; LIN = diet CON including 2.6% added fat from linseed oil; LIN+NIT = diet CON including 2.6% added fat from linseed oil plus 2.3% nitrate from calcium nitrate.

²Linseed = main effect of linseed oil (CON and NIT *versus* LIN and LIN+NIT); Nitrate = main effect of nitrate (CON and LIN *versus* NIT and LIN+NIT); Linseed × nitrate = interaction between main effects of linseed oil and nitrate.

Table 5 Abundance and activity of nitrate (*napA* and *narG*) and nitrite (*nirK*) reductases in the rumen of non-lactating cows supplemented with nitrate fed alone or in association with tea saponin (FD2, n = 4)

Item ²	Diet ¹				SEM	P-Value ²		
	CON	NIT	TEA	TEA+NIT		Nitrate	Saponin	Saponin × nitrate
Nitrate reductase (<i>napA</i>)								
Concentration ($2^{-\Delta Ct} \times 10^6$)	1.33	1.83	1.77	1.52	0.313	0.54	0.74	0.10
Activity ($2^{-\Delta Ct} \times 10^6$)	<LD ²	<LD	<LD	<LD	--	--	--	--
Nitrate reductase (<i>narG</i>)								
Concentration ($2^{-\Delta Ct} \times 10^6$)	14.22	16.18	16.44	14.18	1.503	0.92	0.94	0.21
Activity ($2^{-\Delta Ct} \times 10^6$)	1.31	1.45	1.07	1.41	0.276	0.31	0.55	0.66
Nitrite reductase (<i>nirK</i>)								
Concentration ($2^{-\Delta Ct} \times 10^6$)	22.77	25.92	25.26	22.21	2.329	0.98	0.74	0.13
Activity ($2^{-\Delta Ct} \times 10^6$)	<LD	<LD	<LD	<LD	--	--	--	--

<LD = below limit of detection

¹CON = control; NIT = diet CON including 2.3% nitrate from calcium nitrate; TEA = diet CON including 0.5% saponin from tea; TEA+NIT = diet CON including 0.5% saponin from tea.

² Saponin = main effect of tea saponin (CON and NIT *versus* TEA and TEA+NIT); Nitrate = main effect of nitrate (CON and TEA *versus* NIT and TEA+NIT); Saponin × nitrate = interaction between main effects of tea saponin and nitrate.

Discussion

Absence of dietary treatment effect on total bacteria concentration

The abundance of total bacteria in the rumen of non-lactating cows fed nitrate (2.3% in DM) alone or in association with linseed (2.6% added fat in DM) or tea saponin (0.5% saponin in DM) was similar between diets. Our results are in accordance with the literature since nitrate (2.1% in DM) and lipids from soybean (up to 4.4% added fat in DM) fed individually to sheep [26] or steers [6] did not affect total abundance of ruminal bacteria. The effect of tea saponin on total bacteria has never been studied, but Mao et al., (2010) [17] reported no effect on the concentration of cellulolytic bacteria (*Ruminococcus flavefaciens* and *Fibrobacter succinogenes*) in the rumen of sheep supplemented with the same plant extract at a similar dose (0.5% tea saponin in DM). To our knowledge, this is the first report showing that there was no additional effect on rumen total bacteria abundance when combining nitrate with linseed or tea saponin.

Nitrate toxicity on rumen methanogens

In our study, 2.3% nitrate fed alone reduced CH₄ emissions of non-lactating cows by 25% on average [8], slightly reduced *mcrA* DNA copies in FD1 (-0.17 log₁₀ *mcrA* copies/g DM rumen content) and *mcrA* expression in the two experiments (-2.3 folds). The negative effect of nitrate on methanogens' abundance estimated by qPCR has already been highlighted in sheep supplemented with 2.1% nitrate (-0.7 log₁₀/mL of rumen contents; [26]). The inhibitory effect of nitrate and other derivative N-compounds (nitrite, nitric oxide and nitrous oxide) on *Methanosarcina barkeri*, *Methanobacterium bryantii* and *Methanobacterium formicicum* has also been reported in *in vitro* experiments with soil and salt marsh sediments [3, 13]. However, the negative effect of nitrate on *mcrA* expression in the gastrointestinal tract of animals has never been reported before.

Nitrate is known to reduce CH₄ emissions of ruminants by acting as a H₂-sink during its reduction to nitrite and ammonia [14]. As a consequence, nitrate would have an indirect effect on methanogens activity by decreasing H₂ availability. According to our results, nitrate would also have a direct toxic effect on methanogens as suggested by the rise of dissolved H₂ concentration in the rumen and of gaseous H₂ emissions eructated during the 3 h following nitrate supplementation to sheep [26] and cows [9]. Then, as long as nitrate consumes H₂, rumen H₂ availability is low and methanogens activity decreases. When nitrate has been reduced, the derivative N-compounds act as methanogen inhibitors, and rumen dissolved H₂ concentrations and gaseous H₂ emissions increase. Similar findings have been reported in a previous work studying the effect of nitrate on methane production and fermentation by slurries of human fecal bacteria [1].

To our knowledge, the effect of associating nitrate to linseed or tea saponin on methanogens population has never been studied. While reducing CH₄ emissions by 17% [8], linseed tended to reduce the abundance of methanogens in the rumen of non-lactating cows (-0.09 log₁₀ *mcrA* copies/g DM rumen content) without affecting their activity. This result confirms a previous *in vivo* experiment in which the ruminal concentration of methanogens in dairy cows, fed a corn silage-based diet supplemented with linseed (up to 5% added fat in DM) was significantly reduced 3 h after feeding (-0.47 log₁₀ *mcrA* copies/μg DNA; [20]). In our study, we suggest that methanogens reduction with linseed is associated to a decrease in H₂ availability, as protozoa which are important H₂-producers in the rumen were reduced by 52% in LIN compared to CON [8]. Tea saponin did not change the abundance or activity of rumen methanogens. Our results strengthen previous observations [17, 27] and correlate with the absence of CH₄-mitigating effect of this plant extract supplemented to the same animals

(Guyader et al., personal communication). Diets LIN+NIT and TEA+NIT reduced methanogens abundance and activity to a similar extent than when NIT was fed alone, suggesting that the effect was due to nitrate alone. However, LIN+NIT fed to these same animals additively reduced CH₄ emissions (-32%; [8]).

Absence of nitrate effect on microbial genes coding for nitrate and nitrite reductases

By a culture-based approach, it was already reported that some rumen bacteria (*S. ruminantium*, *V. parvula* and *W. succinogenes*) can reduce nitrate to nitrite and ammonia [11]. Moreover, qPCR data showed that rumen abundance of *S. ruminantium* and *V. parvula* was not affected in goats fed with 1% nitrate in DM [2]. Similarly, *V. parvula* (*rrs* gene copy number) remained stable in steers supplemented with 1.2% of nitrate [15]. Inversely, the number of *W. succinogenes* increased considerably in the rumen of goats supplemented with 1% nitrate in DM (from less than 1.0×10^2 to 1.2×10^3 cells/mL) [2]. Based on the above information, we can affirm that the effect of nitrate supplementation on microbes involved in nitrate metabolism in ruminants remains unclear and needs more investigation.

The present paper is the first one to target particular genes coding for nitrate reductases for assessing the potential activity of nitrate reduction that covers both identified and not-yet identified nitrate-reducing rumen microbes. We focused on the abundance and activity of genes coding for membrane-bound (*narG*) and periplasmic (*napA*) nitrate reductases. We first confirmed the presence of these genes in the rumen ecosystem by interrogating published rumen metagenomes; these genes are also present in the genomes of *S. ruminantium*, *W. succinogenes* and *V. parvula* [18]. However, we cannot exclude that the abundance and expression of targeted genes may be linked with bacterial sediment ingested with feed.

Both nitrate reductase genes *narG* and *napA* were detected but their abundance was not affected by nitrate supplementation. These results confirm a previous work in which *narG* relative abundance from *S. ruminantium* was similar between steers receiving or not 1.2% nitrate in DM [15]. Expression of *narG* was also not affected by diets. The level of expression of *napA* was low suggesting that this gene was not expressed, or that the level of expression was below the detection limits.

A recent work reported that N₂O emissions occurred when dairy cattle were fed up to 2.1% nitrate in DM [22], suggesting that rumen nitrate degradation may partially follow the denitrification pathway (nitrate to nitrite to nitric oxide to nitrous oxide) [12, 23]. In our experiment, abundance and activity of nitrite reductase, performing the reduction of nitrite to nitric oxide, were evaluated by monitoring *nirK*, which is found in bacteria from soil but not clearly annotated in published rumen metagenomes. Although this gene was detected in

rumen microbial DNA, its abundance was not affected by nitrate supplementation and, additionally, its level of expression was below the detection limits. Further work should assess the effect of nitrate supplementation on both N₂O emissions and on the abundance and expression of other genes (e.g. *nirS*, [21]) known to be involved in the reduction of nitrite to N₂O.

Concluding remarks

We showed an inhibitory effect of dietary nitrate on the activity of rumen methanogens in non-lactating cows. Abundance and expression of *narG* and *napA* genes coding for nitrate reductases and *nirK* gene coding for a nitrite reductase were not affected by nitrate supplementation. Further work is required to assess the effect of nitrate on other nitrate and nitrite reductases which have been recently found within the rumen metagenome. The use of high throughput sequencing methods is in progress to assess the effect of dietary nitrate on the rumen microbiota diversity.

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References

1. Allison, C. and G.T. MacFarlane: Effect of nitrate on methane production and fermentation by slurries of Human faecal bacteria. *J Gen Microbiol* 1988, 134:1397-1405.
2. Asanuma, N., M. Iwamoto, M. Kawato and T. Hino: Numbers of nitrate-reducing bacteria in the rumen as estimated by competitive polymerase chain reaction. *Anim Sci J* 2002, 73:199-205.
3. Balderston, W.L. and W.J. Payne: Inhibition of methanogenesis in salt marsh sediments and whole-cell suspensions of methanogenic bacteria by nitrogen oxides. *Appl. Environ. Microbiol.* 1976, 32:264-269.
4. Bustin, S.A., V. Benes, J.A. Garson, J. Hellemans, J. Huggett, M. Kubista, R. Mueller, T. Nolan, M.W. Pfaffl, G.L. Shipley, J. Vandesompele and C.T. Wittwer: The MIQE guidelines: minimum information for publication of quantitative real-time PCR experiments. *Clin. Chem.* 2009, 55:611-622.

5. Denman, S.E., N.W. Tomkins and C.S. McSweeney: Quantitation and diversity analysis of ruminal methanogenic populations in response to the antimethanogenic compound bromochloromethane. *FEMS Microbiol. Ecol.* 2007, 62:313-322.
6. Duarte Messana, J., T. Teresinha Berchielli, P. Braga Arcuri, R. Andrade Reis, R. Carrilho Canesin, A. Ferreira Ribeiro, G. Fiorentini and J.J. de Resende Fernandes: Rumen fermentation and rumen microbes in Nellore steers receiving diets with different lipid contents. *Rev Bras Zootec* 2013, 42:204-212.
7. Edwards, J.E., S.A. Huws, E.J. Kim and A.H. Kingston-Smith: Characterization of the dynamics of initial bacterial colonization of nonconserved forage in the bovine rumen. *FEMS Microbiol. Ecol.* 2007, 62:323-335.
8. Guyader, J., M. Eugène, M. Doreau, Y. Rochette, D.P. Morgavi and C. Martin: Association of nitrate and linseed oil effectively reduces methane emission in ruminants. *Proc Aust Soc Anim Prod* 2014, 30:87.
9. Guyader, J., M. Silberberg, D.P. Morgavi and C. Martin: Daily pattern of hydrogen concentration in the rumen of cows fed nitrate or linseed oil. *Proc Aust Soc Anim Prod* 2014, 30:86.
10. Henry, S., E. Baudoin, J.C. Lopez-Gutierrez, F. Martin-Laurent, A. Brauman and L. Philippot: Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR. *J. Microbiol. Methods* 2004, 59:327-335.
11. Iwamoto, M., N. Asanuma and T. Hino: Ability of *Selenomonas ruminantium*, *Veillonella parvula*, and *Wolinella succinogenes* to reduce nitrate and nitrite with special reference to the suppression of ruminal methanogenesis. *Anaerobe* 2002, 8:209-215.
12. Jeyanathan, J., C. Martin and D.P. Morgavi: The use of direct-fed microbials for mitigation of ruminant methane emissions: a review. *Animal* 2014, 8:250-261.
13. Klüber, H.D. and R. Conrad: Inhibitory effects of nitrate, nitrite, NO and N₂O on methanogenesis by *Methanosarcina barkeri* and *Methanobacterium bryantii*. *FEMS Microbiol. Ecol.* 1998, 25:331-339.
14. Lewis, D.: The metabolism of nitrate and nitrite in the sheep. 1 The reduction of nitrate in the rumen of the sheep. *Biochem. J.* 1951, 48:175-180.
15. Lin, M., W. Guo, Q. Meng, D.M. Stevenson, P.J. Weimer and D.M. Schaefer: Changes in rumen bacterial community composition in steers in response to dietary nitrate. *Appl. Microbiol. Biotechnol.* 2013, 97:8719-8727.
16. Livak, K.J. and T.D. Schmittgen: Analysis of relative gene expression data using real-time quantitative PCR and the 2^{-ΔΔCT} method. *Methods* 2001, 25:402-408.

17. Mao, H.L., J.K. Wang, Y.Y. Zhou and J.X. Liu: Effects of addition of tea saponins and soybean oil on methane production, fermentation and microbial population in the rumen of growing lambs. *Livest. Sci.* 2010, 129:56-62.
18. Meyer, F., D. Paarmann, M. D'Souza, R. Olson, E. Glass, M. Kubal, T. Paczian, A. Rodriguez, R. Stevens, A. Wilke, J. Wilkening and R. Edwards: The metagenomics RAST server - a public resource for the automatic phylogenetic and functional analysis of metagenomes. *BMC Bioinformatics* 2008, 9:386.
19. Mosoni, P., C. Martin, E. Forano and D.P. Morgavi: Long-term defaunation increases the abundance of cellulolytic ruminococci and methanogens but does not affect the bacterial and methanogen diversity in the rumen of sheep. *J. Anim. Sci.* 2011, 89:783-791.
20. Mosoni, P., Y. Rochette, M. Doreau, D.P. Morgavi, E. Forano, A. Ferlay, Y. Chilliard and C. Martin: Effect of increasing levels of extruded linseed in the diet of dairy cows on the number of protozoa, cellulolytic bacteria and methanogenic archaea. In *Joint INRA-RRI Gastrointestinal Tract Microbiology Symposium*, Clermont Ferrand, France; 2008: p 84.
21. Oakley, B.B., C.A. Francis, K.J. Roberts, C.A. Fuchsman, S. Srinivasan and J.T. Staley: Analysis of nitrite reductase (*nirK* and *nirS*) genes and cultivation reveal depauperate community of denitrifying bacteria in the Black Sea suboxic zone. *Environ. Microbiol.* 2007, 9:118-130.
22. Petersen, S.O., A.L.F. Hellwing, M. Brask, O. Højberg, M. Poulsen, Z. Zhu and P. Lund: Nitrous oxide emission partly alleviates the methane mitigation effect of nitrate intake by dairy cattle. In *Livestock, Climate change and food security conference*, Madrid, Spain; 2014: p 46.
23. Philippot, L., S. Hallin and M. Schlöter: Ecology of denitrifying prokaryotes in agricultural soil. *Adv. Agron.* 2007, 96:249-305.
24. Popova, M., C. Martin, M. Eugène, M.M. Mialon, M. Doreau and D.P. Morgavi: Effect of fibre- and starch-rich finishing diets on methanogenic Archaea diversity and activity in the rumen of feedlot bulls. *Anim. Feed Sci. Technol.* 2011, 166:113-121.
25. Smith, C.J., D.B. Nedwell, L.F. Dong and A.M. Osborn: Diversity and abundance of nitrate reductase genes (*narG* and *napA*), nitrite reductase genes (*nirS* and *nrfA*), and their transcripts in estuarine sediments. *Appl. Environ. Microbiol.* 2007, 73:3612-3622.

26. Van Zijderveld, S.M., W.J.J. Gerrits, J.A. Apajalahti, J.R. Newbold, J. Dijkstra, R.A. Leng and H.B. Perdok: Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. *J. Dairy Sci.* 2010, 93:5856-5866.
27. Zhou, Y.Y., H.L. Mao, F. Jiang, J.K. Wang, J.X. Liu and C.S. McSweeney: Inhibition of rumen methanogenesis by tea saponins with reference to fermentation pattern and microbial communities in Hu sheep. *Anim. Feed Sci. Technol.* 2011, 166-167:93-100.

STEP 5: Dose response effect of nitrate on hydrogen distribution between rumen fermentation end-products: an *in vitro* approach

Objective

1/ To study the dose response effect of nitrate on *in vitro* production of rumen fermentation end-products such as gas (CH₄ and H₂), VFA and microbial biomass (estimated from insoluble protein).

2/ To understand the CH₄-mitigating mechanisms of nitrate by estimating metabolic H₂ distribution between rumen fermentation end-products.

Experimental approach

In vitro system → 2 repeated incubations → Dose response: CON (50% hay + 50% concentrate) + 0, 1, 2, 4 or 6 mM nitrate (from ammonium nitrate) Exp 1

→ 2 repeated incubations → Dose response: CON (100% glucose) + 0, 1, 2, 4 or 6 mM nitrate (from ammonium nitrate) Exp 2

HOUR	1	...	3	...	8	...	12	...	24	...	32	...	48
Kinetics of gas production (total, CH ₄ and H ₂ ; exp 1 and 2)													
pH, VFA and NH ₄ ⁺ concentrations (exp 1)													
pH (48 h), kinetics of VFA, NH ₄ ⁺ and insoluble proteins concentrations (exp 2)													

Estimation of metabolic H₂ distribution between rumen fermentation end-products (mmoles):

- H₂ production = 2 × acetate + 2 × butyrate
- H₂ consumption = 4 × CH₄ + 1 × propionate + 4 × NO₃⁻ + 0.41 × microbial biomass (insoluble protein)
- H₂ balance = H₂ production – H₂ consumption – H₂ emissions

Main results

Exp 1

Exp 2

- In exp 1 and 2, total gas and CH₄ production linearly decreased as nitrate doses increased. Nitrate reduced CH₄ production during the first 10 h of incubation. Hydrogen emissions were detected only with high doses of nitrate, after 10 h incubation.
- Rumen fermentation parameters including microbial biomass synthesis (calculated from insoluble protein concentration in exp 2) were poorly affected by nitrate.
- Estimated H₂ balance indicated that 23% (6mM nitrate; exp 1) of H₂ was not used for production of studied rumen fermentation end-products.

Conclusion

Nitrate is an efficient CH₄-mitigating strategy, but with doses higher than 4 mM, *in vitro* fermentations were negatively affected. Estimation of H₂ distribution between studied rumen fermentation end-products suggest that nitrate enhances another H₂ consuming pathway.

Dose response effect of nitrate on hydrogen distribution between rumen fermentation end-products: an *in vitro* approach

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Abstract

The objective of this work was to study the *in vitro* dose response effect of nitrate on hydrogen distribution between rumen fermentation end-products. Five nitrate concentrations (0, 1, 2, 4 and 6 mM) were tested in two *in vitro* experiments. In experiment 1, a mixture of hay and concentrate (50:50) was used to calculate efficiencies of methane reduction and to study differences between fermentation profiles. In experiment 2, glucose was used as the sole protein-free substrate to quantify the effect of nitrate dosage on microbial synthesis. In both experiments, two 48 h-incubations were carried out using bovine rumen contents as inoculum. Total gas production and composition was automatically analyzed throughout the incubations. In experiment 1, volatile fatty acids (VFA) and ammonium concentrations were analyzed from samples taken after 48-h incubation. In experiment 2, VFA, ammonium and insoluble protein concentrations were analyzed from samples collected at various time points. In experiment 1, total gas production was decreased with the highest dose of nitrate ($P=0.019$). Methane emissions tended to linearly decrease as nitrate doses increased ($P=0.079$). Kinetics of methane emissions showed that hydrogen removal via nitrate reduction occurred mainly during the first 10 h-incubation. The apparent yield of methane reduction relative to control incubations exceeded 100% with nitrate doses higher than 4 mM. Gaseous hydrogen production was similar between treatments, despite numerically higher hydrogen emissions for nitrate concentrations above 4 mM. Concentrations and proportions of VFA were not affected by treatments. Proportions of unaccounted hydrogen in total hydrogen produced were similar and positive for all treatments, despite a numerical increase as nitrate doses increased. Experiment 2 showed that insoluble protein concentrations were not affected by nitrate. In this *in vitro* work, we confirmed that nitrate acts as an electron acceptor in the rumen. We also suggest that nitrate or its reduced forms have a direct inhibiting effect towards methanogens, as indicated by the release of gaseous hydrogen and the high efficiencies of methane reduction.

Highlights

- Increasing nitrate dose linearly reduces methane emissions *in vitro*.
- High doses of nitrate inhibit overall gas production.
- High doses of nitrate do not reduce methane by only acting as an electron acceptor.
- Nitrate does not enhance microbial synthesis.

Keywords: hydrogen, *in vitro*, methane, microbial biomass, nitrate, rumen

Introduction

In the rumen, hydrogen (H_2) is produced by bacterial and protozoal hydrogenases after the reoxidation of coenzymes or pyruvate generated during the synthesis of volatile fatty acids (VFA): the production of one mole acetate or butyrate generates two moles H_2 (Hegarty and Gerdes, 1999). Since an increased H_2 concentration inhibits the normal function of microbial enzymes in the rumen, H_2 disposal is essential. Most of the H_2 is used to reduce carbon dioxide (CO_2) to methane (CH_4) consuming 4 moles H_2 per mole CH_4 produced. Then, methanogenesis uses between 48 and 80% of H_2 (Czerkawski, 1986; Mills et al., 2001). Between 19 and 33% of H_2 is used for VFA synthesis, as one mole H_2 is required per mole propionate or valerate produced. And finally, 0.6 to 12% of H_2 is used for microbial growth, as 0.41 moles H_2 are required per kg of microbes.

Considering the importance of H_2 in CH_4 production by ruminants, several CH_4 mitigation strategies aimed at reducing the availability of H_2 for microbial H_2 -users such as methanogens. One of these strategies is to supply nitrate (NO_3^-) to the animals' diet. This additive would act as an electron acceptor reducing the amount of H_2 formed by 4 molar equivalents of H_2 through its reduction to nitrite (NO_2^-) and ammonium (NH_4^+). A recent meta-analysis reported that 1% NO_3^- added to the diet of cattle reduced CH_4 emissions by 10% on average (Lee and Beauchemin, 2014). However, the dose response effect of NO_3^- on both CH_4 emissions and rumen fermentation has not been reported or studied, due to the risks of blood methemoglobinemia for animals supplemented with high doses of this additive (Lewis, 1951).

Recent work also highlighted that NO_3^- reduced the number and/or activity of methanogens (Van Zijderveld et al., 2010; Guyader et al., 2014c), changed fermentation profile towards acetate production (Veneman et al., 2014) and increased dissolved H_2 concentration in the rumen (Guyader et al., 2014b) and H_2 emissions (Van Zijderveld et al., 2011). These results

suggest that NO_3^- may not only act as an electron acceptor in the rumen and that its CH_4 -mitigating effect may involve other mechanisms. The study of H_2 fluxes towards fermentation end-products such as methanogenesis, VFA synthesis and microbial biomass, with different doses of NO_3^- may allow a better understanding of the effect of this additive in the ruminal environment.

The objective of this work was to deepen the understanding of the CH_4 -mitigating mechanisms of NO_3^- by studying its dose response effect on i) CH_4 emissions and microbial fermentation profile, and ii) the distribution of H_2 between fermentation end-products. Due to the risk of blood metHb for animals fed high doses of NO_3^- (Lee and Beauchemin, 2014), an *in vitro* approach was favored and two experiments were carried out. In the first experiment, a hay and concentrate based substrate was used in order to get close to ruminants diet conditions. The apparent yield of CH_4 reduction with different NO_3^- concentrations and their effects on fermentation profiles were studied. In the second experiment, glucose was used as the sole protein-free substrate to quantify the effect of NO_3^- on microbial synthesis.

Material and methods

Two *in vitro* experiments, each one consisting in two repeated incubations, were carried out at AgResearch Grasslands (Palmerston North, New Zealand) with a fully automated incubation system (Muetzel et al., 2014) using ammonium nitrate (NH_4NO_3) as the source of NO_3^- .

Design of experiments

In experiment 1, a general purpose substrate (GP) was composed of a mixture of hay (500 g/kg), barley (290 g/kg), soybean (100 g/kg), molasses (100 g/kg), dicalcium phosphate (5.5 g/kg), salt (3 g/kg) and minerals and vitamins (1.5 g/kg) on a dry matter (DM) basis. The substrate was ground in a Wiley mill to pass a 1-mm screen. Treatments were: 1/ control (10 mg GP/ml medium), 2/ control plus 1 mM NO_3^- , 3/ control plus 2 mM NO_3^- , 4/ control plus 4 mM NO_3^- and 5/ control plus 6 mM NO_3^- . Duplicate bottles for each treatment served as technical replicates.

In experiment 2, the substrate was composed of D-glucose (GLU) only. Treatments were: 1/ control (6.67 mg GLU/ml medium), 2/ control plus 1 mM NO_3^- , 3/ control plus 2 mM NO_3^- , 4/ control plus 4 mM NO_3^- and 5/ control plus 6 mM NO_3^- . Four bottles were prepared per treatment: two bottles served as technical replicates for gas analysis whereas the two other ones served as technical replicates for frequent sample collection.

Batch culture incubations

Rumen contents were obtained from two ruminally fistulated cows. Within the two experiments, one different donor cow was used per incubation. The donor animals were kept on pasture at Grasslands animal facility. Samples were taken manually at 0830 h from the dorsal part of the rumen and were immediately placed in pre-warmed thermos and transported to the laboratory. The rumen contents were then strained through one layer of cheesecloth and diluted (20% v/v) with a warm (39°C), reduced and CO₂-saturated buffer solution (Mould et al., 2005). The medium was continuously subjected to a CO₂ stream and maintained at 39°C in a water bath before starting incubations. Treatments were incubated in pre-warmed (39°C) bottles filled with 60 ml buffered rumen fluid and purged with a CO₂ stream. Immediately after filling with the medium, the bottles were sealed with a butyl rubber stopper and placed on a shaker in an incubator and connected via a 23-gauge needle to the pressure sensor and valve setup. Samples were incubated for 48 h at 39°C.

Sampling and gas measurement

Before starting the incubation, a sample (1.8 ml) of the medium was collected for subsequent analysis of NH₄⁺, VFA (experiment 1 and 2) and insoluble protein (experiment 2 only).

In the two experiments, kinetics of gas production and composition were determined throughout the incubations using an automated *in vitro* gas production system with a gas chromatograph attached for automatic CH₄ and H₂ analysis (Muetzel et al., 2014). In experiment 1, gas kinetics were determined in all bottles for 48 h. After 48 h incubation, the bottles were removed from the incubator, opened and pH was immediately measured. Samples (1.8 ml) were taken for subsequent analysis of NH₄⁺ and VFA. In experiment 2, gas kinetics were determined for 48 h in two bottles out of the four bottles per treatment. The two other bottles were used for sampling (1.8 ml) after 1, 3, 8, 12, 16, 24, 32, and 48 h incubation for NH₄⁺, VFA and insoluble protein analysis. At 48 h, the remaining bottles from the gas measurement were also collected as described above and pH was measured.

All samples were centrifuged (21,000×g at 4°C for 10 min). For NH₄⁺ and VFA analysis, 0.9 ml of the supernatant was transferred in a micro centrifuge tube containing 0.1 ml of internal standard solution (19 mM ethyl butyrate in 20% (v/v) phosphoric acid), mixed well, and kept at -20°C over night. When insoluble protein concentration was analyzed, the remaining supernatant was discarded and the pellet was washed once with a saline solution (0.85% NaCl, w/v) and stored at -20°C until processed.

Thawed fluid samples were clarified by centrifugation (21,000×g at 4°C for 10 min) and 0.8 ml of the supernatant was transferred into a 2 ml crimp cap gas chromatography vial for VFA analysis and 0.1 ml was collected for NH_4^+ analysis. Volatile fatty acids were analyzed by gas chromatography (Attwood et al., 1998) and NH_4^+ was analyzed by a colorimetric method (Chaney and Marbach, 1962). Thawed pellets were suspended in 750 μl SDS (1%, w/v), using an Eppendorf MixMate at 2,000 rpm for 10 min. The samples were then heated to 100°C for 10 min to solubilize the proteins and then centrifuged (21,000×g at room temperature for 10 min). The supernatant (300 μl) was then transferred in a micro centrifuge tube containing 1 ml acetone (100%, w/w) for protein precipitation. After incubation (-30°C for 2 h), precipitated proteins were centrifuged (21,000×g at 4°C for 10 min) and washed with 600 μl acetone (75%, w/w). The final pellet was suspended in 300 μl SDS (1%, w/w) and the concentration of insoluble protein was determined using the Pierce BCA assay (Thermo Scientific, Rockford, USA).

Calculations and statistical analyses

For each incubation, a logistic model (France et al., 2000) was fitted to the 48 h gas production (total, CH_4 and H_2) data using least squares regression. The resulting logistic parameters were used to calculate gas production at 32 h. Yield of CH_4 reduction for a treatment was calculated as the ratio between observed CH_4 reduction for this treatment (ml/g) relative to its expected CH_4 reduction (ml/g) based on stoichiometry. Expected CH_4 reduction was calculated assuming that one mole NO_3^- reduces CH_4 production by one mole. Concentrations of NH_4^+ were corrected for the amount of NH_4^+ added from ammonium nitrate. Insoluble protein production was calculated by subtracting the initial insoluble protein concentrations in the medium from the concentrations of each bottle.

The VFA production data (at 48 and 32 h in experiments 1 and 2, respectively) were used to calculate net H_2 production (mmol/bottle) assuming that i) the formation of VFA was solely derived from carbohydrates fermentation to hexoses and pentoses; ii) the production of one mole acetate or one mole butyrate generates two moles H_2 . Methane (at 32 h in both experiments) and propionate (at 48 and 32 h in experiments 1 and 2, respectively) production, NO_3^- reduction and microbial biomass synthesis (at 32 h in experiment 2 only) were considered as H_2 consuming pathways. The amount of H_2 directed towards these pathways (mmol/bottle) was calculated considering that the synthesis of one mole CH_4 and propionate requires four and one mole H_2 respectively and that NO_3^- reduction to NH_4^+ requires four

moles H_2 . The amount of H_2 required for microbial biomass synthesis (mmol/bottle) was calculated using the following equation:

$$H_2 \text{ towards microbial biomass} = (ISP \times a \times b)/c$$

With ISP = insoluble protein concentrations (mg/ml), a = the volume of medium in each bottle (60 ml), b = the microbial requirement of H_2 when they grow without preformed amino acids (0.41 moles H_2 /kg microbes; Mills et al., 2001) and c = the percentage of proteins in bacteria (54.46 g proteins/100g dry bacterial cells; Reichl and Baldwin, 1975). Finally unaccounted H_2 was calculated as the difference between estimated H_2 production (mmol/bottle) and H_2 consumption and gaseous H_2 (mmol/bottle).

Data from duplicate bottles were averaged for statistical analyses. The dose effect of NO_3^- on gas production at 32 h (total gas, ml/g; CH_4 , ml/g and % of total gas; H_2 , ml/g and % of total gas; yield of CH_4 reduction), on fermentation parameters at 48 h for experiment 1 and 32 h for experiment 2 (pH; NH_4^+ , mM; VFA, mmol/g; insoluble protein, mg/ml; acetate, propionate and butyrate, %; acetate/propionate and (acetate+butyrate)/propionate) and on H_2 metabolism (H_2 produced, consumed, emitted and unaccounted, mmol/bottles) was analyzed using the MIXED procedure of SAS (Version 9.4; SAS Institute, 2009). The statistical model included the fixed effect of NO_3^- dosage ($n = 5$), and run ($n = 2$) was considered as a random effect. Differences between diets were tested using the PDIFF option. The effect of increasing level of NO_3^- was assessed through linear, quadratic and cubic orthogonal contrasts using the CONTRAST statement of SAS. As NO_3^- doses were not equidistant, the IML procedure was used to calculate coefficients for unequally spaced contrasts. Cubic effect was not significant and consequently its effect was not presented in the tables of results. Data were considered significant at $P < 0.05$, and trends were discussed at $0.05 < P < 0.1$.

Table 1 *In vitro* dose response effect of nitrate on gas production and composition after 32 h incubation, and on fermentation parameters after 48 h incubation with GP substrate (50% hay and 50% concentrate; experiment 1)

Item	Nitrate dose (mM)					SED	Dose	P-Value ¹	
	0	1	2	4	6			L	Q
Gas production and composition									
Total gas production (ml/g)	259.7 ^a	253.4 ^a	248.4 ^a	236.5 ^a	191.6 ^b	20.15	0.019	0.003	0.114
CH ₄ production (ml/g)	42.9 ^a	42.1 ^a	38.6 ^a	30.3 ^{ab}	18.7 ^b	9.67	0.079	0.013	0.460
CH ₄ production (% of total gas)	16.5	16.6	15.5	12.6	9.2	3.75	0.229	0.044	0.589
H ₂ production (ml/g)	0.26	0.25	0.26	1.17	1.90	1.160	0.460	0.114	0.628
H ₂ production (% of total gas)	0.10	0.10	0.11	0.53	1.12	0.671	0.451	0.114	0.524
Efficiency of CH ₄ reduction (%)	--	26.8	80.0	119.1	152.5	--	--	--	--
Fermentation parameters									
pH	6.18 ^a	6.21 ^a	6.24 ^{ab}	6.27 ^b	6.29 ^b	0.066	0.042	0.007	0.295
NH ₄ ⁺ (mM)	30.31	27.95	30.37	29.75	32.42	2.984	0.666	0.354	0.501
Total VFA (mmol/g)	6.71	6.65	6.45	6.99	6.48	0.642	0.408	0.898	0.568
Acetate (% of total VFA)	60.2	61.1	61.7	62.1	62.2	3.66	0.838	0.353	0.645
Propionate (% of total VFA)	19.7	19.3	19.2	20.0	21.1	3.41	0.857	0.406	0.577
Butyrate (% of total VFA)	14.9	14.5	14.1	13.6	12.4	1.39	0.182	0.032	0.768
Acetate/butyrate	3.07	3.18	3.23	3.20	3.10	0.702	0.989	0.989	0.648
(Acetate+butyrate)/propionate	3.83	3.93	3.97	3.88	3.71	0.747	0.964	0.687	0.606

^{a, b} Means in the same row with different superscripts differ (P<0.05).¹ Orthogonal contrasts for L = linear and Q = quadratic effects.

Results

Experiment 1

After 32 h incubation with GP substrate, total gas production was not different between control and 1, 2 and 4 mM NO_3^- , and was reduced by 26% with 6 mM NO_3^- ($P=0.019$; Table 1). Methane production expressed in ml/g tended to linearly decrease as NO_3^- concentrations increased ($P=0.079$), whereas no difference between treatments was observed when CH_4 was expressed as a percentage of total gas produced. Yield of CH_4 reduction was lower than 100% with 1 and 2 mM NO_3^- , but exceeded 100% with concentrations higher than 4 mM. The kinetics of CH_4 production (ml/g) indicated that the decrease in CH_4 emissions occurred during the first 10 h of incubation (Figure 1) and after 10 h, the rate of CH_4 production appeared similar to the control treatment.

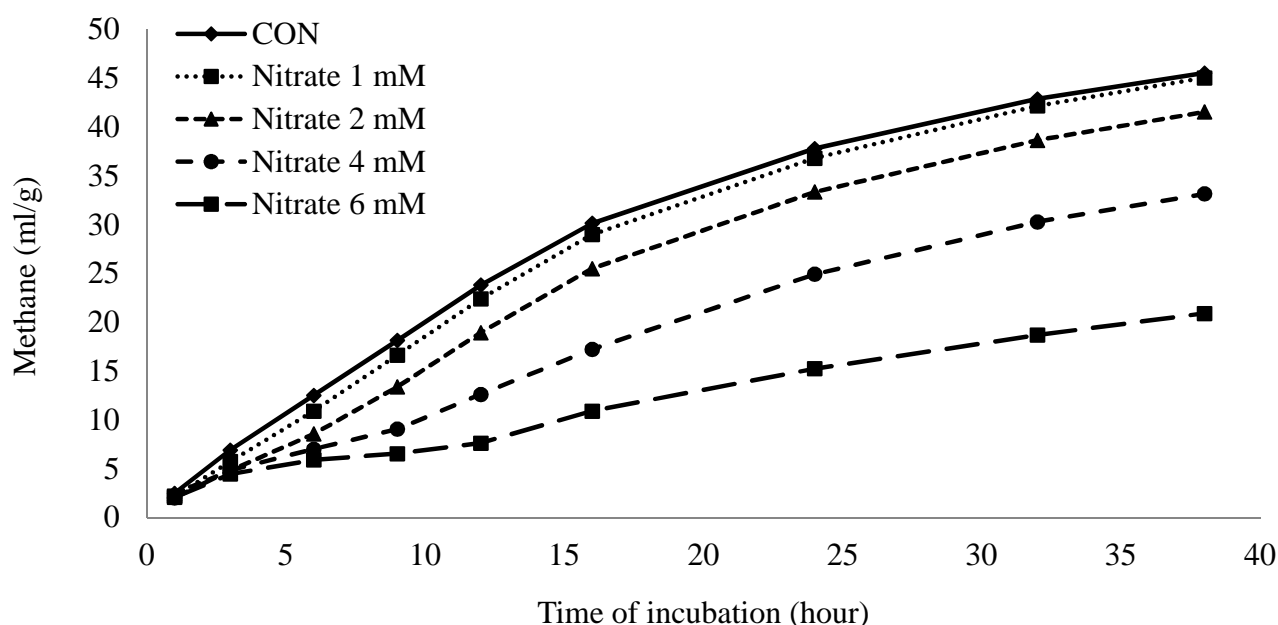


Figure 1 *In vitro* dose response effect of nitrate on kinetics of methane production during 48 h incubation with GP substrate (50% hay and 50% concentrate; experiment 1)

Nitrate did not alter gaseous H_2 emissions expressed in ml/g or as a percentage of total gas produced (Table 1). However, more H_2 emissions occurred after 10 h and 15 h incubation for NO_3^- doses of 4 and 6 mM, respectively (Figure 2).

The final pH linearly increased from 6.18 in the control to 6.29 for 6 mM NO_3^- ($P=0.042$; Table 1). The concentrations of NH_4^+ and of total VFA production were not affected by NO_3^- and averaged 30.2 mM and 6.7 mmol/g, respectively. Nitrate levels did not affect proportions of acetate, propionate, and butyrate, which averaged 61.5%, 19.9%, and 13.9%, respectively.

Consequently, the ratios acetate/propionate and (acetate+butyrate)/propionate were similar between treatments.

Total production and consumption of H_2 was not affected by treatments and averaged 5.11 and 4.43 mmol/bottle, respectively (Table 2). Unaccounted H_2 was positive and tended to increase with increasing levels of nitrate (from 11% in control to 23% with 6 mM NO_3^- ; $P=0.099$).

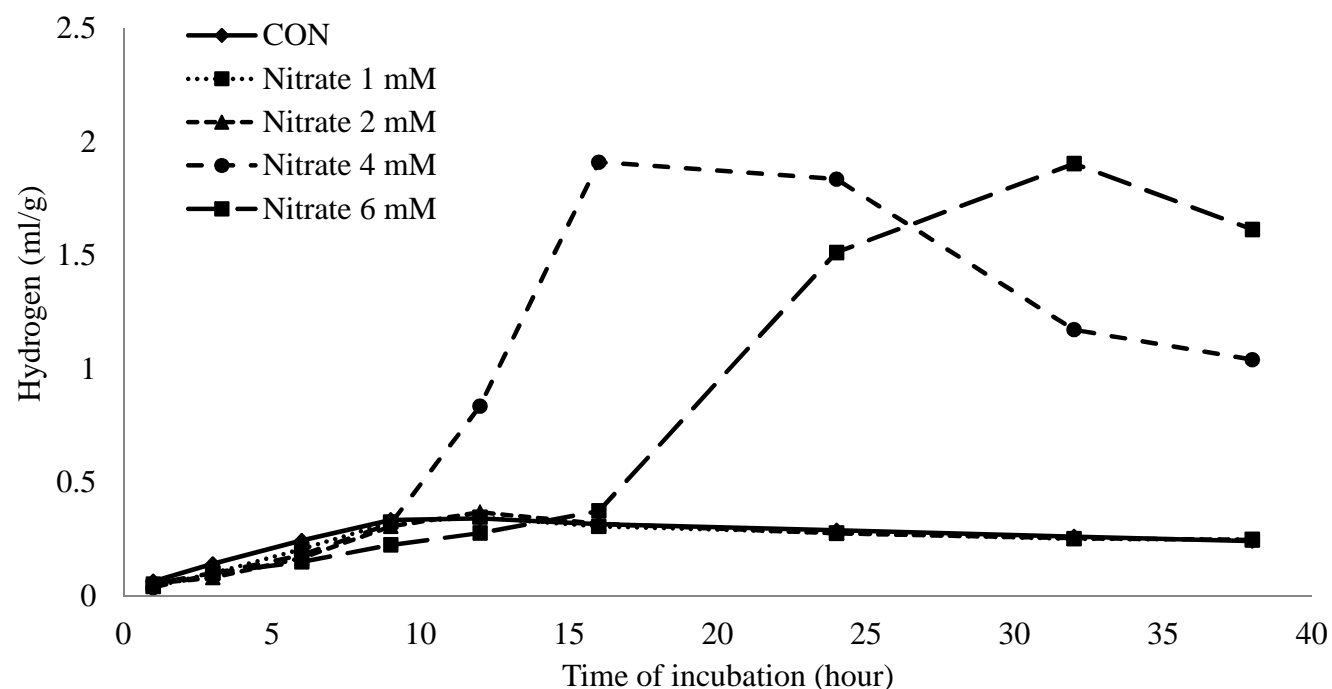


Figure 2 *In vitro* dose response effect of nitrate on kinetics of hydrogen production during 48 h incubation with GP substrate (50% hay and 50% concentrate; experiment 1)

Table 2 *In vitro* dose response effect of nitrate on calculated hydrogen production and distribution between fermentation end-products with GP substrate (50% hay and 50% concentrate; experiment 1)

Item	Nitrate dose (mM)					SED	Dose	P-Value ¹	
	0	1	2	4	6			L	Q
H ₂ production (mmol/bottle)									
From acetate	4.11	4.14	4.05	4.44	4.13	0.652	0.748	0.655	0.603
From butyrate	1.02 ^a	0.98 ^a	0.92 ^a	0.96 ^a	0.81 ^b	0.035	0.025	0.006	0.397
Total	5.13	5.12	4.97	5.39	4.94	0.649	0.612	0.887	0.524
H ₂ consumption (mmol/bottle)									
For methane	3.89 ^a	3.83 ^a	3.51 ^a	2.75 ^{ab}	1.70 ^b	0.879	0.079	0.013	0.460
For propionate	0.67	0.65	0.63	0.70	0.68	0.052	0.457	0.342	0.644
For nitrate reduction	0.00	0.24	0.48	0.96	1.44	0.000	--	--	--
Total	4.56	4.72	4.62	4.41	3.82	0.828	0.578	0.188	0.451
H ₂ emission (mmol/bottle)	0.01	0.01	0.01	0.03	0.04	0.026	0.459	0.114	0.629
H ₂ unaccounted (mmol/bottle)	0.56	0.39	0.35	0.96	1.08	0.231	0.099	0.026	0.341
H ₂ unaccounted (% of produced H ₂)	10.9	7.6	6.9	18.3	23.0	6.67	0.227	0.059	0.389

^{a, b} Means in the same row with different superscripts differ (P<0.05).¹ Orthogonal contrasts for L = linear and Q = quadratic effects.

Table 3 *In vitro* dose response effect of nitrate on gas production and composition, and fermentation parameters after 32 h incubation with GLU substrate (100% glucose; experiment 2)

Item	Nitrate dose (mM)					SED	Dose	P-Value ¹	
	0	1	2	4	6			L	Q
Gas production and composition									
Total gas production (ml/g)	350.8 ^a	339.4 ^a	323.8 ^{ab}	290.5 ^{bc}	274.5 ^c	18.33	0.022	0.003	0.631
CH ₄ production (ml/g)	31.0 ^a	26.2 ^{ab}	22.1 ^b	12.8 ^c	11.2 ^c	6.01	0.002	<0.001	0.067
CH ₄ production (% of total gas)	8.8 ^a	7.7 ^{ab}	6.8 ^b	4.3 ^c	4.1 ^c	1.56	0.002	0.001	0.048
H ₂ production (ml/g)	0.29	0.20	0.34	0.35	0.22	0.153	0.809	0.948	0.531
H ₂ production (% of total gas)	0.09	0.06	0.11	0.12	0.08	0.051	0.774	0.760	0.468
Efficiency of CH ₄ reduction (%)	--	136.7	126.0	129.2	93.5	--	--	--	--
Fermentation parameters									
pH	6.08 ^a	6.09 ^a	6.14 ^b	6.17 ^b	6.21 ^c	0.062	0.003	<0.001	0.538
NH ₄ ⁺ (mM)	9.44 ^a	10.11 ^a	12.56 ^{ab}	17.14 ^c	15.09 ^{bc}	1.737	0.018	0.004	0.063
Insoluble protein (mg/ml)	0.26	0.20	0.22	0.22	0.17	0.053	0.641	0.276	0.923
Total VFA (mmol/g)	7.08	6.92	6.84	5.30	4.82	1.168	0.155	0.036	0.826
Acetate (% of total VFA)	42.9 ^a	45.3 ^{ab}	44.8 ^a	51.6 ^{bc}	57.8 ^c	2.04	0.020	0.004	0.250
Propionate (% of total VFA)	42.8 ^a	41.6 ^{ab}	41.5 ^{ab}	36.9 ^b	31.0 ^c	2.03	0.027	0.006	0.175
Butyrate (% of total VFA)	13.4	11.8	13.0	11.3	11.2	1.27	0.100	0.041	0.614
Acetate/propionate	1.01 ^a	1.10 ^{ab}	1.08 ^a	1.41 ^b	1.87 ^c	0.096	0.012	0.003	0.069
(Acetate+butyrate)/propionate	1.33 ^a	1.39 ^{ab}	1.40 ^{ab}	1.71 ^b	2.23 ^c	0.112	0.013	0.003	0.061

^{a, b} Means in the same row with different superscripts differ (P<0.05).¹ Orthogonal contrasts for L = linear and Q = quadratic effects.

Table 4 *In vitro* dose response effect of nitrate on calculated hydrogen production and distribution between fermentation end-products with GLU substrate (100% glucose; experiment 2)

Item	Nitrate dose (mM)					SED	Dose	P-Value ¹	
	0	1	2	4	6			L	Q
H ₂ production (mmol/bottle)									
From acetate	2.31	2.33	2.33	2.06	2.11	0.441	0.692	0.289	0.934
From butyrate	0.72	0.63	0.68	0.47	0.42	0.152	0.085	0.021	0.996
Total	3.03	2.96	3.01	2.53	2.53	0.589	0.393	0.123	0.948
H ₂ consumption (mmol/bottle)									
For methane	2.11 ^a	1.78 ^{ab}	1.50 ^b	0.87 ^c	0.76 ^c	0.408	0.002	<0.001	0.067
For propionate	1.15	1.14	1.08	0.74	0.57	0.154	0.076	0.016	0.609
For nitrate reduction	0.00	0.24	0.48	0.96	1.44	0.000	--	--	--
For microbial biomass	0.012	0.009	0.010	0.010	0.008	0.0021	0.604	0.276	0.958
Total	3.27	3.12	3.07	2.58	2.78	0.507	0.065	0.021	0.144
H ₂ emission (mmol/bottle)	0.01	0.00	0.01	0.01	0.00	0.003	0.819	0.869	0.520
H ₂ unaccounted (mmol/bottle)	-0.24	-0.15	-0.07	-0.06	-0.25	0.265	0.902	0.986	0.408
H ₂ unaccounted (% of produced H ₂)	-7.8	-4.7	-1.8	-3.3	-13.3	11.88	0.870	0.659	0.415

^{a, b} Means in the same row with different superscripts differ (P<0.05).¹ Orthogonal contrasts for L = linear and Q = quadratic effects.

Experiment 2

When glucose was used as substrate, total gas production linearly decreased from 350.8 to 274.5 ml/g as NO_3^- doses increased from 0 to 6 mM NO_3^- (Table 3). Methane production (ml/g and % of total gas) was linearly reduced with NO_3^- , ranging from 8.8% of total gas produced in the control treatment to 4.1% of total gas produced for 6 mM NO_3^- ($P=0.002$). Except for 6 mM NO_3^- , the reduction in CH_4 emission was higher than the stoichiometrically calculated reduction. Nitrate did not affect gaseous H_2 emissions expressed in ml/g or as a percentage of total gas produced.

After 32 h incubation, the pH linearly increased from 6.08 to 6.21 with increasing nitrate concentrations ($P=0.003$). Nitrate increased NH_4^+ concentrations ($P=0.018$) but did not affect the production of insoluble protein which averaged 0.22 mg/ml. Kinetics of insoluble protein production also confirmed the absence of treatment effect throughout the incubation (Figure 3). Nitrate did not affect total VFA production, but increased the proportion of acetate ($P=0.020$) while reducing the proportion of propionate ($P=0.027$). These results led to a linear increase of the ratios acetate/propionate ($P=0.012$) and (acetate+butyrate)/propionate ($P=0.013$).

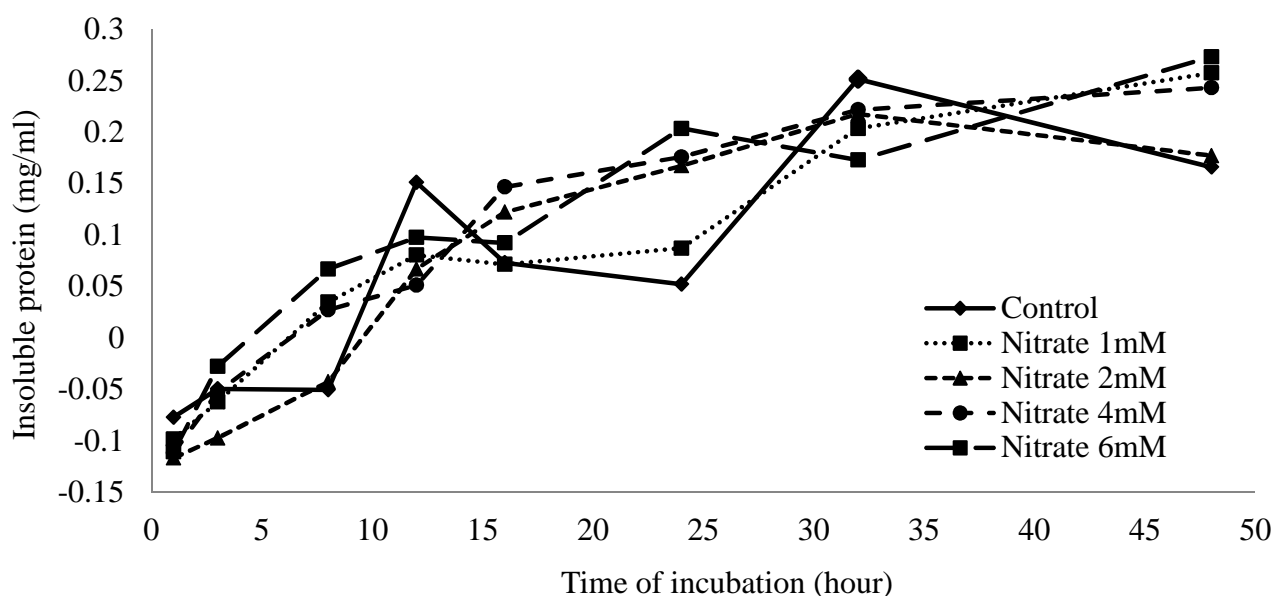


Figure 3 *In vitro* dose response effect of nitrate on kinetics of insoluble protein during 48 h incubation with GLU substrate (100% glucose; experiment 2)

Treatments did not affect total H_2 production which averaged 2.82 mmol/bottle, but total H_2 consumption tended to be different between treatments ($P=0.065$; Table 4). The quantities of

H₂ used for microbial biomass were similar between treatments and averaged 0.010 mmol/bottle. Finally, unaccounted H₂ was similar and close to zero for all treatments.

Discussion

Dose response effect of nitrate on gaseous emissions and rumen fermentation profile

With 6 mM NO₃⁻, total gas production was decreased by 26% and 22% with GP and GLU substrates, respectively. Similar observations were made in previous *in vitro* experiments testing similar or higher NO₃⁻ doses with alfalfa hay (13 mM; Bozic et al., 2009), wheat straw and concentrate (5 and 10 mM; Sakthivel et al., 2012) or alfalfa hay and concentrate (5 and 10 mM; Patra and Zhongtang, 2013; 2014). These results indicate that NO₃⁻ at a level greater than 5 mM inhibits *in vitro* rumen fermentation. However, NO₃⁻ did not affect production and composition of VFA with GP substrate. This is in accordance with Patra and Zhongtang (2014), but in contrast to Bozic et al. (2009) where *in vitro* NO₃⁻ supplementation reduced propionate proportion in total VFA. In the present work, the stability of propionogenesis may be explained by an equilibrium between two opposite actions of nitrate on the H₂ pool: i) a reduction of H₂ availability for nitrate reduction (electron sink); ii) an increase of H₂ availability via its direct toxic effect towards methanogens as indicated by the observed higher gaseous H₂ emissions (Janssen, 2010).

In the two *in vitro* experiments of this study, CH₄ emissions were linearly reduced with increasing concentrations of NO₃⁻. The kinetics of CH₄ emissions with GP substrate indicated that NO₃⁻ acts rapidly during the first 10 h. This observation can be related to the quick absorption of NO₃⁻ by rumen microbes: *in vitro*, microbes used NO₃⁻ within 10 h incubation in the medium (Shi et al., 2012). *In vivo* on sheep fed 1.3 g NaNO₃/kg metabolic weight, the concentration of NO₃⁻ was decreased by 50% within 5 h postfeeding (Sar et al., 2004). In the rumen of cows fed 3% calcium nitrate, NO₃⁻ was not even detected 3 h after feeding (Guyader et al., 2014a).

When GP diet was used as a substrate, the observed CH₄ inhibition was higher than the stoichiometrically calculated inhibition at levels of NO₃⁻ exceeding 4 mM, and increased as NO₃⁻ doses increased. In contrast, when GLU was used, the apparent yield of CH₄ reduction was higher than 100%, independent of the NO₃⁻ concentration. This observation indicated that the assumption that NO₃⁻ only acts as an electron acceptor is not sufficient enough to understand the CH₄-mitigating mechanisms of NO₃⁻. While the conversion of NO₃⁻ to NH₄⁺ requires electrons, a release of gaseous H₂ is an indicator for a direct inhibition of

methanogens, and the decrease in gas production indicates a direct inhibitory effect on fermentative microorganisms. As long as NO_3^- is deviating electrons, no H_2 emissions will be observed, and only after 10 to 15 h when the NO_3^- is exhausted, gaseous H_2 is observed. These results confirm previous *in vitro* (Zhou et al., 2011) and *in vivo* (Van Zijderveld et al., 2010; Guyader et al., 2014c) results showing that NO_3^- have a toxic effect towards methanogens. However, the direct toxicity of NO_3^- against methanogens is dose and substrate dependent, and becomes evident only at concentrations above 4 mM with GP substrate and no such a toxic effect was observed when GLU was used as a substrate.

Dose response effect of nitrate on H_2 metabolic fluxes

Total H_2 production calculated from acetate and butyrate concentrations was similar between treatments, when GP substrate was used. Differences were only observed for H_2 consuming pathways such as methanogenesis. For the control treatment, 76% of H_2 was directed towards methanogenesis and 13% towards propionate synthesis. These percentages were in the range of previous estimations of H_2 distribution between fermentation end-products (Czerkawski, 1986; Mills et al., 2001).

Unaccounted H_2 represented between 6.9 and 23.0%, which may be either captured in microbial biomass (Czerkawski, 1986; Mills et al., 2001) or derived from substrates other than glucose on which the calculation is based on (Wolin, 1960). In this balance, we assumed a full transformation of NO_3^- to NH_4^+ , which is supported by the high efficiencies of NO_3^- reduction. Unaccounted H_2 was similar to the control at low level of NO_3^- , but higher levels increased the percentage of unaccounted H_2 . Two hypotheses were tested in order to understand how missing H_2 can be used. Firstly, we assumed that formate which production may require H_2 via the formate-hydrogen lyase, accumulated in the medium, as shown in previous monoculture of *Ruminococcus flavefaciens* (Wolin et al., 1997). This intermediate of rumen fermentation was also observed *in vitro* when CH_4 emissions were inhibited with propynoic acid or ethyl 2-butyrate (Ungerfeld et al., 2006). However, although in the present incubation formate was not determined, no formate was found in response to 2 and 8 mM NO_3^- in separate *in vitro* incubations with GP substrate (data not shown).

Assuming that microbes require 0.41 moles H_2 per kg microbes (Mills et al., 2001), it was expected that microbial biomass was increased with high doses of NO_3^- , using a part of unaccounted H_2 . However, treatments did not affect insoluble protein concentrations, showing that NO_3^- did not enhance microbial synthesis. This result confirms previous *in vitro* experiments reporting an absence of NO_3^- (5 and 10 mM doses) effect on bacterial and

protozoal concentrations (Zhou et al., 2011; Patra and Zhongtang, 2013; 2014). This is also in accordance with an *in vivo* experiment reporting no difference in microbial protein supply based on urinary excretion of purine derivatives of non-lactating cows supplemented with NO_3^- (2.3% in DM; Guyader et al., 2014a). In addition, the estimated percentage of H_2 directed towards microbial biomass was very low and ranged between 0.3 and 0.3%, confirming the low contribution of microbes to H_2 consumption (Mills et al., 2001).

Conclusions

Nitrate is an efficient CH_4 -mitigating strategy but it can be used only to a limited extent, before fermentation is negatively affected. The *in vitro* threshold appears to be between 2 to 4 mM, which would correspond to a supplementation to animals situated between 1.2 and 2.5% of DM. The difference between observed and theoretical CH_4 production shows that this additive acts as an electron acceptor, but its mechanisms of action must also involve a direct toxic effect on methanogens. In addition, the study of H_2 distribution between fermentation end-products shows that NO_3^- must enhance another unknown H_2 consuming pathway, different from H_2 emitted or captured for NO_3^- reduction, and for production of CH_4 , VFA or microbial biomass.

Conflict of interest

None.

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References

- Attwood, G.T., Klieve, A.V., Ouwerkerk, D., Patel, B.K.C., 1998. Ammonia-hyperproducing bacteria from New Zealand ruminants. *Appl. Environ. Microbiol.* 64, 1796-1804.
- Bozic, A.K., Anderson, R.C., Carstens, G.E., Ricke, S.C., Callaway, T.R., Yokoyama, M.T., Wang, J.K., Nisbet, D.J., 2009. Effects of the methane-inhibitors nitrate, nitroethane, lauric acid, Lauricidin and the Hawaiian marine algae *Chaetoceros* on ruminal fermentation in vitro. *Bioresour. Technol.* 100, 4017-4025.

- Chaney, A.L., Marbach, E.P., 1962. Modified reagents for determination of urea and ammonia. *Clin. Chem.* 8, 130-132.
- Czerkawski, J.W., 1986. An introduction to rumen studies. Pergamon Press, Oxfordshire and New York.
- France, J., Dijkstra, J., Dhanoa, M.S., Lopez, S., Bannink, A., 2000. Estimating the extent of degradation of ruminant feeds from a description of their gas production profiles observed in vitro: derivation of models and other mathematical considerations. *Br. J. Nutr.* 83, 143-150.
- Guyader, J., Eugène, M., Meunier, B., Doreau, M., Morgavi, D.P., Silberberg, M., Rochette, Y., Gerard, C., Loncke, C., Martin, C., 2014a. Additive effect between dietary linseed oil and nitrate as methane emission-reducer in cattle. *J. Anim. Sci.* Accepted.
- Guyader, J., Silberberg, M., Morgavi, D.P., Martin, C., 2014b. Daily pattern of hydrogen concentration in the rumen of cows fed nitrate or linseed oil. *Proc. Aust. Soc. Anim. Prod.* 30, 86.
- Guyader, J., Silberberg, M., Popova, M., Seradj, A.R., Morgavi, D.P., Martin, C., 2014c. Dietary nitrates decrease methane emission by inhibiting rumen methanogenic archaea without influencing nitrate reducing bacteria, *Gut microbiology: From sequence to function*, Aberdeen, UK.
- Hegarty, R.S., Gerdes, R., 1999. Hydrogen production and transfer in the rumen. *Recent Adv. Anim. Nutr. Aust.* 12, 37-44.
- Hungate, R.E., Smith, W., Bauchop, T., Yu, I., Rabinowitz, J.C., 1970. Formate as an intermediate in the bovine rumen fermentation. *J. Bacteriol.* 102, 389-397.
- Janssen, P.H., 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Anim. Feed Sci. Technol.* 160, 1-22.
- Lee, C., Beauchemin, K.A., 2014. A meta-analysis of effects of feeding nitrate on toxicity, production, and enteric methane emissions in ruminants, *Linking animal science and animal agriculture: Meeting the global demands of 2050*, Kansas City, USA, pp. 845-846.
- Lewis, D., 1951. The Metabolism of Nitrate and Nitrite in the Sheep. 1 The reduction of nitrate in the rumen of the sheep. *Biochem. J.* 48, 175-180.
- Mills, J.A., Dijkstra, J., Bannink, A., Cammell, S.B., Kebreab, E., France, J., 2001. A mechanistic model of whole-tract digestion and methanogenesis in the lactating dairy cow: model development, evaluation, and application. *J. Anim. Sci.* 79, 1584-1597.

- Mould, F.L., Morgan, R., Kliem, K.E., Krystallidou, E., 2005. A review and simplification of the in vitro incubation medium. *Anim. Feed Sci. Technol.* 123, 155-172.
- Muetzel, S., Hunt, C., Tavendale, M.H., 2014. A fully automated incubation system for the measurement of gas production and gas composition. *Anim. Feed Sci. Technol.* 196, 1-11.
- Patra, A.K., Zhongtang, Y., 2013. Effective reduction of enteric methane production by a combination of nitrate and saponin without adverse effect on feed degradability, fermentation, or bacterial and archaeal communities of the rumen. *Bioresour. Technol.* 148, 352-360.
- Patra, A.K., Zhongtang, Y., 2014. Combinations of nitrate, saponin, and sulfate additively reduce methane production by rumen cultures in vitro while not adversely affecting feed digestion, fermentation or microbial communities. *Bioresour. Technol.* 155, 129-135.
- Reichl, J.R., Baldwin, R.L., 1975. Rumen modeling: rumen input-output balance models. *J. Dairy Sci.* 58, 879-890.
- Sakthivel, P.C., Kamra, D.N., Agarwal, N., Chaudhary, L.C., 2012. Effect of sodium nitrate and nitrate reducing bacteria on in vitro methane production and fermentation with buffalo rumen liquor. *Asian Australas. J. Anim. Sci.* 25, 812-817.
- Sar, C., Santoso, B., Mwenya, B., Gamoa, Y., Kobayashi, T., Morikawa, R., Kimura, K., Mizukoshi, H., Takahashi, J., 2004. Manipulation of rumen methanogenesis by the combination of nitrate with b1-4 galacto-oligosaccharides or nisin in sheep. *Anim. Feed Sci. Technol.* 115, 129-142.
- Shi, C., Meng, Q., Hou, X., Ren, L., Zhou, Z., 2012. Response of ruminal fermentation, methane production and dry matter digestibility to microbial source and nitrate addition level in an in vitro incubation with rumen microbes obtained from wethers. *J. Anim. Vet. Adv.* 11, 3334-3341.
- Ungerfeld, E.M., Rust, S.R., Burnett, R., 2006. Effects of butyrate precursors on electron relocation when methanogenesis is inhibited in ruminal mixed cultures. *Lett. Appl. Microbiol.* 42, 567-572.
- Van Zijderveld, S.M., Gerrits, W.J.J., Apajalahti, J.A., Newbold, J.R., Dijkstra, J., Leng, R.A., Perdok, H.B., 2010. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. *J. Dairy Sci.* 93, 5856-5866.

- Van Zijderveld, S.M., Gerrits, W.J.J., Dijkstra, J., Newbold, J.R., Hulshof, R.B.A., Perdok, H.B., 2011. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. *J. Dairy Sci.* 94, 4028-4038.
- Veneman, J.B., Muetzel, S., Hart, K.J., Faulkner, C.L., Moorby, J.M., Molano, G., Perdok, H.B., Newbold, J.R., Newbold, C.J., 2014. Dietary nitrate but not linseed oil decreases methane emissions in two studies with lactating dairy cows, *Livestock, Climate Change and Food Security Conference*, Madrid, Spain. p 38.
- Wolin, M., Miller, T., Stewart, C., 1997. Microbe-microbe interactions., In: Hobson, P.N., Stewart, C.S. (Eds.), *The rumen microbial ecosystem*, Chapman & Hall, London, pp. 467-491.
- Wolin, M.J., 1960. A theoretical rumen fermentation balance. *J. Dairy Sci.* 43, 1452-1459.
- Zhou, Z., Meng, Q., Yu, Z., 2011. Effects of methanogenic inhibitors on methane production and abundances of methanogens and cellulolytic bacteria in in vitro ruminal cultures. *Appl. Environ. Microbiol.* 77, 2634-2639.

General discussion

Methane released by ruminants is the main greenhouse gas at the farm level (Veysset et al., 2010) and constitutes an energetic loss for the animal, ranging from 2 to 12% of its GEI (Johnson and Johnson, 1995). Therefore, this PhD thesis takes part in the global context of CH₄ mitigation, in order to reduce the negative environmental impacts of ruminants while improving their feed efficiency.

Knowing that H₂ is the limiting substrate of methanogenesis in the rumen, the objective of this PhD thesis consisted in studying the importance of the different H₂ metabolic pathways (production and consumption), in order to determine the more efficient way to manipulate H₂ pool in the rumen. The final purpose of this work consists in proposing new dietary CH₄-mitigating strategies. We assumed that acting on both reduction of H₂ production and stimulation of H₂ consumption by a competitive pathway to methanogenesis decreases CH₄ production to a higher extent than when acting on a single pathway.

Our scientific approach was divided in two parts. Firstly, the bibliographical approach detailed the biological and thermodynamic mechanisms of H₂ production and utilization in the rumen *via* a classic literature review. In addition, a meta-analysis reported the relationship between rumen protozoa and CH₄ emissions. Secondly, the experimental approach assessed the effect of association of dietary strategies on CH₄ emissions of non-lactating and lactating cows. The originality of our work consisted in associating dietary treatments with different mechanisms of action on H₂ pool (reducing H₂ production or consuming H₂). Moreover, the distribution of H₂ between fermentation end-products was estimated *in vitro* with a strategy acting on H₂ utilization.

In the following discussion, we will focus on the main original results obtained during this PhD thesis. This section will be divided into three parts:

- 1/ we will give an experience feedback on new equipment acquired during this PhD thesis (CH₄-open chambers and H₂-sensors), and we will assess precision and accuracy of CH₄ emissions and rumen dissolved H₂ concentrations obtained in cows fed control diets.
- 2/ we will assess the relevance of the tested CH₄-mitigating strategies on methanogenesis, but also on overall digestive and zootechnical performances. Rumen fermentation mechanisms of these CH₄-mitigating strategies will be highlighted, by relating them with distribution of H₂ in the different fermentation end-products and with modification in the microbiota.
- 3/ we will discuss the possibility of a practical use at the farm scale of the most efficient CH₄-mitigating dietary strategy tested in this PhD thesis.

I. PRECISION AND ACCURACY OF METHANE EMISSIONS AND RUMINAL DISSOLVED HYDROGEN CONCENTRATIONS IN COWS FED CONTROL DIETS

During this PhD thesis, two new methods were implemented in the team to monitor cows' individual kinetics of i) enteric CH₄ emissions using open chambers and ii) rumen dissolved H₂ concentrations using H₂-sensors. In the following section, we will give an experience feedback on these two devices and we will assess the precision and accuracy of our data obtained on cows fed control diets by comparing them with the literature.

1.1. Precision and accuracy of methane emissions

Table 11 Compiled data of methane emissions obtained in the experiments of this PhD thesis with non-lactating and lactating cows

Experiment	Experimental design				Methane emissions (± SD)		
	Animal (n)	Forages (% of DM)	Methane measurement technique	Days in chambers	g/day	g/kg DMI	% of GEI
1 & 3	Non-lactating cows (8)	Grass hay (50)	Open chambers	4	310.5 (± 16.50)	25.2 (± 1.56)	7.2 (± 0.45)
2 & 3	Lactating cows (15)	Corn silage, grass hay (60)	Open chambers	4 or 2	450.9 (± 111.77)	21.2 (± 3.50)	6.0 (± 0.99)

n: number of animals; SD: Standard deviation

In this PhD thesis (Table 11), 8 non-lactating cows (experiments 1 and 3) were fed a same control hay-based diet in restricted conditions (90% of *ad libitum* intake). Their CH₄ emissions were measured in open chambers for 4 consecutive days. Fifteen lactating cows (experiments 2 and 3) were fed *ad libitum* a same control corn silage-based diet. During measurement of their CH₄ emissions in open chambers for 4 or 2 days, animals were restricted fed (95% of *ad libitum* intake).

1.1.1. Experience feedback on open chambers for cattle

Our open chambers allowed to measure daily kinetics of enteric CH₄ emissions in cattle (see experiment 1 for a detailed description of chambers). Chambers were designed to be spacious and comfortable for the animals (4-cm thick mattress, 2.5-m² lying area) in order to avoid disturbance of cows' behavior and performances during their stay inside. In addition, animals were used to be attached in the barn before to be moved in open chambers. The levels of DMI in chambers averaged 12.4 (day-to-day coefficient of variation, CV = 1.3%) for non-lactating and 21.2 (day-to-day CV = 2.0%) kg/day for lactating cows, and were similar to the levels of DMI measured the week preceding or following CH₄ measurement (12.4 kg/day, day-to-day CV = 1.1% for non-lactating cows; 20.8 kg/day, day-to-day CV = 3.6% for lactating cows). The constant DMI and milk production of cows between inside and outside chambers reflected that animals easily adapted to open chambers and that our experimental conditions are good enough to measure accurate CH₄ emissions in cows.

In our experimental conditions, the day-to-day variability of CH₄ emissions (g/day) within animals was low and similar between non-lactating and lactating cows (4.1%, on average). This variability level was comparable with data reported in the literature, which ranged between 4.3 and 7.2% for animals placed in respiration chambers for a minimum of 3 consecutive days (dairy and beef cattle, n = 87, Blaxter and Clapperton, 1965; dairy cattle, n = 16, Grainger et al., 2007).

Variability of CH₄ emissions (g/day) between animals averaged 5.3% for non-lactating cows fed a hay-based diet and 24.8% for lactating cows fed a corn silage-based diet. These levels remained comparable with reviews cited previously (8.1%, no indication about diets, Blaxter and Clapperton, 1965; 17.8%, 75% forage in diet, Grainger et al., 2007). The higher variability of CH₄ emissions between lactating cows is consistent with their higher DMI variability (18.6% for lactating cows fed sub *ad libitum* versus 7.9% for dry cows restricted fed). Then, we also confirmed a previous study reporting that the CV between animals is larger when intake is not restricted (Grainger et al., 2007).

Table 12 Equations used to estimate CH₄ emissions of cows fed control diets in all experiments of this PhD thesis

Reference	n	Animals	Methane measurement technique	Forage (% of DM)	DMI% BW [min-max]	CH ₄ (g/day) [min-max]	Equation ¹	RMSE (unit)	R ²
Sauvant et al., 2011 [1]	976	Dairy and beef cattle, sheep and goat ²	Chambers (n=976)	60	1.61 [0.56-4.01]	NA ⁴	CH ₄ (g/day) = (7.14 + 0.22 × DOM) / DMI	2.70 (g/kg DMI)	0.81
Mills et al., 2003 [2]	159	Dairy cattle (n=159)	Chambers (n=159)	55	NA ³	479.5 [325.0-605.9]	CH ₄ (g/day) = (5.93 + 0.92 × DMI) × Z	1.82 (MJ/day)	0.60
Ellis et al., 2007 [3]	172	Dairy (n=89) and beef cattle (n=83)	Chambers (n=101) SF6 (n=42) Others (n=29)	75	2.25 [NA]	236.8 [56.4-499.6]	CH ₄ (g/day) = (3.27 + 0.74 × DMI) × Z	0.28 (MJ/day)	0.68
Ellis et al., 2007 [4]	83	Beef cattle (n=83)	Chambers (n=44) SF6 (n=37) Others (n=2)	80	1.99 [NA]	183.2 [56.4-345.1]	CH ₄ (g/day) = (3.96 + 0.561 × DMI) × Z	0.26 (MJ/day)	0.44
Ellis et al., 2007 [5]	89	Dairy cattle (n=89)	Chambers (n=57) SF6 (n=5) Others (n=27)	70	2.37 [NA]	286.9 [86.5-499.6]	CH ₄ (g/day) = (3.23 + 0.809 × DMI) × Z	0.26 (MJ/day)	0.65
Ramin and Huhtanen, 2013 [6]	207	Dairy (n=145) and beef (n=62) cattle	Chambers (n=207)	70	2.18 [0.78-5.23]	218.7 [9.2-541.7]	CH ₄ (g/day) = (20 + 35.8 × DMI – 0.50 × DMI ²) × 0.71427	NA	--
Sauvant and Nozière, 2013 [7]	450	Dairy and beef cattle, sheep and goat ²	Chambers (n=450)	60	1.61 [0.56-4.01]	NA ⁴	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	2.3 (g/kg DOM)	--

n: number of treatments; RMSE: residual mean square error; NA: non-available

¹ Z = conversion factor between CH₄ expressed in MJ/day to CH₄ expressed in g/day = 20.0638; DOM (% of DM) = digestible OM in diet = OM content of the diet (% of DM) × OM digestibility (0-1); DMI (kg/day) = dry matter intake; PC = concentrate proportion (0-1); DOMI = digestible OM intake (kg/day) = DOM × DMI

² Proportions not available

³ DMI = 19.6 kg/day, with minimum and maximum: 12.5 and 28.4 kg/day

⁴ CH₄ = 18.3 g/kg DMI, with minimum and maximum: 13.6 and 23.0 g/kg DMI

1.1.2. Comparison of methane emissions with the literature

Daily pattern of CH₄ emissions. In the experiments of this PhD thesis, animals were fed twice daily and the daily patterns of CH₄ emissions were similar between cows fed the control diets. Methane emissions increased quickly following feed intake to reach a peak 2 h after feeding, and then decreased progressively until the next feeding. These daily patterns of methanogenesis according to feeding frequency are in accordance with previous observations (Grainger et al., 2007; Janssen, 2010; Van Zijderveld et al., 2010).

Difference between non-lactating and lactating cows. Expressed in g/day, CH₄ emissions of non-lactating cows fed a hay-based diet were lower than lactating cows fed a corn silage-based diet (310.5 *versus* 450.9 g/day). This expected result is explained by the lower intake level of non-lactating cows compared to lactating cows (12.4 *versus* 21.2 kg DMI/day). Indeed, the positive correlation between CH₄ emissions (g/day) and DMI is well known (Reynolds et al., 2011; Ramin and Huhtanen, 2013).

Inversely, when expressed in g/kg DMI or as a percentage of GEI, CH₄ emissions of non-lactating cows (hay-based diet) were higher than lactating cows (corn silage-based diet; 25.2 *versus* 21.2 g/kg DMI; 7.2 *versus* 6.0% GEI). This difference may be explained by two confounded effects. The first one is related to the higher intake level of lactating cows compared to non-lactating cows, which decreased the feed retention time in the rumen, lowering the time for microbial fermentation of feed substrates (Reynolds et al., 2011). The second one is related to the forage nature of the basal diet. Forage preservation may affect enteric CH₄ production which tends to be lower when forages are ensiled than when they are dried (Martin et al., 2010). From direct comparisons, Doreau et al. (2011) also reported that lactating cows fed silage-based diets produce less CH₄ (g/per kg milk) than those fed hay-based diets.

Comparison of observed and predicted CH₄ emissions. In order to assess the coherence of our CH₄ emissions, data from individual cows fed control diets were confronted to CH₄ emissions estimated with equations from the literature. To predict enteric CH₄ emissions, several equations are available in the literature, which are based on various criteria such as intake level, diet composition, production level of animals or rumen fermentation parameters. In the present work, we selected 7 predictive equations (Table 12) for the following reasons:

1/ They predict CH₄ emissions from the level of DMI (Ellis et al., 2007; Mills et al., 2003; Ramin and Huhtanen, 2013) which is the main determinant of CH₄ production (Reynolds et al., 2011; Ramin and Huhtanen, 2013), and/or from the level of digestible OM (DOM) in the diet (Sauvant et al., 2011; Sauvant and Nozière, 2013) which is a good predictor of CH₄ emissions, as it is statistically related with the level of fermented OM in the rumen (Sauvant et al., 2011).

2/ They were developed from large database built with *in vivo* data collected from animals fed diets containing proportions of concentrate (20-50%) overlying those of our experimental diets (40-50% concentrate).

3/ They were developed from data of CH₄ emissions mostly measured with chambers, as realized in this work (93% with chambers *versus* 4% with SF6 *versus* 3% with other techniques).

We adopted two approaches for comparison of observed and predicted CH₄ emissions (g/day). To compare **absolute values**, a T-test was applied between observed and predicted CH₄ emissions for each equation. To check **variations in CH₄ emissions**, the relationship between observed and predicted CH₄ emissions was tested for each equation using the general linear model (GLM) procedure:

$$\text{Observed CH}_4 = \alpha + \beta \times \text{predicted CH}_4$$

Where α = the overall intercept and β = the overall slope. Non-significant intercepts were considered as equal to 0. Slopes were compared to 1 by calculating T ($T = (\text{slope} - 1)/\text{SD}_{\text{slope}}$), which was compared to t_{α} obtained from the T-Student table ($\alpha = 0.05$). If $T > t_{\alpha}$, the slope was considered different from 1. Statistical analyses were performed with Minitab (Version 16).

When considering all data from lactating or non-lactating cows fed control diets (Table 13; Figure 20), we showed that absolute data between observed and predicted CH₄ emissions were positively correlated (average $R^2 = 76.6\%$ on average) whatever the equation ($P < 0.001$). Absolute CH₄ emissions between observed and predicted were similar ($P > 0.05$) with equations 2, 5, 6 and 7, whereas observed CH₄ emissions were significantly higher than predicted with equations 1, 3 and 4 (average bias = +142.5, +68.0 and +119.1 g/day, respectively; $P < 0.05$). Concerning variations between observed and predicted CH₄ emissions, all intercepts tended or were equal to 0, and slopes of regressions were significantly equal to 1 ($P < 0.05$) for all equations, except for equations 4 and 6. The differences between observed and predicted CH₄ emissions with equations 1, 3 and 4 may be explained by i) the different animal species and type of cattle production (equation 1 is proposed for sheep and cattle,

equation 4 is proposed for beef cattle), and ii) the higher concentrate proportion in our experiments (40-50%) compared with the average concentrate proportion in the dataset used to generate the equations 3 and 4 (20-25%).

Table 13 Comparison and relationship between observed and predicted CH₄ emissions (g/day) of lactating and non-lactating cows (n=23) used in the experiments of this PhD thesis.

Equation	Predicted CH ₄ (±SD) ¹	<i>P-Value</i> (T-test)	Intercept (SE)	Slope (SE)	Slope = 1	R ²	RMSE
[1]	259.6 (±78.4)	<0.001	74.2 [†] (40.70)	1.26 ^{***} (0.150)	Yes	0.77	55.3
[2]	452.8 (±98.7)	0.112	-53.3 ^{ns} (54.83)	1.01 ^{***} (0.118)	Yes	0.77	54.8
[3]	334.1 (±79.4)	0.023	-15.7 ^{ns} (50.50)	1.25 ^{***} (0.147)	Yes	0.77	54.8
[4]	283.0 (±60.2)	<0.001	-64.7 ^{ns} (56.14)	1.65 ^{***} (0.194)	No	0.77	54.8
[5]	358.3 (±86.8)	0.148	-7.8 ^{ns} (49.60)	1.14 ^{***} (0.135)	Yes	0.77	54.8
[6]	350.2 (±65.6)	0.064	-116.8 [†] (67.56)	1.48 ^{***} (0.190)	No	0.74	58.4
[7]	362.4 (±90.6)	0.195	6.7 ^{ns} (48.68)	1.09 ^{***} (0.131)	Yes	0.77	55.5

SE: Standard error

¹ Observed CH₄ emissions averaged 402 ± 112.8 g/day.

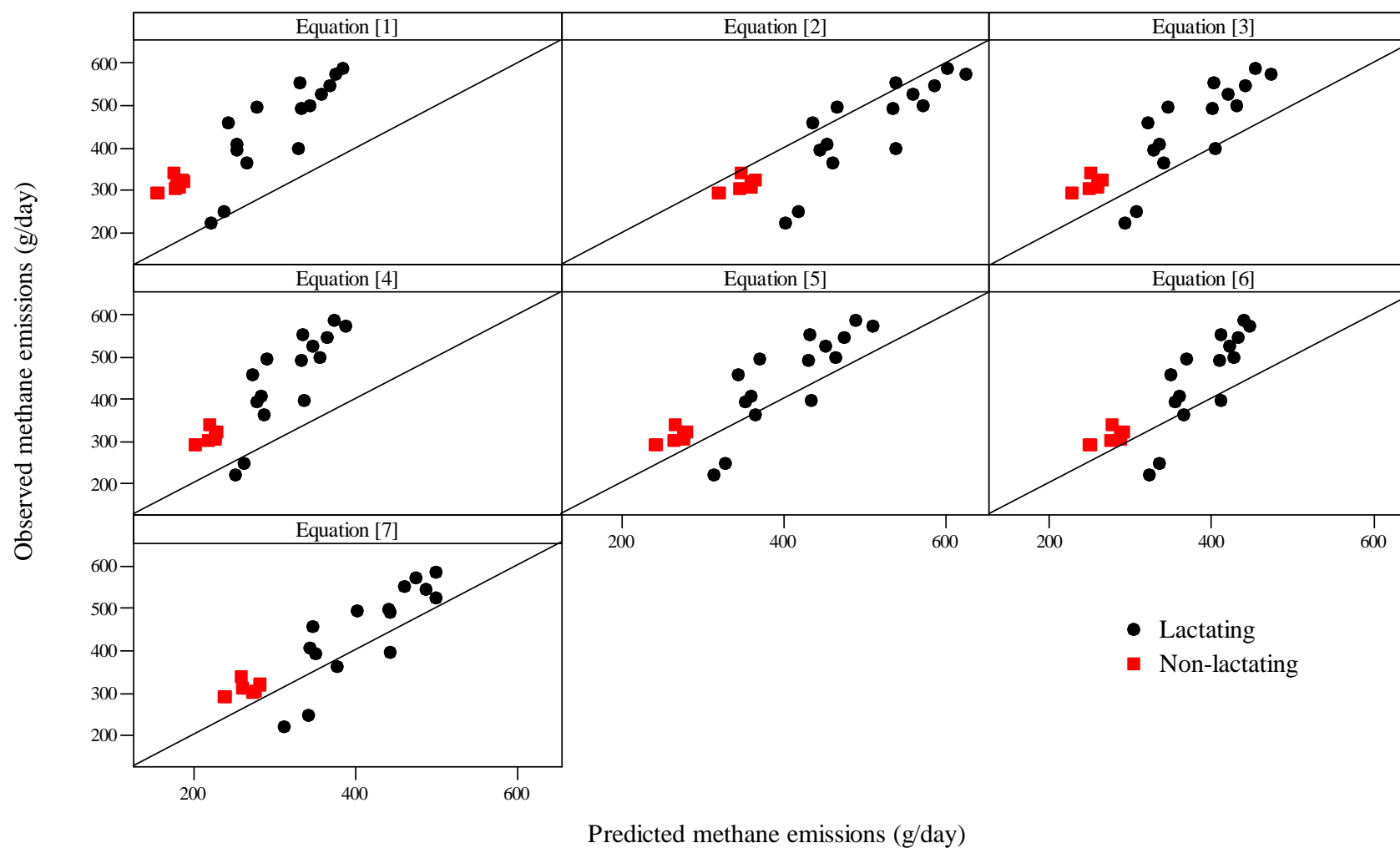


Figure 20 Relationship between observed and predicted methane production (g/day) of lactating (n=15) and non-lactating (n=8) cows fed control diets in the different experiments of this PhD thesis

1.2. Precision and accuracy of ruminal dissolved hydrogen concentrations

This PhD thesis is the first work reporting kinetics of dissolved H₂ concentrations in the rumen of fistulated cows using indwelling H₂-sensors (experiment 1).

1.2.1. Experience feedback on H₂-sensors

We succeeded to adapt an H₂-sensor (commonly used in marine research) for continuous and *in situ* measurement of dissolved H₂ concentration in the rumen. This system counteracted the main disadvantages of previous H₂-measurement devices (detailed in Materials and methods section of this manuscript): i) it measured kinetics of dissolved H₂ concentrations, ii) it detected quick modification of H₂ concentrations (90% response in 15 sec) which was important knowing that the turnover time of H₂ in the rumen is ~0.08 sec, and iii) it had a low limit of H₂ quantification (0.3 μ M).

From a practical point of view, the full system did not require important equipment, and the sensor size was rather small which did not disturb the ruminal environment. However, the glass-made tip of the sensor was very fragile, and the sensor required a strong home-made protection prior to its insertion into the rumen, and this was very challenging if the animal just ate. To counteract this issue, we inserted the sensor before morning feeding and removed it before afternoon feeding. Kinetics of dissolved H₂ concentrations were measured for 5 h postfeeding.

As we only had one available and functional H₂-sensor, measurements were carried out only one day per cow per experimental period. Then, for the whole experiment, we collected 4 daily kinetics obtained on 4 cows fed the control diet. For this reason, variability between days was impossible to estimate for this measure.

Rumen dissolved H₂ concentrations of the 4 non-lactating cows fed a same control diet presented an important inter-animal variability. Figure 21 shows that the highest variability levels were observed during the time outside feeding time (from 2.5 to 3 h after feeding). This result highlights the importance of repeating the measurement for several days for a same animal. In our case, this was impossible because of limitation in H₂-sensors availability.

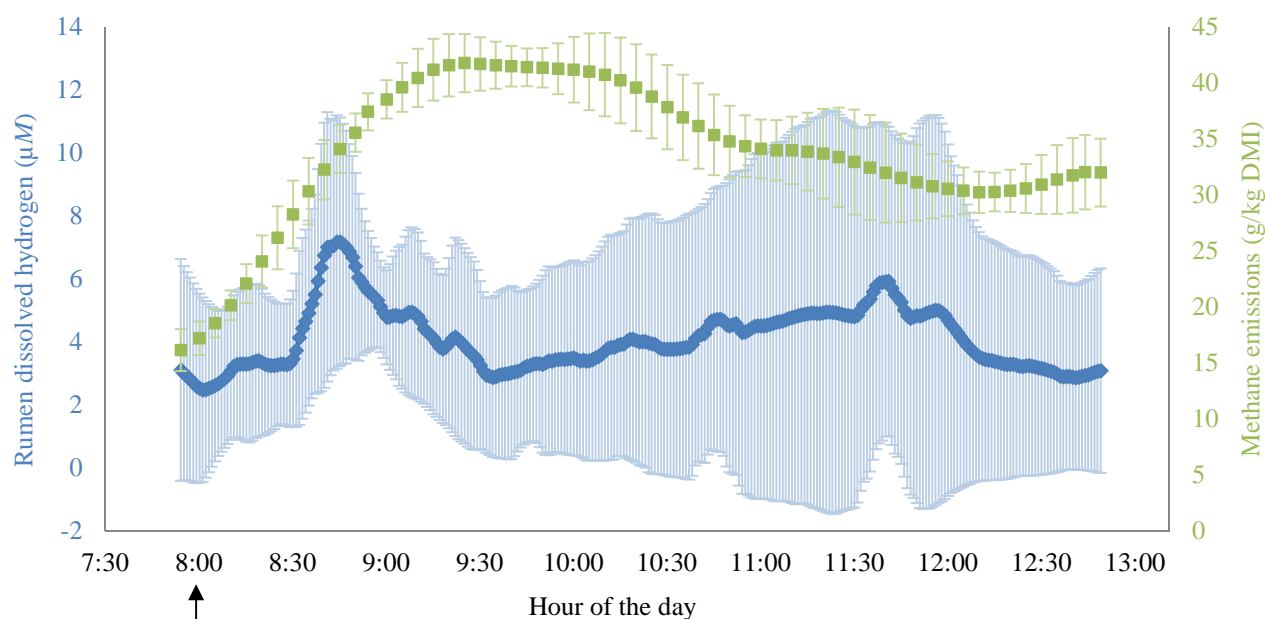


Figure 21 Average rumen dissolved hydrogen concentrations and methane emissions up to 5 h after feeding a similar hay-based diet to four non-lactating cows. Errors bars indicate SD. The arrow indicates time of feeding.

1.2.2. Comparison of ruminal dissolved hydrogen concentrations with the literature

During the 5 h after feeding, dissolved H_2 concentration in the rumen of cows fed a hay-based diet averaged $4.1 \mu M$ with an interval situated between 2.5 and $7.2 \mu M$. These concentrations were low, but in the range of previous observations (0.1 - $50 \mu M$) given by Janssen (2010).

The maximum ruminal dissolved H_2 concentrations ($7.2 \mu M$) was observed less than 1 h after feeding. This postprandial peak of rumen dissolved H_2 concentration was situated upstream of the observed postprandial peak of CH_4 emissions, coinciding with previous observations (Swainson et al., 2011). This postfeeding H_2 peak probably corresponded to the release of H_2 coming from fermentation of fresh feed ingested. This pattern was in accordance with previous studies on cattle or sheep, which also observed a rise of ruminal dissolved H_2 concentrations between 10 min and 3 h after feeding (Morgavi et al., 2012; Robinson et al., 1981; Smolenski and Robinson, 1988). As for CH_4 emissions, we assume that the extent and time of the postfeeding H_2 peak is dependent on the fermentation rate of diet components and on the feeding frequency.

The aim of this PhD thesis was to propose new dietary strategies to mitigate CH_4 emissions in ruminants via a modification of H_2 availability in the rumen. Quantification of individual CH_4 emissions was an essential measurement in this work, as well as

dissolved H_2 concentrations in the rumen as indicator of H_2 availability. Monitoring kinetics of these two parameters allowed getting a better insight of mechanisms involved in CH_4 mitigation. Consequently, four open CH_4 chambers for cattle were implemented in the team and H_2 -sensors were adapted to the rumen environment. Overall results indicate that in our experimental conditions, kinetics of CH_4 emissions were precise and accurate. Data on H_2 kinetics were original, but additional research is required to assess the reproducibility and repeatability of measurements. In conclusion, we confirm that these two devices were adapted to evaluate the efficiency and understand the mechanisms of actions of the selected dietary CH_4 -mitigating strategies.

Table 14 Compiled data from the literature on the effects of lipids from linseed supplementation to cattle or sheep on methane emissions, total tract digestibility and nitrogen balance

Reference	Animal species	Forages (% of DM)	Linseed form	Added fat ¹ (% of DM)	Methane reduction (g/kg DMI, % per 1% added C18:3)	Effect on total tract digestibility	Effect on nitrogen balance
Experiment 1, this PhD thesis	Dry cows	Grass hay (50)	Linseed oil	2.6	-6.6	No effect	No effect
Machmüller et al., 2000	Lambs	Corn silage, grass hay (76)	Crude linseed	2.4	-6.0	No effect	NA
Martin et al., 2008	Dairy cows	Corn silage, grass hay (65)	Crude linseed	4.2	-2.5	dOM ³ : -4%	NA
			Extruded linseed	4.4	-6.0	dNDF ³ : -7%	
			Linseed oil	5.8	-9.0	dADF ³ : -6%	
Chung et al., 2011	Dry cows	Grass hay (48)	Crude linseed	5.6	-0.91	dOM: -3%	NA
	Dry cows	Barley silage (48)	Crude linseed	4.8	-6.8	dNDF: -7%	
Martin et al., 2011	Dairy cows	Grass silage/hay (57)	Extruded linseed	3.0	-4.7	dADF: -18%	NA
	Dairy cows	Pasture (79)	Extruded linseed	2.0	-8.1	dOM: -7%	
Veneman et al., 2014	Dairy cows	Grass/maize silage (NA)	Linseed oil	2.6 ²	-0.59	dNDF: -20%	NA
	Dairy cows	Corn silage (NA)	Linseed oil	2.6 ²	+0.4	dADF: -28%	
AVERAGE					-4.6		

NA: Data not available

¹ Based on ether extract content of the diet² Values based on estimation, knowing that in our experiment, 4% linseed oil = 2.6% added fat³ Extent of reduction in total tract digestibility similar between treatments

Table 15 Compiled data from the literature on the effects of tea saponin (*C. sinensis* or *assamica*) supplementation to cattle or sheep on methane emissions, total tract digestibility and nitrogen balance

Reference	Animal species	Forages (% of DM)	Distribution method of tea saponin powder	Tea saponin (% of DM) (% of active saponin compound)	Methane reduction (g/kg DMI, % per 1% added saponin)	Effect on total tract digestibility	Effect on nitrogen balance
Experiment 3, this PhD thesis	Dry cows	Grass hay (50)	In a pellet	0.77 (0.52)	-4.0	No effect	No effect
	Dairy cows	Corn silage, grass hay (60)	In a pellet	0.76 (0.52)	+17.9	dADF: +8%	No effect
Yuan et al., 2007	Adult sheep	Lucerne hay (60)	Mixed with feed	0.5 (NA)	-17.4	NA	NA
Mao et al., 2010	Lambs	Chinese wild rye (60)	Mixed with feed	0.4 (NA)	-68.7	NA	NA
Zhou et al., 2011	Adult sheep	Chinese wild rye (60)	Mixed with feed	0.4 (0.24)	-26.5 ¹	NA	NA
Li and Powers, 2012	Steers	Corn silage (46)	Mixed with feed	0.25 (0.06) 0.50 (0.12)	-29.2 -1.0	NA	No effect
AVERAGE					-26.4		

NA: Data not available

¹ DMI was not available. We assumed that DMI was similar to Mao et al. (2010)

Table 16 Compiled data from the literature on the effects of nitrate supplementation to cattle or sheep on methane emissions, total tract digestibility and nitrogen balance

Reference	Animal species	Forages (% of DM)	Nitrate source	Nitrate dose (% of DM)	Methane reduction (g/kg DMI, % per 1% added nitrate)	Effect on total tract digestibility	Effect on nitrogen balance
Experiment 1, this PhD thesis	Dry cows	Grass hay (50)	Calcium nitrate	2.3	-9.6	No effect	No effect
Experiment 3, this PhD thesis	Dry cows	Grass hay (50)	Calcium nitrate	2.3	-11.7	No effect	No effect
Nolan et al., 2010	Adult sheep	Oaten hay (100)	Potassium nitrate	2.5	-9.5	No effect	NA
Van Zijderveld et al., 2010	Adult sheep	Corn silage, barley straw (90)	Calcium nitrate	2.6	-12.2	NA	NA
Van Zijderveld et al., 2011	Dairy cows	Corn silage, dried alfalfa, barley straw (66)	Calcium nitrate	2.1	-7.9	No effect	No effect
Hulshof et al., 2012	Steers	Sugar cane (60)	Calcium nitrate	2.2	-12.3	NA	NA
Li et al., 2012	Lambs	NA	Calcium nitrate	2.3	-15.4	No effect	No effect
El-Zaiat et al., 2014	Lambs	Grass hay (60)	Calcium nitrate	3.4	-9.7	NA	NA
Lee et al., 2014a	Steers	Forage (55)	Calcium nitrate	2.3	-8.0	NA	No effect
de Raphélis-Soissan et al., 2014	Adult sheep	Oaten hay (100)	Calcium nitrate	2.0	-7.5	NA	NA
Lund et al., 2014	Dairy cows	Grass/clover/corn silage (58)	Calcium nitrate	2.0	-12.5	NA	NA
Veneman et al., 2014	Dairy cows	Grass/corn silage (NA)	Calcium nitrate	2.0	-6.8	NA	NA
	Dairy cows	Corn silage (NA)	Calcium nitrate	2.0	-8.2	NA	NA
AVERAGE					-10.1		

NA: Data not available

II. OVERALL EFFECT OF DIETARY STRATEGIES ON METHANE EMISSIONS AND COWS' PERFORMANCES

2.1. Additive methane-mitigating effect of strategies acting on hydrogen production and consumption: validation of our initial hypothesis

The purpose of this PhD thesis consisted in proposing new efficient dietary CH₄-mitigating strategies acting on H₂ availability for methanogens. We assumed that decreasing H₂ production AND stimulating H₂ consumption by a competitive pathway to methanogenesis, reduce CH₄ production to a higher extent than when acting on a single pathway.

To reduce methanogenesis *via* a reduction of H₂ production, we chose to test lipids from linseed and tea saponin. Indeed, our meta-analysis (Guyader et al., 2014) and previous reviews (Beauchemin et al., 2008; Doreau et al., 2011; Gerber et al., 2013a) highlighted that lipids and plant extracts would have a toxic effect towards protozoa, which are the main H₂-producers in the rumen. For lipids, we focused on PUFA from linseed (linolenic acid, C18:3), which have been reported as the most efficient PUFA to mitigate CH₄ (Doreau et al., 2011). In addition, linseed supplemented to ruminants has nutritional benefits by improving milk and meat fatty acids profiles (Chilliard et al., 2009; Scollan et al., 2001). Concerning plant extracts, we selected tea saponin, as it would be the most promising saponin source reducing CH₄ among the large family of plant extracts (Gerber et al., 2013a; Wang et al., 2012). Moreover, an *in vitro* experiment showed its positive effect on OM digestibility (Wei et al., 2012).

To reduce H₂ availability for methanogenesis, the other strategy consisted in supplementing animals with additives consuming H₂ (instead of methanogens) and without affecting protozoa. In our literature review, we reported that nitrate may act as a H₂-sink in the rumen, and recent reviews showed that all published experiments using this additive resulted in CH₄ mitigation (Doreau et al., 2014a; Lee and Beauchemin, 2014b).

The doses of linseed, tea saponin and calcium nitrate used in the present work were determined in order to reach a 15-20% CH₄ reduction when these treatments were fed individually (Doreau et al., 2011; Lee and Beauchemin, 2014a; Mao et al., 2010). Assuming an additive effect on H₂ availability in the rumen, their association (nitrate plus linseed and nitrate plus tea saponin) was expected to reduce CH₄ emissions by 30-40%.

To be adopted by farmers and consumers, a feeding strategy reducing CH₄ emissions must do so, without adverse effects on animals' digestive efficiency, performances, quality of products and health. For these reasons, the overall effect of the different selected dietary strategies was assessed by considering not only methanogenesis but also all the parameters cited above. For linseed and tea saponin, we closely monitored their effect on diet digestibility, knowing that more than 5% added fat may reduce *in vivo* total tract digestibility of diets (Martin et al., 2010; Martin et al., 2008), and that tea saponin may improve *in vitro* nutrients digestibility (Wei et al., 2012). In addition, having in mind that nitrate is a N source with a potential toxicity for animals (methemoglobinemia; Lee et al., 2014b) and human health (nitrate and nitrite accumulation in animals products), we also carefully assessed nitrate effect on N release, animals' health and the concentration of N-derivatives compounds in milk.

2.1.1. Effect of linseed fed individually to reduce hydrogen production in the rumen on methane emissions and overall cows' performances

Lipids from linseed (4% linseed oil in DM corresponding to 2.6% added fat) fed individually to non-lactating cows (n = 4 in experiment 1) did not affect intake, total tract diets digestibility and N balance. These results were in accordance with the literature, reporting that less than 4% added fat in a diet does not alter animals' intake, digestive processes and performances (Table 14). However, Martin et al. (2011) observed a reduction of DMI (-7%) without effect on milk yield of lactating cows fed grass silage supplemented with extruded linseed (3% added fat in DM).

Daily pattern of CH₄ emissions indicated that linseed acted all along the day (Figure 3, experiment 1). Its supplementation decreased daily CH₄ emissions (g/kg DMI) by 17.2% on average, corresponding to a CH₄ reduction of 6.6% per percent added fat in the diet. This result was in accordance with the majority of previous *in vivo* studies (Table 14) and with a meta-analysis reporting that 1% additional linolenic acid in the diet induces a 5.6% CH₄ reduction (Doreau et al., 2011).

The CH₄-mitigating effect of lipids is not systematic (Chung et al., 2011; Veneman et al., 2014). The extent of CH₄ decrease with lipids is proportional to the level and availability of lipids supply (Martin et al., 2010; Doreau et al., 2011), but these two factors did not explain data of Chung et al., (2011) and Veneman et al., (2014). In the trial of Veneman et al. (2014), lactating cows from New Zealand were used. We assumed that the CH₄-mitigating effect of linseed is dependent on the rumen microbiota, which is related to animals' environmental

growing and living conditions. This hypothesis was confirmed in an *in vitro* experiment, using rumen inoculum from NZ cows fed pasture, in which we reported that linseed oil used in our *in vivo* experiment (experiment 1) did not modify *in vitro* CH₄ production or rumen fermentation parameters (Muetzel et al., unpublished data). This supports the interest of current international programs such as the Global Rumen Census, which compare the diversity of microbial communities from rumen samples taken on a large diversity of ruminants throughout the world.

In conclusion, we confirmed that linseed oil supplementation to cattle (2.6% added fat in DM) is an efficient CH₄-mitigating strategy without reducing digestive efficiency in cows.

2.1.2. Effect of tea saponin fed individually to reduce hydrogen production in the rumen on methane emissions and overall cows' performances

Tea saponin (0.5% saponin in DM) failed to reduce CH₄ emissions (g/kg DMI) in the experiment with non-lactating cows (n = 4 in experiment 3) and enhanced methanogenesis in the experiment with lactating cows (n = 8 in experiment 3). These results were in contradiction with previous data on sheep or cattle supplemented with tea saponin doses ranging between 0.05% up to ~0.40% of DM (Table 15). These differences may come from a bad quality of our tea saponin product and/or an alteration of the active compound during pelleting. Plant maturity, geographical area of production and extraction methods are three parameters affecting the final concentration and quality of the saponin (Li and Powers, 2012). In our experiments, we estimated the quantity of active compound in the tea saponin extract from the origin certificate of the Chinese supplier, but assessing the activity of our extract would have been useful before starting the trials. Moreover, we included the tea saponin extract in pelleted concentrates, as handling of the powder form led to respiratory irritation problems for users and feed refusals for animals. This issue has never been highlighted previously, whereas this plant extract was distributed as a powder and mixed with the diet in other studies (Table 15). We assume that pelleting denatured the active compound of tea saponin during heating (~40°C). A modification of the miscellaneous structure of *Quillaja* saponin was already observed after heating between 20 and 60°C (Mitra and Dungan, 1997).

In the present work, diet digestibility and N balance of non-lactating cows were unchanged by tea saponin supplementation (0.5% in DM). Inversely, ADF digestibility was improved by 8% when lactating cows were fed the same dose of this plant extract. To our

knowledge, this is the first report showing a positive effect of tea saponin on *in vivo* fiber digestibility. A positive effect of tea saponin on OM digestibility was reported *in vitro* (+21%; Wei et al., 2012), whereas no effect on nutrients digestibility was reported *in vivo* with goats supplemented with lower doses than those tested in our experiments (0.04 to 0.08% of DM; Zhou et al., 2012).

Tea saponin supplemented to lactating cows tended to reduce feed intake by 12% and significantly reduced milk yield by 18% compared to control, whereas this same plant extract did not affect DMI of non-lactating cows restricted fed. We assume that the lower intake explained the lower milk production as feed efficiency was similar between cows fed control with or without tea saponin. This finding agreed with Li and Powers (2012) who reported that tea saponin (0.11% in DM) reduced DMI of growing steers by 27% leading to a drop of their average daily weight gain of 80%. However, 0.4% tea saponin did not affect feed intake and growth of lambs (Mao et al., 2010).

Overall results on the effects of tea saponin supplementation in diets of ruminants are contrasted. Additional research is necessary to give a reliable conclusion about its effect on animals' performances, diet digestibility and CH₄ emissions.

2.1.3. Effect of nitrate fed individually to modify hydrogen consumption in the rumen on methane emissions and overall cows' performances

Nitrate (2.3% of DM) fed individually to non-lactating cows (n = 4 in experiment 1; n = 4 in experiment 3) never affected intake, total tract digestibility and N balance. Previous studies on sheep or cattle also reported the absence of nitrate effect on these parameters and animals' performances (Table 16), except for Hulshof et al. (2012; -6% DMI without affecting growth performance).

Nitrate decreased CH₄ emissions to a similar extent in our two experiments, with a reduction averaging 10.7% of CH₄ yield (g/kg DMI) per percent added nitrate. This result was in accordance with the literature: on average, CH₄ emissions were reduced by 10% per percent added nitrate, whatever the animal species and the nature of the basal diet (Table 16). Then, overall results show the efficiency and repeatability of the nitrate CH₄-mitigating effect between studies. Moreover, a recent meta-analysis reported a linear dose-response effect of nitrate (0.3 to 1.2 g/kg BW/day) on enteric CH₄ emissions with a reduction of 12% of CH₄ yield (g/kg DMI) per 0.1 g added nitrate/kg BW/day (Lee and Beauchemin, 2014b).

Kinetics of CH₄ emissions measured in open chambers indicated that dietary nitrate affected methanogenesis during the 3 h postfeeding in our experimental conditions (Figure 2, experiment 1). This result agreed with previous observations on sheep and cattle (Van Zijderveld et al., 2010; Van Zijderveld et al., 2011) and suggests that nitrate acts as a H₂-sink shutting down postprandial CH₄ production which is normally at its maximum.

Overall results show that nitrate effect on CH₄ emissions is systematic and repeatable between studies, without altering digestive performances and N balance.

2.1.4. Effect of association of strategies acting on hydrogen production and consumption in the rumen on methane emissions and overall cows' performances

We assumed that supplementing ruminants with CH₄-mitigating strategies acting on both production and use of H₂ reduces methanogenesis to a larger extent than when these strategies are fed individually. To test this hypothesis, two associations of strategies were tested on non-lactating cows: linseed plus nitrate (1.0% added fat plus 2.3% nitrate in DM; n = 4 in experiment 1) and tea saponin plus nitrate (0.5% saponin plus 2.3% nitrate in DM; n = 4 in experiment 3).

As tea saponin fed individually failed to decrease methanogenesis, we assumed that the observed CH₄ reduction (g/kg DMI; -28%) with tea saponin plus nitrate was fully explained by the nitrate effect. Consequently, this association of feeding strategies did not allow us to test our hypothesis and will not be further discussed.

For the first time, we observed a positive interaction between linseed and nitrate, as their association reduced CH₄ yield (g/kg DMI) by 32%. As these dietary strategies have different mechanisms of action, we expected a 39% CH₄ reduction for a fully additive effect (-17% and -22% CH₄ reduction for linseed and nitrate fed alone, respectively). According to stoichiometry and considering that control CH₄ emissions is equal to 100, CH₄ emissions corrected for the CH₄-mitigating effect of linseed fed individually (17%) would be $100 - 100 \times 0.17 = 83$. Then, these CH₄ emissions corrected for the CH₄-mitigating effect of nitrate fed individually (22%) would be $83 - 83 \times 0.22 = 65$. In total, this corresponds to an expected CH₄ reduction of 35% with linseed plus nitrate. But, the fat content in linseed plus nitrate was lower than in linseed fed individually (-1.6 % of DM), corresponding to a CH₄ mitigation potential of 10.7%. When applying the same stoichiometry estimation than previously, we obtained an expected CH₄ reduction of 27% with linseed plus nitrate. In both cases, observed CH₄ reduction with this dietary association was close to expected, and confirmed a fully

additive effect between these two strategies. This result is original and supports our initial assumption according to which decreasing H_2 pool in the rumen by acting on both H_2 production and consumption decreases CH_4 production to a higher extent than when acting on a single pathway. In addition, we showed that linseed plus nitrate (3.5% added fat plus 1.8% nitrate in DM) fed to lactating cows ($n = 8$ in experiment 2) during 4 months induced a constant reduction of CH_4 yield (g/kg DMI; -29%). This persistent effect showed the absence of adaptation of rumen microbiota. These results also suggest that the CH_4 -mitigating effect of linseed plus nitrate is repeatable whatever the physiological stage of the cows.

Association of linseed (1.0% added fat in DM) to nitrate (2.3% of DM) did not modify N balance and total tract digestibility of non-lactating cows, confirming the effect observed when these dietary strategies were individually fed. Similarly, nitrate (1.8% of DM) plus linseed (3.5% added fat in DM) fed to lactating cows did not affect N balance, but tended to reduce ADF digestibility (-8%). This highlights the importance of studying the dose-response effect of this association on cattle digestibility.

Linseed plus nitrate supplemented to lactating cows tended to reduce *ad libitum* intake and milk production throughout our 4-month experiment. As feed efficiency (kg of milk per kg of feed) was similar between diets, we assumed that the lower intake explained the lower milk production. This is in contradiction with our results on non-lactating cows, for which we did not observe a detrimental effect of linseed plus nitrate on intake. As shown previously, in some cases, nitrate or linseed fed individually can reduce intake. Consequently, we showed that linseed plus nitrate is an efficient CH_4 -mitigating strategy without improving cows' performances. We suggest that further studies should focus on the dose-response effect of this association on animals' performances.

In conclusion, linseed plus nitrate is an efficient strategy to reduce CH_4 emissions in the long-term without altering digestive processes. However, the energetic benefits from the decreased CH_4 emissions did not appear beneficial for the dairy cows.

2.2. Rumen fermentative and microbial mechanisms involved in selected methane-mitigating strategies

To understand the mechanisms involved in the regulation of H_2 availability and CH_4 emissions, we studied the effects of the selected dietary strategies on rumen fermentation and microbiota.

2.2.1. Relationship between observed methane emissions and VFA profile

In the rumen, H_2 is mainly produced during acetate (C2) and butyrate (C4) synthesis, as two moles H_2 are generated per mole C2 or C4 produced. Inversely, the synthesis of one mole propionate (C3) or valerate (C5) consumes one mole H_2 . Then a rise of C2 and/or C4 concentrations may indicate a higher H_2 availability in the rumen, whereas a rise of C3 and/or C5 concentrations may indicate a lower H_2 availability. As a result, knowing the key role of H_2 availability in methanogenesis, the ratio C2/C3 is positively correlated with CH_4 emissions expressed as a percentage of GEI (Sauvant et al., 2011; Figure 22).

Linseed fed alone to non-lactating cows (experiment 1) reduced C2/C3 ratio compared to control, *via* an increase of C3 concentration in the rumen. This result may explain a part of the observed CH_4 -mitigating effect of linseed. To our knowledge, we are the first ones to report this effect, as most studies reported an absence of effect of linseed on rumen VFA composition (Chung et al., 2011; Doreau et al., 2009; Martin et al., 2011).

Tea saponin, fed alone to non-lactating or lactating cows (experiment 3) did not modify VFA profiles, except that it tended to increase C2/C3 ratio *via* a higher C2 concentration for lactating cows. This effect may explain why, for this particular group of cows, this plant extract led to higher CH_4 emissions compared to cows fed control treatment. Previous studies did not observe changes in VFA profiles in the rumen of sheep and goats supplemented with similar dosage of this plant extract (Mao et al., 2010; Yuan et al., 2007; Zhou et al., 2011).

Nitrate fed alone or in association with tea saponin (experiment 3) or linseed (experiments 1 and 2) to non-lactating or lactating cows increased C2/C3 ratio by increasing C2 or reducing C3 concentrations. These results confirmed previous findings reporting an increase of C2 and C4 concentrations and/or a decrease of C3 concentration in the rumen of animals fed this H_2 -sink at a similar dosage (Hulshof et al., 2012; Nolan et al., 2010; Veneman et al., 2014). We assumed that this pattern is due to a reduction of H_2 availability in the rumen because of H_2 consumption for nitrate reduction. Acetate concentration may increase to compensate for the H_2 deficiency, and C3 concentration may decrease because of the lack of H_2 (Janssen, 2010).

For a more global approach, we related the observed C2/C3 ratios with CH_4 emissions (% of GEI) for each experiment and dietary treatment of this PhD thesis, and we compared these results with the relationship of Sauvant et al. (2011; Figure 22). Data from diets without CH_4 -mitigating effect fit with the relationship, as low C2/C3 ratios were associated to low CH_4 emissions. Inversely, the highest C2/C3 ratios were observed with diets presenting the

best CH₄-mitigating effect (nitrate and/or linseed-supplemented diets). Then, the curvilinear positive relationship between CH₄ and C2/C3 ratio was not applicable in those cases.

Results on control and tea saponin-supplemented diets confirmed the positive relationship between CH₄ emissions and VFA profiles. Nevertheless, this equation may be inaccurate with CH₄-mitigating dietary treatments such as linseed and nitrate-supplemented diets. This finding suggests that, in those specific cases, others interfering fermentative and/or microbial processes need to be taken into account to estimate CH₄ emissions.

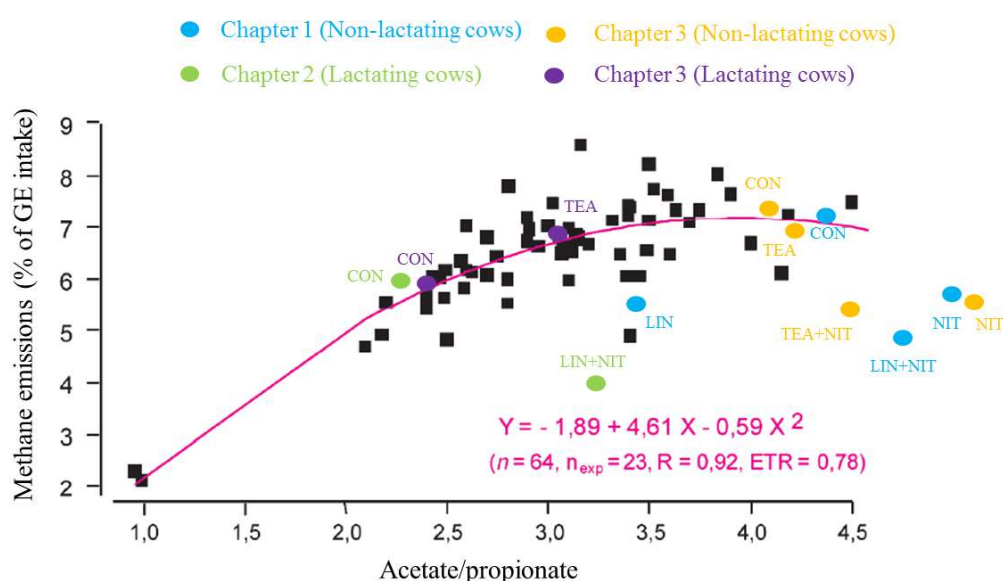


Figure 22 Relationship between C2/C3 ratio and methane emissions (adapted from Sauvant et al., 2011). Colored points indicate the position of our data obtained in the four experiments of this PhD thesis (rumen samples taken 3 to 3.5 h following the morning meal).

2.2.2. Relationship between observed methane emissions and rumen microbiota

To our knowledge, the relationship between rumen microbial biomass synthesis and CH₄ emissions has never been studied. In this work, CH₄ emissions were reduced by linseed and nitrate fed alone or in association with linseed or tea saponin, whereas excretion of purine derivatives in the urine of non-lactating cows (experiments 1 and 3), as indicator of microbial biomass synthesis in the rumen, was not affected by dietary treatments (data not shown). We concluded that there was no relationship between rumen microbial biomass synthesis and CH₄ emissions in non-lactating cows (Figure 23).

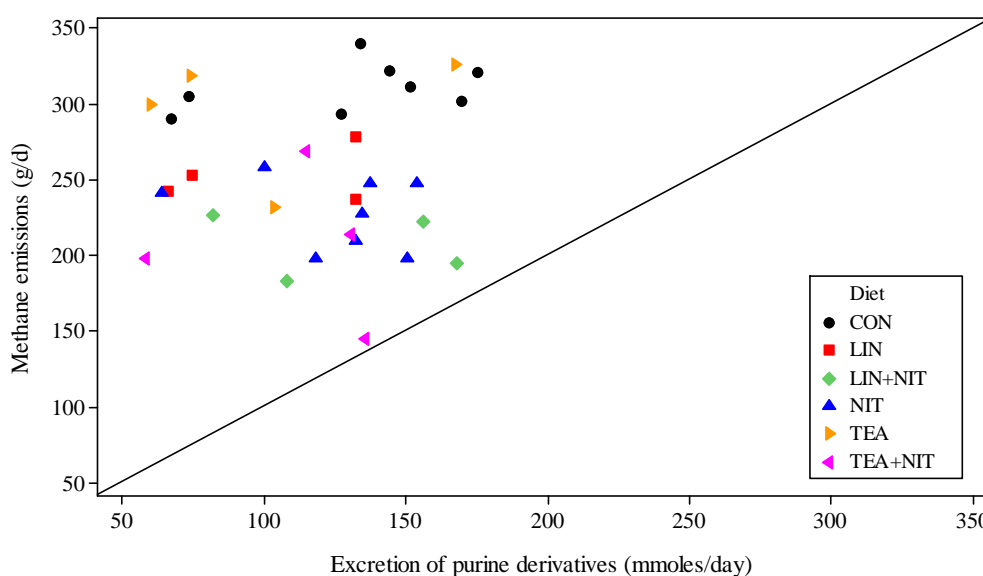


Figure 23 Relationship between methane emissions and excretion of purine derivatives in the urine of non-lactating cows fed different dietary CH₄-mitigating strategies acting on the rumen hydrogen pool (experiments 1 and 3)

When looking at specific rumen microbial populations of these same animals, we observed that total bacteria concentrations were never affected by treatments (experiments 1 and 3). Compared to control diets, linseed fed alone to non-lactating cows (experiment 1) reduced protozoa (before feeding, -53%) and methanogens (after feeding, -8%) concentrations, while reducing CH₄ emissions (g/kg DMI) by 17%. The inhibiting effect of linseed towards protozoa was not observed when associating it with nitrate. For non-lactating cows, this may be caused by the lower dose of added fat in this diet (1.0% added fat in linseed plus nitrate *versus* 2.6% added fat in linseed). For lactating cows, this may be linked to a lower representativity of rumen samples taken by stomach tubing. In addition, rumen content was sampled after feeding whereas the defaunating effect of linseed fed alone was only observed before feeding. When relating observed CH₄ emissions with rumen protozoa concentrations obtained in cows fed linseed alone, we confirmed *in vivo* the positive relationship between these two parameters that we already highlighted in our meta-analysis (Guyader et al., 2014; Figure 24).

Tea saponin did not modify methanogens concentration or activity (experiment 3). Moreover, we did not observe the expected inhibiting effect on protozoa, explaining the absence of CH₄-mitigating effect of this plant extract fed to non-lactating and lactating cows after 4 weeks of feeding saponin. These results suggest an adaptation of rumen microbiota. Indeed, in sheep, a decrease of protozoa number after 4 days of feeding saponins (*Sesbania sesban*) was reported but this population recovered 10 days later (Newbold et al., 1997).

Nitrate fed alone or in association with linseed or tea saponin did not modify protozoa concentrations whereas CH₄ emissions (g/kg DMI) were reduced from -22 to -32%. Then, those dietary treatments confirmed that protozoa concentrations is not the only factor regulating methanogenesis and that other factors may be implied. Besides, quantity and activity of methanogens were reduced in the rumen of non-lactating cows fed diets including nitrate.

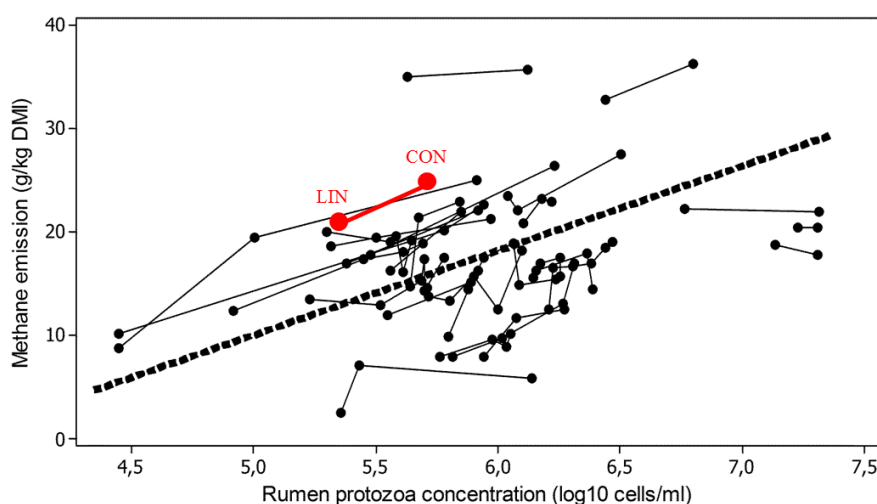


Figure 24 Position of observed rumen protozoa concentration and methane emissions from non-lactating cows fed control diet (CON) or CON supplemented with 2.6% added fat (LIN) (experiment 1, this PhD thesis) among the experiments selected to study the relationship between these two parameters by meta-analysis (adapted from Guyader et al., 2014).

These results confirm the importance of detailing rumen microbiota composition to understand the mechanisms involved in CH₄-mitigation. Such approaches should take into account the interactions between microbes and should describe the microbial populations in terms of quantity, activity and diversity.

2.2.3. Nitrate reduction and lipids biohydrogenation: stoichiometric yield of methane reduction

In the rumen, it is commonly accepted that nitrate follows Dissimilatory Nitrate Reduction to Ammonium (DNRA), which consists in the reduction of 1 mole nitrate to 1 mole nitrite which is further reduced to 1 mole ammonia. The overall process consumes 4 moles H₂. Knowing that 4 moles H₂ are also required to produce 1 mole CH₄, it is considered that one mole added nitrate reduced CH₄ production by 1 mole, assuming a full conversion of nitrate to ammonia. In this PhD thesis, nitrate reduction (2.3% in DM) fed alone or in

association with tea saponin to non-lactating cows explained 82% of observed CH₄ reductions (g/day), which is close to the reported efficiencies in the literature (88% on average; Hulshof et al., 2012; Van Zijderveld et al., 2011; Veneman et al., 2013).

Rumen biohydrogenation of 1 mole C18:1, C18:2 or C18:3 consumes 1, 2 or 3 moles H₂, respectively. Then, assuming that a full biohydrogenation of unsaturated fatty acids occurs, one mole added C18:1, C18:2 or C18:3 reduced CH₄ production by 0.25, 0.50 or 0.75 moles. In the present work, biohydrogenation of lipids from linseed (2.6% added fat) fed alone to non-lactating cows only explained 11% of observed CH₄ reductions. This result is in accordance with a previous study on dairy cows fed a corn silage-based diet supplemented with 4.2 to 5.8% added fat from linseed (10% on average; Martin et al., 2008).

Fed to non-lactating cows, nitrate reduction (2.3% in DM) plus linseed biohydrogenation (1.0% added fat in DM) explained 72% of observed CH₄ reduction. Similarly, nitrate reduction (1.8% in DM) plus linseed biohydrogenation (3.5% added fat in DM) fed to lactating cows explained 46% of observed CH₄ reduction.

We conclude that nitrate has a higher potential for H₂ consumption than PUFA. Nevertheless, this sole mechanism cannot fully explain the CH₄-mitigating effect of these dietary strategies.

2.2.4. Relationship between methane emissions and gaseous hydrogen losses

In the literature, few studies simultaneously measured *in vivo* gaseous H₂ losses and CH₄ emissions on the same animals. Nevertheless, a negative relationship would exist *in vivo* between these two parameters. Indeed, sheep fed pelleted diets presented higher H₂ emissions than sheep fed fresh perennial ryegrass (0.115 *versus* 0.019% GEI), while emitting less CH₄ (Pinares-Patiño et al., 2010). Similarly, lactating cows supplemented with nitrate presented lower CH₄ emissions and higher H₂ emissions (0.017 *versus* 0.006% GEI) than when they were fed a control diet (Van Zijderveld et al., 2011). We assumed that gaseous H₂ emissions come from an excess of dissolved H₂ concentrations in the rumen. In addition to measuring CH₄ emissions, we monitored ruminal dissolved H₂ concentrations and gaseous H₂ emissions (data not show) of non-lactating cows fed a control diet with or without linseed (LIN, 2.6% added fat), nitrate (NIT, 2.3% nitrate) or linseed plus nitrate (LIN+NIT, 1.0% added fat plus 2.3% nitrate) (experiment 1). Animals fed diets including nitrate (NIT and LIN+NIT) presented higher dissolved H₂ concentrations (33.1 *versus* 3.8 µM on average, respectively; Figure 5 in experiment 1) and gaseous H₂ emissions (4.5 L/h *versus* 0 L/h on average 1 h after

feeding, respectively) than animals fed CON and LIN. Then, similarly to gaseous H_2 , we observed a significant negative relationship between CH_4 emissions (g/kg DMI) and dissolved H_2 concentrations (μM) in the rumen of these animals (Figure 25): $CH_4 = 22.6^{***} - 0.181^{**} \times H_2$, with RMSE = 2.56 and $R^2 = 0.46$. This pattern may be explained by the toxic effect of nitrate on quantity and activity of methanogens, as reported for the first time in this work (experiments 1 and 3).

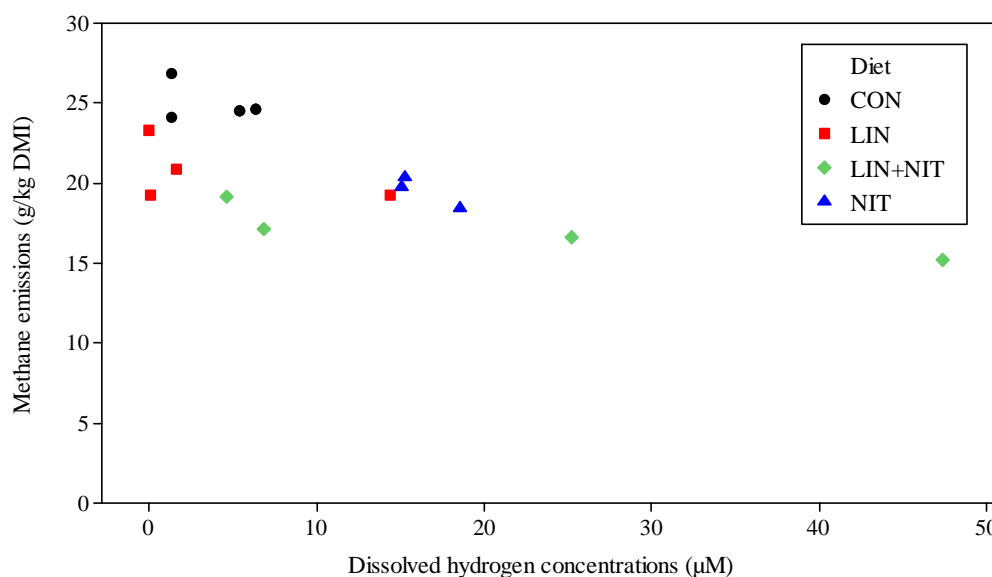


Figure 25 Relationship between methane emissions and dissolved hydrogen concentrations in the rumen of non-lactating cows fed different dietary CH_4 -mitigating strategies acting on the rumen hydrogen pool (experiment 1)

Consequently, gaseous H_2 losses can occur when feeding animals with CH_4 -mitigating strategies, but they represent small energetic losses and cannot by themselves explain observed CH_4 reductions.

2.3. Overview of the mechanisms of action of dietary strategies: estimation of hydrogen distribution between rumen fermentation end-products

To get a global view on the mechanisms of action of selected CH_4 -mitigating dietary strategies (experiments 1, 2 and 3), we calculated the production and distribution of H_2 in the different rumen fermentation end-products (Figure 26). Production of H_2 was estimated from VFA and microbial biomass synthesis, knowing that 2 moles H_2 are generated per mole C_2 or C_4 produced, and 0.58 moles H_2 are produced per kg dry microbial matter growing on AA (Mills et al., 2001). Daily productions of individual VFA and dry microbial matter were

estimated from rumen fermentable organic matter content in diets and from microbial proteins production in the rumen (Nozière et al., 2010; Sauvant and Nozière, 2013). To estimate H_2 consumption, five pathways were considered: methanogenesis (4 moles H_2 / mole CH_4), VFA synthesis (1 mole H_2 / mole C3 or C5), microbial biomass synthesis (0.41 moles H_2 / kg dry microbial matter growing on NPN; Mills et al., 2001), nitrate reduction (1 mole H_2 / mole reduced nitrate) and lipids biohydrogenation (1, 2, and 3 moles H_2 / mole saturated C18:1, C18:2 and C18:3). We assumed that the totality of nitrate intake was reduced to ammonia *via* DNRA, and that the totality of C18:1, C18:2 and C18:3 intake was saturated during biohydrogenation. Detailed methods of calculations are given in Annex 2.

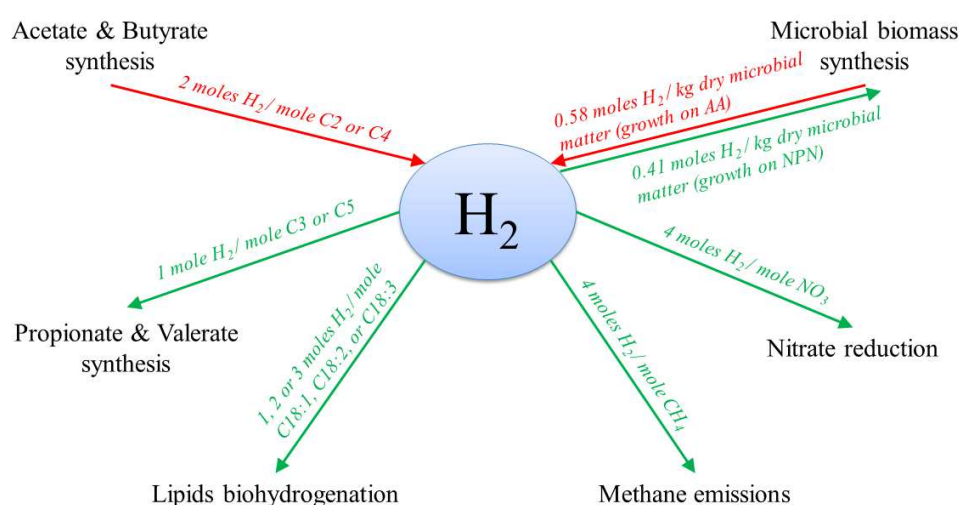


Figure 26 Selected hydrogen producing (red arrows) and consuming (green arrows) pathways for estimation of hydrogen distribution between rumen fermentation end-products

To our knowledge, this work is the first to calculate production and distribution of H_2 in rumen fermentation end-products, when CH_4 -mitigating strategies acting on ruminal H_2 availability are fed to non-lactating and lactating cows. The recovery rate of H_2 averaged 104 ± 11.2 %, which means that H_2 consuming pathways considered in our approach fully explained total H_2 produced. Among the different potential bias in each calculation step, one may come from the fact that H_2 production during dietary proteins fermentation was not considered, as this estimation would require more information on AA profile. Then, we can use these H_2 balances to summarize the mechanisms involved in the regulation of H_2 availability and CH_4 emissions by our tested CH_4 -mitigating strategies (Figure 27; detailed data are provided in Annex 2)

In control diets, methanogenesis, C3 and C5 production, and microbial biomass synthesis respectively consumed 97.8% (91.1-102.1), 14.4% (10.5-18.7), and 0.33% (0.31-

0.36) of total produced H₂. Our *in vitro* approach (experiment 4) also gave similar results with control treatment (50% hay - 50% concentrate; 95 and 16% of produced H₂ were consumed for CH₄ and VFA production, respectively). These results were close to previous data obtained by a modelling approach, which reported that these fermentation pathways consume 48-80%, 19-33% and 0.6-12% of total consumed H₂ (Czerkawski, 1986; Mills et al., 2001). Overall results agree with the low contribution of microbial biomass synthesis in H₂ consumption and regulation of H₂ availability in the rumen.

As tea saponin supplementation poorly affected CH₄ emissions and rumen fermentation pathways, we logically did not find differences in the distribution of H₂ between fermentation end-products, compared to control treatments. Inversely, as reported in the analysis of VFA profiles, linseed fed alone (experiment 1) modified H₂ distribution between rumen fermentation end-products, as 13.3% of produced H₂ was directed towards C3 and C5 synthesis *versus* 10.5% in control treatment. A small part of produced H₂ was also used for lipids biohydrogenation (1.90%), confirming the low contribution of lipids biohydrogenation in direct H₂ consumption (1 to 2.6% reported in Czerkawski, 1986). To improve rumen H₂ balance with linseed, further approach should consider the inhibiting effect of PUFA on protozoa, which induced a lower H₂ production not taken into account in applied equations.

Nitrate reduction pathway consumed on average 21% of produced H₂ in the rumen. In diets including this H₂-sink, the sum of H₂ proportions directed towards nitrate reduction and methanogenesis was almost equal to the H₂ proportion directed towards methanogenesis in control diets. This highlights the equilibrium in the distribution of H₂ between these two pathways. To get a more precise rumen H₂ balance with nitrate, gaseous H₂ losses should be taken into account. Indeed, we observed that nitrate supplementation increased dissolved H₂ concentrations in the rumen (experiment 1), probably because of a direct toxic effect towards quantity and activity of methanogens (experiments 1 and 3). We assume that excess H₂ in the rumen was released in a gaseous form. Moreover, quantities of consumed H₂ during nitrate reduction in the rumen should be adjusted to take into account that a part of nitrate may have been converted to gaseous N₂O produced *via* denitrification, as recently reported in cows (Neumeier et al., 2014; Petersen et al., 2014) and sheep (de Raphélis-Soissan et al., 2014). During denitrification, 2 moles nitrate are reduced to 1 mole nitrous oxide, while consuming 5 moles H₂.

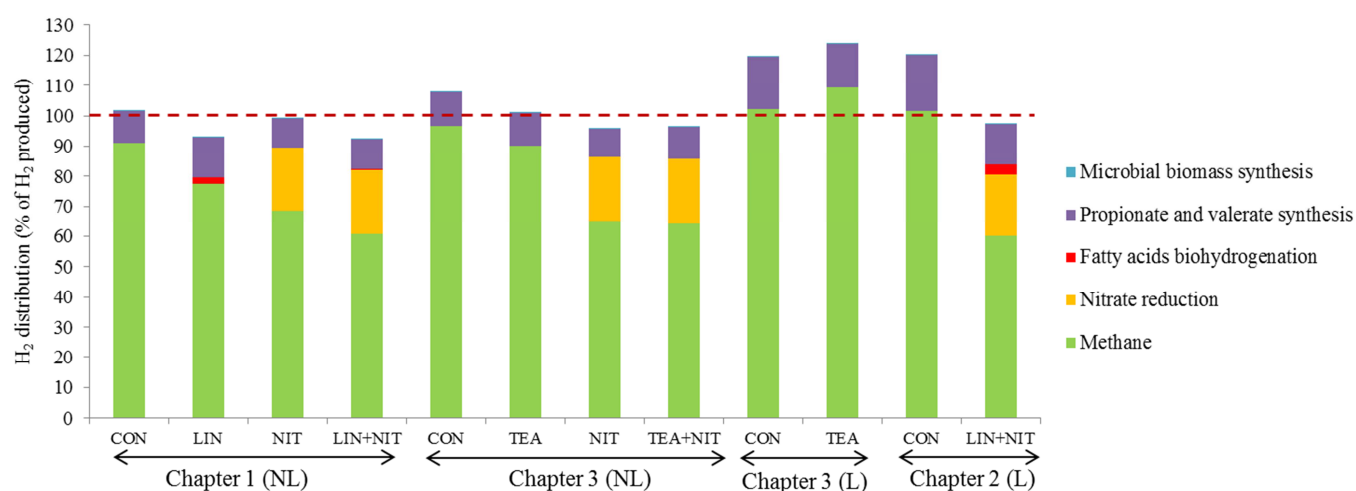


Figure 27 Estimation of hydrogen distribution (% of produced hydrogen) between rumen fermentation end-products in the experiments of this PhD thesis. Detailed figures are given in Annex 2.

In conclusion, mechanisms of selected CH₄-mitigating strategies involved modifications in rumen fermentation processes related to shifts in microbiota. This work confirms the interest of simultaneous study of fermentative and microbial parameters, in order to understand the mechanisms involved in the regulation of rumen H₂ availability.

III. PRACTICAL USE OF ASSOCIATION OF METHANE MITIGATING STRATEGIES ACTING ON HYDROGEN PRODUCTION AND CONSUMPTION: FOCUS ON LINSEED PLUS NITRATE

While testing the CH₄-mitigating effect of linseed plus nitrate, we showed that acting on both H₂ production and consumption decreased methanogenesis to a higher extent than when acting on a single pathway. However, before considering the practical use of this dietary strategy at the farm scale, several recommendations deserve to be highlighted.

3.1. Animals' health and zootechnical performances

3.1.1. Is nitrate a safe non-protein nitrogen source in substitution for urea ?

Knowing the low efficiency of N utilization in ruminants (25% on average; Calsamiglia et al., 2010), one may ask about the effect of feeding nitrate on animals' N metabolism. Indeed, potential risk of using nitrate as a NPN source substituting urea is a low

utilization by the animal resulting in additional N release in the form of nitrate, nitrite or ammonia, which would contribute to N pollution from agriculture. However, we confirmed that nitrate supplemented to non-lactating or lactating cows did not increase the quantity of N excreted in urine, feces and milk compared to cows supplemented with urea (Van Zijderveld et al., 2011). Then, to avoid excessive N losses, we recommend nitrate supplementation in substitution for urea to animals fed diets not already containing nitrate (such as nitrate-fertilized pasture) or diets deficient in degradable N (such as corn silage, sugar cane, sugar beet, molasses or cassava-based diets) (Leng, 2008).

Potential risk of nitrate poisoning of animals is one of the major limitations of its utilization in animal nutrition. Indeed, in the rumen, nitrate is converted to nitrite and then ammonia. If nitrite accumulates in the rumen, it can pass through the rumen wall into the blood and convert Hb to metHb, which cannot then transport oxygen to the tissues (Lewis, 1951). The level of blood metHb determines the symptoms severity: first symptoms are depressed feed intake, milk production and weight gain, then animals become more susceptible to infections, have more reproductive failure and present brown mucous membrane discoloration, to finish with respiratory distress, coma, cyanosis, and even death (Bruning-Fann and Kaneene, 1993).

In this work, nitrate was gradually introduced in the diet of cows (up to 2.3% in DM) during a 10 to 15-day adaptation period. During this period, we observed a gradual increase of blood metHb levels, without apparition of clinical symptoms. Following this period, blood metHb recovered low levels situated between 1.2 and 10.5% on average. We also showed the absence of nitrate poisoning during its long-term (4 months) supplementation to lactating cows. Our data were in accordance with the literature on cattle, but higher than data reported on sheep fed doses close to our experimental conditions (Table 17). These high values of metHb in our experiments are difficult to explain, but may come from a combination of several factors such as animal species, length of adaptation period, and feeding frequency. Cattle would be more susceptible to nitrate poisoning compared to sheep (Leng, 2008). In addition, within a species, some animals would have more risks of developing methemoglobinemia: erythrocytes (red blood cells) phenotype would affect activity of the enzyme responsible for metHb reduction (Godwin, 2014). Our adaptation period was shorter than in other experiments from the literature, and animals acclimatized to nitrate during a long adaptation period have lower risks of blood metHb (Lee and Beauchemin, 2014b). In the present work, a restricted feeding was always applied. However, for a same amount of nitrate in diet, a fractionated feeding throughout the day limits the risk of blood metHb *via* a slow

release of the additive in the rumen (Figure 28; Callaghan et al., 2014). With the same mechanism, *ad libitum* feeding reduces the risk of blood metHb compared to restricted feeding.

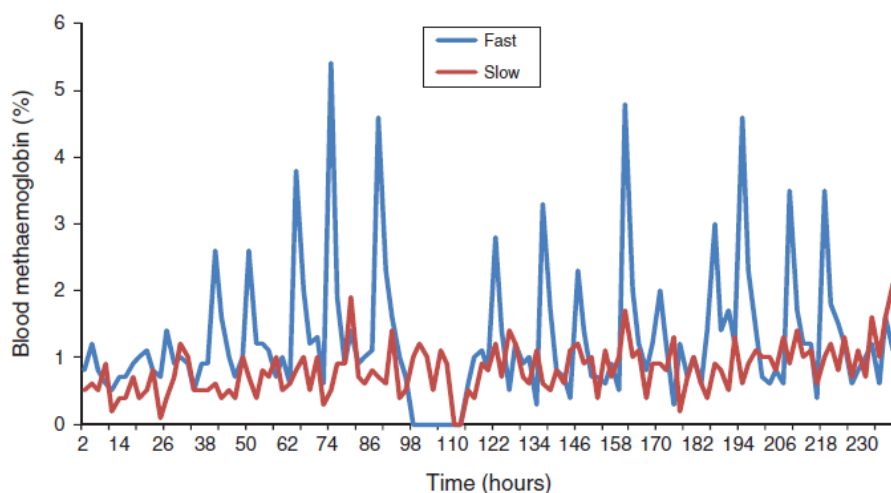


Figure 28 Effect of feeding a same amount of nitrate to steers consuming the dose within 5 min (fast) or 45 min (slow) on blood methemoglobin (from Callaghan et al., 2014)

Then, from a practical point of view, we emphasize the importance that farmers do not directly deal with nitrate utilization, to avoid its excessive and uncontrolled distribution. Solutions must be thought according to feeding frequency, in order to match rumen H_2 production from feed fermentation with nitrate concentration. For animals continuously eating small quantities of feed, solutions would consist in the use of nitrate-supplemented lick blocks. However, because of the uncontrolled and variable access between animals, the use of slow-release encapsulated nitrate may be a safer solution, and gave similar extent of CH_4 abatement without raising blood metHb levels (El-Zaiat et al., 2014). For animals eating their meals in a fractionated manner, one may consider including the nitrate in the TMR in its raw form or included in pellets as performed in this PhD thesis.

Table 17 Compiled data from the literature on the effects of nitrate supplementation to cattle or sheep on blood methemoglobin levels

Reference	Animal species	Forages (% of DM)	Voluntary intake (%)	Time before full dose (days)	Nitrate dose (% of DM)	Blood sampling (h after feeding)	Average metHb ¹ (%)	Maximum metHb ² (%)
Experiment 1, this PhD thesis	Dry cows	Grass hay (50)	90	10	2.3	3	10.5	26.3
Experiment 2, this PhD thesis	Dairy cows	Corn silage, grass hay (60)	95 ³	14	1.8	3.5	1.2	30.8
Experiment 3, this PhD thesis	Dry cows	Grass hay (50)	90	10	2.3	3	4.5	25.9
Sar et al., 2004	Adult sheep	Timothy/lucerne hay (80)	Maintenance level	7	0.7	NA	9.2	18.4
Nolan et al., 2010	Adult sheep	Oaten hay (100)	NA	18	2.5	NA	0.6	2.8
Van Zijderveld et al., 2010	Adult sheep	Corn silage, barley straw (90)	95 ³	21	2.6	3	0.5	7.0
Van Zijderveld et al., 2011	Dairy cows	Corn silage, dried alfalfa, barley straw (66)	95	21	2.1	3	3.9	19.0
Li et al., 2012	Lambs	NA	Ad libitum	7	2.3	3	0.6	1.2
de Raphélis-Soissan et al., 2014	Adult sheep	Oaten hay (100)	Maintenance level	14	2.0	2.5	14.0	45.0
El-Zaiat et al., 2014	Lambs	Grass hay (60)	Ad libitum	21	3.4	6	1.08	<1.1
Lee et al., 2014b	Steers	Forage (55)	75	20	0.7	3	<1	<1
					1.3-1.9 2.6-3.9		8.6 3.3	23.6 13.6
	Steers	Forage (55)	Ad libitum	21	2.0	NA	8.4	23.6

NA: Data not available

¹ After adaptation period² Throughout the experiment³ *Ad libitum* during adaptation period, 95% restricted during measurement weeks

3.1.2. Required research on nutrients digestibility and zootechnical performances

In this PhD thesis, linseed plus nitrate fed to non-lactating (1.0% added fat plus 2.3% nitrate in DM) or lactating (3.5% added fat plus 1.8% nitrate in DM) cows did not modify total tract digestibility of DM, OM and NDF compared to control diets. Nevertheless, this association tended to reduce ADF digestibility, intake and milk production of dairy cows, even if feed efficiency was similar between diets. Then, before using association of linseed plus nitrate as a CH₄-mitigating feeding strategy at the breeding scale, an additional dose response study is required to determine the optimal dosage for maintaining animals' performances.

To our knowledge, the impact of nitrate supplementation on reproduction performances of cows still requires further research. Indeed nitrate has been reported to lower conception rate (0.7 mg/kg BW; Davison et al., 1964) and to cause abortions in beef and dairy cattle (Sonderman and Odde, 1993). The death of the fetus would be induced by a decrease in oxygen concentration in fetal arterial blood in dams fed nitrate and by a rise of nitrate concentration in the placenta.

3.2. *Quality of animals' products and societal perception*

3.2.1. Benefits of linseed and nitrate for quality of animals' products

In addition to be an efficient CH₄-mitigating strategy, linseed plus nitrate may improve the quality of milk and meat from ruminants. Indeed, previous studies reported that linseed supplementation improves milk and meat fatty acids profiles by increasing the quantities of PUFA, which have well-known positive effects on human health (anticarcinogenic and antiatherogenic; Chilliard et al., 2009; Scollan et al., 2001). Besides, the advantages of using linseed in animal feed are largely promoted by private companies, such as in the French initiative "Bleu-Blanc-Coeur". To complete the present work, characterization of milk fatty acids profile of samples taken from dairy cows fed linseed plus nitrate is under progress.

Concerning nitrate, a potential risk of its supplementation would be the accumulation of nitrate and nitrite in animals' products for human consumption. Indeed, even if nitrite is a common food preservative, an excess of nitrite in humans diet may promote gastric inflammation (Weitzberg and Lundberg, 2013). For the first time, we reported the absence of nitrate and nitrite residues in milk and home-made milk products (yoghurts, whey, curd and 6-wk ripened Saint-Nectaire cheese) from cows fed nitrate (1.8% of DM) plus linseed (3.5% added fat in DM; experiment 2) during 4 months. This result completes the work carried out

by El-Zaiat et al. (2014), who also did not detect nitrate and nitrite residues in meat of lambs fed nitrate (3.4% of DM). Consequently, based on current knowledge, the consumption of milk and meat from animals fed linseed plus nitrate does not seem to be an issue for human health.

3.2.2. Negative perception of nitrate by consumers and farmers

In public opinion, nitrate is viewed as a chemical product used as a crop fertilizer, and is frequently associated with water pollution and health hazards. Then, despite the absence of risks in the consumption of milk and meat from cows fed linseed plus nitrate, one may expect some hesitation of consumers to buy such products. Trainings and dialogue with them may reduce their time for acceptance of this dietary strategy. From farmers point of view, knowing the severe legislation on agricultural nitrate release (EU nitrate directive 91/676/EEC), they may apprehend using this additive in animals' diets, even if it does not induce additional N losses. Moreover, as the relationship between CH₄ emissions abatement and improvement of animals' performances has never been reported, farmers' willingness to participate in the global effort of CH₄ mitigation may be only enhanced if they receive direct governmental subsidies. However, in the case that emissions taxes would be implemented, the major difficulty for governments would be the on-farm measurement of CH₄-emissions (Gerber et al., 2010). Anyway, to our knowledge, in the French and European legislations, nitrate has been authorized as a raw feed material, but not as an animal feed additive, even if several reports support its utilization as a CH₄-mitigating strategy at national (Doreau and Benoît, 2013) or international (Gerber et al., 2013a) levels.

3.3. *Environmental benefits of using linseed plus nitrate: importance of a global approach*

We reported the long-term (4 months) CH₄-mitigating effect of linseed plus nitrate, which suggests that rumen microbiota do not adapt to this dietary treatment and supports its application at the farm scale. However, to consider applicability of this dietary strategy, two other environmental criteria remain to be discussed.

3.3.1. Nitrous oxide emissions

Nitrate supplementation may induce N₂O emissions from the ruminants and/or from manure fermentation, if excessive dietary nitrate is released in urine (de Raphélis-Soissan et al., 2014; Neumeier et al., 2014; Petersen et al., 2014). Nitrous oxide is the third GHG at the

global level (8% of total GHG produced), with a GWP of 298 (IPCC, 2007). Then, we highly encourage further studies to monitor N₂O emissions to assess the global GHG mitigating efficiency of linseed plus nitrate. To our knowledge, only one study used this type of approach, and showed that the CH₄-mitigating efficiency of nitrate was lowered by 18% due to the rise in N₂O emissions from eructation or manure of sheep supplemented with this additive (de Raphélis-Soissan et al., 2014).

3.3.2. Environmental effectiveness of linseed and nitrate production

To assess applicability of wide scale supplementation of linseed plus nitrate in ruminants' nutrition, it will be important to analyze its global effect on GHG emissions at the chain level (from feed production to the farm gate) *via* a life cycle assessment (LCA). By this approach, one study already reported the effect of individual supplementation of extruded linseed (1.1% added fat in DM for summer; 2.8% added fat in DM in winter) and nitrate (1.0% in DM in summer and winter) on GHG changes at the farm scale, using a Dutch dairy farm model (Van Middelaar et al., 2014). They assume that 1% added fat or nitrate reduced enteric CH₄ emissions by 6.1 and 9.4% on average, respectively. Compared to a reference dairy farm (840 kg CO₂-equivalents/T fat and protein-corrected milk), supplementation of extruded linseed reduced emissions by 9 kg CO₂-equivalents/T fat and protein-corrected milk, whereas supplementation of nitrate reduced emissions by 32 kg CO₂-equivalents/T fat and protein-corrected milk.

With a more global approach, Doreau et al. (2014) assessed the national potential abatement of CO₂-equivalents up to year 2030 if French cattle was supplemented with either additional fat (whatever the source; 3.5% added fat in DM only for cows receiving more than 1 kg concentrate daily) or nitrate (1% in DM only for cows receiving diets short in fermentable protein). They assumed a mean abatement of enteric CH₄ emissions of 4 and 10% per percent added fat and nitrate, respectively. They resulted that, at the French scale, fat may present a higher GHG abatement potential than nitrate (1.89 M T CO₂-equivalents *versus* 0.48 M T CO₂-equivalents in 2030). Similar approaches need to be considered to assess the global environmental impact of linseed plus nitrate supplementation to cattle, but we assumed an additive positive effect between these two dietary strategies.

3.4. Economical aspect

The final aspect to assess applicability of a CH₄-mitigating strategy is the cost effectiveness (€/T CO₂-equivalents reduced) of its application, which is calculated by dividing the decrease in labor income of farm (€/year) by the decrease in GHG emissions at the chain level (kg CO₂-equivalents/year) (Van Middelaar et al., 2014). At the farm scale with a Dutch dairy farm model, nitrate (1.0% in DM in summer and winter) supplementation would be more cost-effective than extruded linseed (1.1% added fat in DM for summer; 2.8% added fat in DM in winter) supplementation (241€/T CO₂-equivalents reduced *versus* 2,594€/T CO₂-equivalents reduced; Van Middelaar et al., 2014).

At the national scale, Doreau et al. (2014) confirmed these results using the French model. They first showed that fat supplementation to ruminants is the best strategy for global abatement of GHG emissions in French agriculture, even if it is the most expensive one (Figure 29). This would be mainly due to high production costs and poor availability of raw material causing high importation costs. They also reported that nitrate (1% in DM only for cows receiving diets short in fermentable protein) supplementation is more cost-effective than fat (3.5% added fat in DM only for cows receiving more than 1 kg concentrate daily) supplementation (38€/T CO₂-equivalents reduced *versus* 267€/T CO₂-equivalents reduced). Both studies cited above highlighted a range of uncertainties in their calculations, because of variability in feed prices which has a strong impact on costs of option (Doreau et al., 2014a). From these results, we suggest that linseed plus nitrate supplementation to cattle would be an expensive CH₄-mitigating option, even if it would result in a high annual abatement of CO₂-equivalents.

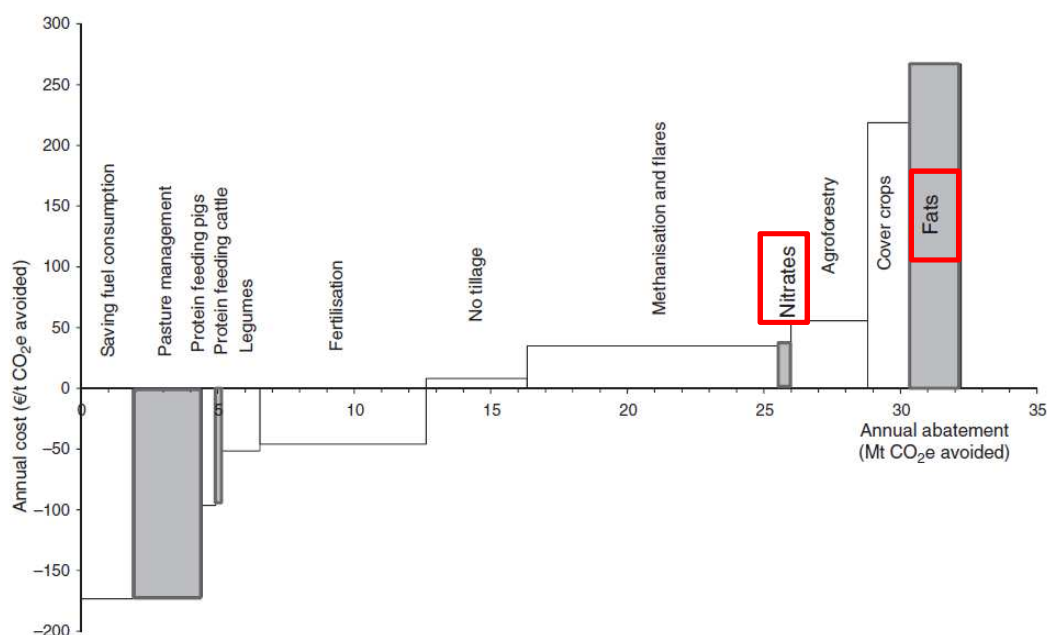


Figure 29 Place of several options for enteric methane mitigation in a global abatement cost curve for French agriculture (from Doreau et al., 2014a)

We conclude that linseed plus nitrate can be proposed as a CH₄-mitigating strategy in ruminant nutrition under controlled conditions. Linseed already has a good public image thanks to its positive effect on quality of ruminants' end-products. For the first time, we showed that the consumption of dairy products from nitrate-fed animals does not seem an issue for the human health. Further work should detail the cost-effectiveness of this strategy.

Conclusions and perspectives

This PhD thesis deepened the knowledge about the importance of the different metabolic pathways of H_2 in the rumen, in order to propose and evaluate new dietary strategies to mitigate CH_4 emissions in ruminants. We assumed that manipulating at the same time production AND utilization of H_2 in the rumen allows a more important reduction of CH_4 emissions than acting on a single pathway (production OR utilization). With the bibliographical approach, we selected dietary strategies with different modes of action on rumen H_2 metabolism: lipids from linseed or tea saponin for their potential to decrease H_2 production through their toxic effect on protozoa, and chemical components such as nitrate for their potential to consume H_2 without affecting protozoa. To test our hypothesis, these strategies were fed alone or in association to non-lactating and lactating cows. Tea saponin plus nitrate did not allow us to accept or refuse our hypothesis, as tea saponin had no effect on rumen protozoa concentrations. On the contrary, we reported a fully additive and long term CH_4 -mitigating effect of linseed plus nitrate. To complete this work, several perspectives can be drawn to improve knowledge on involved mechanisms, and to study the on-farm applicability of using association of dietary treatments acting differently on the rumen H_2 pool.

I. DEEPER CHARACTERIZATION OF THE RUMEN MICROBIOTA INVOLVED IN HYDROGEN METABOLISM AND METHANE PRODUCTION

Few studies have related in the same trial variations of CH_4 production with the characteristics of the ruminal microbial ecosystem. The originality of our approach will be to combine a quantitative approach (daily production of CH_4) to a cognitive approach (microbial parameters) of digestive processes in order to understand the observed phenomena. We already reported that linseed reduced protozoa (H_2 -producers) and methanogens (H_2 -consumers) concentrations, and that nitrate inhibited quantity and activity of methanogens without influencing genes coding for microbial nitrate or nitrite reductases. To deepen this work, we aimed at assessing the effect of the different tested CH_4 -mitigating strategies tested on non-lactating cows (experiments 1 and 3) on rumen meta-transcriptome (functional diversity, ARN) using the MiSeq technology of Illumina and by targeting together bacteria, archaea and protozoa as applied previously on DNA (Kittelmann et al., 2013; Annex 1). This approach was unfortunately unsuccessful, for unknown reasons. Work is now under progress to analyze, by the same approach, the rumen meta-genome (sequences diversity, DNA) of

bacteria, archaea and protozoa in these same samples. The integration of overall collected data will allow a better understanding of ruminal methanogenesis and associated biological phenomena.

II. STUDY OF THE EFFECT OF PELLETING PROCESS ON TEA SAPONIN

Tea saponin included in a pellet failed to reduce methanogenesis of non-lactating and lactating cows. We explained this result by the absence of effect of this plant extract on protozoa. We suspect that the plant active compound was denatured during granulation. To check this hypothesis, an *in vitro* experiment will be carried out soon. The effect of two tea saponin forms (powder *versus* pelleted) at different doses will be tested on CH₄ production and protozoa concentrations after 24 h *in vitro* incubation with rumen inoculum from cattle. If it turns out that it is the pelleting process which denatured the substance, one can consider further research to develop solutions for a better ingestion of tea saponin by animals without prior process.

III. IMPROVEMENT OF LINSEED PLUS NITRATE ACCEPTABILITY

Linseed plus nitrate persistently decreased methanogenesis. However, the energetic benefits from the decreased CH₄ emissions did not appear beneficial for the animal. On the contrary, linseed plus nitrate tended to reduce animals' digestibility and performances. Solving this issue is essential for on-farm acceptance of this dietary strategy. More studies are also required to secure the mode of distribution of nitrate, which may lead to animals' health issues when quickly ingested. Additional research on genetic selection of animals presenting lower risks of developing metHb may also be considered. At the consumer level, acceptance of linseed plus nitrate in ruminants' nutrition will be facilitated if the beneficial effect of linseed on the nutritional value of animals' products and if the absence of nitrate residues in animal products is confirmed. In this objective, systematic control of the quality of animals' products has to be considered.

IV. OPENING TO OTHER ASSOCIATION OF DIETARY STRATEGIES ACTING ON THE RUMEN HYDROGEN POOL

This PhD thesis showed that the association of dietary strategies having different mechanisms of action to reduce H_2 availability in the rumen reduced CH_4 emissions to a greater extent than when strategies were fed individually. Then, this work opens up the field of possibilities about testing other association of strategies. Linseed may be replaced by other lipids sources such as grape marc, sunflower or canola seeds, which CH_4 -mitigating effect has already been reported. Nitrate may be replaced by other additives known to modify H_2 consumption such as sulfate, nitro-ethane or nitro-oxypropanol. Electrons acceptors such as iron or manganese still require further research. In any cases, for on-farm applicability, a CH_4 -mitigating dietary strategy has to be efficient on the long term with no adverse effect on animals' health, performances and products quality for human consumption. In addition, life cycle assessment should be applied to analyze the cost and environmental effectiveness of the selected dietary strategy.

List of publications

Peer-reviewed international scientific journals:

Guyader J, Eugène M, Nozière P, Morgavi DP, Doreau M, Martin C 2014. Influence of rumen protozoa on methane emissions in ruminants: A meta-analysis approach. *Animal* 8: 1816-1825.

Guyader J, Eugène M, Meunier B, Doreau M, Morgavi DP, Silberberg M, Rochette Y, Gerard C, Loncke C, Martin C. Additive effect between dietary linseed oil and nitrate as methane emission-reducer in cattle. *Journal of Animal Science* (Accepted, in revision).

Guyader J, Doreau M, Morgavi DP, Gerard C, Loncke C, Martin C. Long-term methane mitigating effect of linseed plus nitrate supplemented to dairy cows. *Journal of Dairy Science* (Accepted, in revision).

Guyader J, Eugène M, Doreau M, Morgavi DP, Gerard C, Loncke C, Martin C. Absence of methane mitigating effect of tea saponin fed to non-lactating and lactating cows. (In progress).

Guyader J, Silberberg M, Popova M, Morgavi DP, Seradj AR, Gerard C, Loncke C, Martin C. Dietary nitrate inhibits rumen methanogenic archaea without influencing genes coding for microbial nitrate or nitrite reductases. (In progress).

Guyader J, Tavendale M, Martin C, Muetzel S. Dose response effect of nitrate on hydrogen distribution between rumen fermentation end-products: an in vitro approach. *Animal Feed Science and Technology* (In preparation).

Scientific communications in international congress:

Guyader J, Eugene M, Doreau M, Rochette Y, Morgavi DP, Martin C 2014. Association of nitrate and linseed oil effectively reduces methane emission in ruminants. Proc. Aust. Soc. Anim. Prod. 30, 87. ISNH-ISRP, 8-12 September 2014, Canberra, Australia (poster)

Guyader J, Silberberg M, Morgavi DP, Martin C 2014. Postprandial kinetics of dissolved hydrogen in the rumen of cows fed nitrate and/or linseed oil. Proc. Aust. Soc. Anim. Prod. 30, 86. ISNH-ISRP, 8-12 September 2014, Canberra, Australia (poster)

Guyader J, Eugène M, Nozière P, Morgavi DP, Doreau M, Martin C. Influence of rumen protozoa on methane emissions in ruminants: a meta-analysis approach. Greenhouse Gases and Animal Agriculture, 24-26 June 2013, Dublin, Ireland (oral communication)

Scientific communications in national congress:

Guyader J. Manipulation du pool d'hydrogène disponible dans le rumen pour réduire la production de méthane chez le bovin. Conseil Scientifique Unité Mixte de Recherche sur les Herbivores de l'INRA Theix, 1st June 2012 (oral communication)

Guyader J, Eugène M, Nozière P, Morgavi DP, Doreau M, Martin C. Influence des protozoaires du rumen sur les émissions de méthane par les ruminants: Approche par méta-analyse. Conseil Scientifique Unité Mixte de Recherche sur les Herbivores de l'INRA Theix, 5 february 2013 (oral communication)

Guyader J, Eugène M, Nozière P, Morgavi DP, Doreau M, Martin C. Influence des protozoaires du rumen sur les émissions de méthane par les ruminants: Approche par méta-analyse. Journées de l'Ecole Doctorale SVSAE, Université Blaise Pascal, Clermont-Ferrand, 13 june 2014 (oral communication)

Annex

I. ANNEX 1 - ANALYSIS OF RUMEN MICROBIOTA DIVERSITY BY HIGH THROUGHPUT SEQUENCING METHODS

Total nucleic acids (DNA and RNA) were co-extracted from rumen samples taken from non-lactating cows (experiments 1 and 3) preserved with *RNAlater*® and stored at -80°C (Popova et al., 2011). RNA reverse-transcribed to cDNA was used to describe the rumen functional diversity by targeting the cDNA copies of 16S (bacteria, archaea) or 18S (protozoa) rRNA.

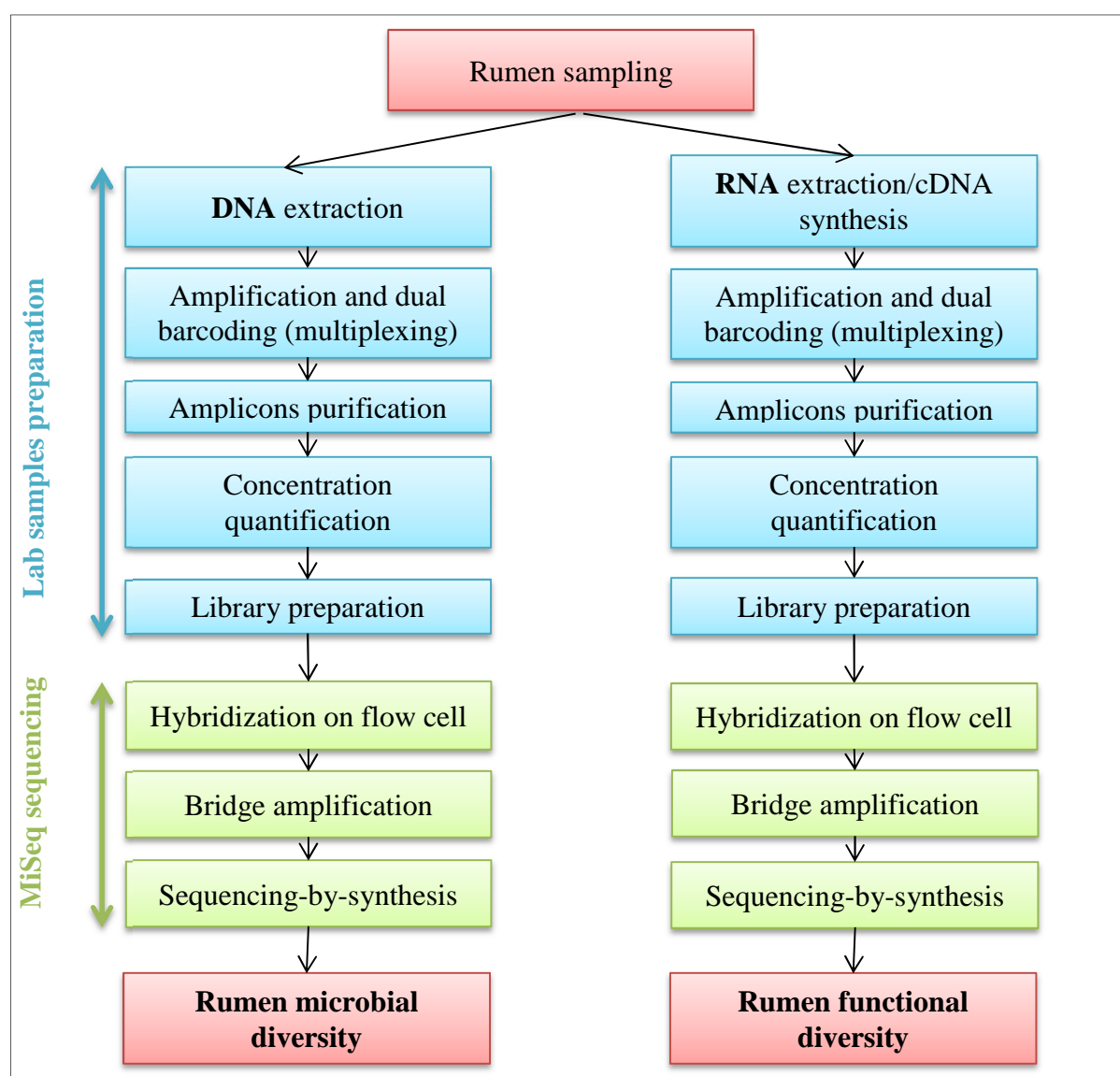


Figure 1 Framework of samples preparation and analysis of rumen microbiota diversity with MiSeq technology (Illumina)

1.1. Samples preparation (Figure 1)

Separate PCR were run in duplicate for each target species (bacteria, archaea and protozoa) and using for each sample: 5 µL PCR Buffer (10X), 6 µL MgCl₂ (25 mM), 1 µL dNTPs, 2.5 µL forward and 2.5 µL reverse primers (10 pM), 0.25 µL HotStar Taq DNA polymerase Taq, 1 µL cDNA template and 31.75 µL water molecular biology grade. Each forward and reverse primers contained (Figure 2): i) an Illumina adaptor (5'- AAT GAT ACG GCG ACC ACC GAG ATC TAC AC-3' and 5'- CAA GCA GAA GAC GGC ATA CGA GAT-3', respectively) common to the three target species, ii) a unique 8-base barcode for multiplexed sample identification (Kozich et al., 2013), iii) a 10-base pad common to the three target species for limiting primer dimmers (5'-TAT GGT AATT-3' and 5'-AGT CAG TCAG-3', respectively), and iv) the group-specific primer (Table 1) with a 2-base linker specific for each target species. The pad sequence was selected so that the combined pad, linker, and gene-specific primer would have a melting temperature over 60°C. Amplification program consisted of one denaturation step (95°C, 15 min), 30 cycles of denaturation (95°C, 20 sec), touchdown annealing (65°C to 55°C, 30 sec) and elongation (72°C, 5 min), and one final elongation step (72°C, 10 min). Theoretical lengths of amplicons were ~364, 309 and 355 base pairs (bp) for bacteria, archaea and protozoa, respectively. The duplicate PCR products were pooled to obtain a final volume of 100 µL.



Figure 2 Dual barcoded primers used for multiplexed sequencing with MiSeq technology. Forward and reverse barcodes combination is different for each sample and target species. Linker and primers are similar among samples but different between target species.

Table 1 Primers used for analysis of diversity of rumen microbiota by MiSeq technology

Organism-Target region (Reference)	Primer set	(Linker)-Primer sequences 5'-3'
Bacteria-16S (Klindworth et al., 2013)	S-DBact-0564-a-S-15 S-DBact-0785-b-A-18	(GT)-AYTGGGYDTAAAGNG (CC)-TACNVGGGTATCTAATCC
Archaea-16S (Klindworth et al., 2013)	S-DArch-0349-a-S-17 S-DArch-0519-a-A-16	(CT)-GYGCASCAGKCGMGA AW (CC)-TTACCGCGGCKGCTG
Protozoa-18S (Sylvester et al., 2004)	Syl316-F Syl539-R	(GC)-GTCTTCGWTGGTAGTGTATT (CT)-CTTGCCCTCYAATCGTWCT

Amplicons were purified and concentrated to a final 30 μL volume using the QIAquick PCR purification kit (Qiagen, Valencia, CA, USA). They were then loaded onto a 2% agarose gel into three separate pools ($3 \times 10 \mu\text{L}$). Bands were visualized, excised under ultraviolet radiation, and gel purified with the GENECLAN Turbo kit (Qiagen) according to manufacturer instruction. Concentration of gel-purified amplicons loaded onto a 2% agarose gel was estimated using a low DNA mass Ladder (Invitrogen Corporation, Carlsbad, CA, USA), and an imaging system Chemimager (Alpha Innotech, San Leandro, CA, USA).

1.2. Construction of the library and sequencing steps (Figure 1)

The final library was constructed by pooling samples with a mixing ratio of 8:1:1 for bacteria, protozoa and archaea, respectively (Kittelman et al., 2013). The library was loaded for one Nanorun on a MiSeq sequencer (Illumina). Analysis steps consisted in (Illumina, 2010):

- 1/ Hybridization on flow cell: Double stranded DNA or cDNA were denaturated and single stranded fragments attached to the inside surface of a flow cell.
- 2/ Bridge amplification: This pre-sequencing amplification step allowed creation of millions of single stranded copies from template DNA or cDNA.
- 3/ Sequencing-by-synthesis: Each sequencing cycle consisted in i) addition of the four different labeled nucleotides and a DNA polymerase; ii) ligation of the labeled nucleotides to the first base of a single stranded fragment thanks to the enzyme; iii) laser excitation, lecture of the fluorescence emitted for each ligated nucleotide, identification of the first base of the fragment, knowing that fluorescence was different between nucleotides; iv) washout of non-used nucleotides. Each cycle added one nucleotide to each single stranded fragment. Sequencing cycles were 250-times repeated to get a minimum of 500 000 single stranded sequences (reads). The minimum number of single reads per sample was then calculated as the ratio between the minimum number of single reads generated during the run, out of the number of samples in the original library.

This approach was recently developed in the laboratory. In this experiment, we faced some difficulties during library preparation and sequencing. Though several optimizations (new design of sequencing primer, PCR optimization) were made, we never obtained good sequencing yield. This approach gave good results with DNA libraries from other projects, which suggest that the reverse transcription of RNA to cDNA may be a step introducing biases in further sample manipulation. Currently, work is under progress to assess, by an

automated method (fluidigm amplification followed by MiSeq sequencing) the diversity of bacteria, archaea and protozoa in the same rumen samples, by targeting genes coding for 16S and 18S rRNA from genomic DNA.

1.3. Advantages and disadvantages of the technique (Di Bella et al., 2013; Kozich et al., 2013)

In terms of samples preparation, multiplex sequencing is a cost-effective method, which allowed simultaneous processing of a large number of samples in a single run. Concerning sequencing, until recently, the Roche 454-sequencing technique was widely applied to assess rumen microbiota diversity. This expensive technique provides a small number of long reads (until 700 bp) allowing a high precision for species identification. Inversely, the MiSeq technology as used in this thesis gives the largest number of sequences per euro, which allowed covering a larger diversity of microbiota. However, compared to 454-sequencing, species identification is less precise, due to the shorter reads length.

II. ANNEX 2 - CALCULATIONS USED TO ESTIMATE *IN VIVO* RUMINAL HYDROGEN PRODUCTION AND CONSUMPTION

2.1. Hydrogen consumption during methane production

Quantities of H₂ (moles/day) consumed in the rumen for methanogenesis (H_{2utilCH₄}) were estimated knowing that 4 moles H₂ are required to produce 1 mole CH₄:

$$H_{2utilCH_4} = (m_{CH_4}/M_{CH_4}) \times 4$$

Where m_{CH₄}: daily CH₄ production (g/day); M_{CH₄} = molecular weight of CH₄ (16 g/mol).

2.2. Hydrogen production and consumption during VFA synthesis

Quantities of H₂ produced and consumed during VFA synthesis were calculated from observed rumen VFA profile and total VFA production estimated from the rumen fermentable organic matter content in diets (Nozière et al., 2010).

2.2.1. Calculation of rumen fermentable organic matter in diets

Rumen fermentable organic matter (MOF, g/kg DM) was calculated according to the equation 42 in Sauvant and Nozière, 2013:

$$\text{MOF} = \text{MOD} - (\text{PDIA} + \text{AMDint} + \text{NDFDint} + \text{AGDint} + \text{PF})$$

Where MOD: digestible organic matter in diet (g/kg DM); PDIA: protein digestible in the intestine (g/kg DM); AMDint: starch digestible in the intestine (g/kg DM); NDFDint: NDF digestible in the intestine (g/kg DM); AGDint: fatty acid digestible in the intestine (g/kg DM); PF: products from silage fermentation. MOD was calculated by multiplying the organic matter content of the diets (OM, g/kg DM) by *in vivo* measurement of total tract organic matter digestibility (dOM, %). PDIA was estimated from diets composition and from the levels of PDIA in the individual ingredients given by INRA tables (INRA, 2010). AMDint was estimated from the level of starch reaching the duodenum (equation 31; Sauvant and Nozière, 2013), which was estimated by subtracting the theoretical amount of degraded starch in the rumen (equation 13; Sauvant and Nozière, 2013) to the total starch content of the diets. NDFDint was estimated from dOM (equations 33, 34 and 35; Sauvant and Nozière, 2013). AGDint was estimated from the fatty acid content of the diets (equations 36 and 37; Sauvant and Nozière, 2013). PF was estimated from INRA tables (INRA, 2010) and from the percentage of silage in diets.

2.2.2. Calculation of total and individual VFA production

Total VFA produced ($\text{tVFA}_{\text{prod}}$, moles/day) were calculated according to Nozière et al., 2010:

$$\text{tVFA}_{\text{prod}} = [(8.36 - 1.1 \times (\text{PCO} - 0.43)) \times \text{MOF}/1000] \times \text{DMI}$$

Where PCO: percentage of concentrate in the diets; DMI: daily DM intake (kg/day). From $\text{tVFA}_{\text{prod}}$, individual VFA productions (Cx_{prod} , moles/day) were calculated with the observed *in vivo* VFA profile in the rumen:

$$\text{C2}_{\text{prod}} = \text{tVFA}_{\text{prod}} \times \text{C2 proportion in the rumen}$$

$$\text{C3}_{\text{prod}} = \text{tVFA}_{\text{prod}} \times \text{C3 proportion in the rumen}$$

$$\text{C4}_{\text{prod}} = \text{tVFA}_{\text{prod}} \times \text{C4 proportion in the rumen}$$

$$\text{C5}_{\text{prod}} = \text{tVFA}_{\text{prod}} \times \text{C5 proportion in the rumen}$$

2.2.3. Calculation of hydrogen production and consumption during VFA synthesis

The amount of H₂ produced during VFA synthesis (H₂_{prodVFA}, moles/day) was finally calculated knowing that 2 moles H₂ are generated per mole C₂ or C₄ produced:

$$H_{2\text{prodVFA}} = 2 \times C_{2\text{prod}} + 2 \times C_{4\text{prod}}$$

The quantities of H₂ consumed during VFA synthesis (H₂_{utilVFA}, moles/day) were calculated knowing that 1 mole H₂ is required to produce 1 mole C₃ or C₅:

$$H_{2\text{utilVFA}} = 1 \times C_{3\text{prod}} + 1 \times C_{5\text{prod}}$$

2.3. *Hydrogen production and consumption during microbial biomass synthesis*

Microbes growing on amino acids would produce 0.58 moles H₂ per kg dry microbial matter whereas microbes growing on NPN would consume 0.41 moles H₂ per kg dry microbial matter (Mills et al., 2001). Then, to calculate the amount of H₂ produced and consumed by microbes in our experiments, we first estimated the production of dry microbial matter from calculated microbial proteins production in the rumen (MAMIC, kg/day; equation 47, Sauvant and Nozière, 2013):

$$MAMIC = (40.7 + 75.6 \times 10^{-3} \times MOF + 8.07 \times PCO) \times DMI$$

Where MOF, PCO and DMI were as previously defined.

The production of microbial organic matter (MOM, kg/day) was then calculated knowing that the factor of conversion between microbial protein and nitrogen content is 6.25, and that 100 g MOM is made of ~9 g N (lab database):

$$MOM = (MAMIC/6.25) \times (100/9)$$

The production of microbial dry matter (MSM, kg/day) was finally estimated knowing that 100 g microbial dry matter would be made of 87.1 g microbial organic matter (Dijkstra et al., 1992):

$$MSM = ((MOM \times 100)/87.1)/1000$$

We estimated that 70% of N supplied in diets of our experiments came from amino acids, the rest coming from NPN. Then, production of H₂ from microbes growing on amino acids (H₂_{prodMIC}, moles/day) was estimated as follow:

$$H_{2\text{prodMIC}} = MSM \times 0.58 \times 0.70$$

Quantities of H₂ consumed by microbes growing on NPN (H₂_{utilMIC}, moles/day) were calculated as follow:

$$H_{2\text{utilMIC}} = MSM \times 0.41 \times 0.30$$

2.4. *Hydrogen consumption during nitrate reduction and lipids biohydrogenation*

Quantities of H₂ consumed during nitrate reduction (H_{2utilNO3}, moles/day) or lipids biohydrogenation (H_{2utilFA}, moles/day) were estimated for diets including nitrate or lipids, knowing that the reduction of one mole nitrate to one mole ammonia requires 4 moles H₂ and that biohydrogenation of 1 mole C18:1, C18:2 or C18:3 requires 1, 2 or 3 moles H₂:

$$H_{2utilNO3} = (m_{NO3}/M_{NO3}) \times 4$$

$$H_{2utilFA} = (m_{C18:1}/M_{C18:1}) + 2 \times (m_{C18:2}/M_{C18:2}) + 3 \times (m_{C18:3}/M_{C18:3})$$

Where m_{NO3} : added nitrate (g/day); M_{NO3} : molecular weight of nitrate (62 g/mol); $m_{C18:1}$: added C18:1 (g/day); $M_{C18:1}$: molecular weight of C18:1 (282.5 g/mol); $m_{C18:2}$: added C18:2 (g/day); $M_{C18:2}$: molecular weight of C18:2 (280.5 g/mol); $m_{C18:3}$: added C18:3 (g/day); $M_{C18:3}$: molecular weight of C18:3 (278.5 g/mol).

2.5. *Estimated quantities of produced and consumed hydrogen in the three in vivo experiments of this thesis*

Details of estimated quantities of produced and consumed H₂ in the three *in vivo* experiments of this thesis are presented in Table 2.

Table 2 Estimated quantities of produced and consumed hydrogen in the four *in vivo* experiments of this thesis testing methane-mitigating strategies having different effects on the rumen hydrogen pool

Experiment	Production of H ₂ (moles/day)			Consumption of H ₂ (moles/day)					Total
	From C2 and C4 synthesis	From microbes synthesis	Total	For CH ₄ synthesis	For C3 and C5 synthesis	For microbes synthesis	For nitrate reduction	For lipids biohydrogenation	
Experiment 1 (N.L. cows)									
CON	83.9	0.85	84.7	77.2	8.8	0.26	0.0	0.00	86.3
LIN	80.6	0.86	81.5	63.2	10.8	0.26	0.0	1.55	75.8
NIT	85.9	0.85	86.8	59.5	8.3	0.26	18.3	0.00	86.3
LIN+NIT	84.3	0.85	85.2	51.7	8.5	0.26	18.3	0.14	78.8
Experiment 2 (L. cows)									
CON	113.2	1.35	114.6	116.2	21.4	0.41	0.0	0.00	138.0
LIN+NIT	103.0	1.15	104.2	62.7	13.9	0.35	21.0	3.85	101.7
Experiment 3 (N.L. cows)									
CON	80.0	0.84	80.9	78.1	9.1	0.25	0.0	0.00	87.4
TEA	80.9	0.83	81.7	73.5	8.9	0.25	0.0	0.00	82.6
NIT	83.3	0.83	84.1	54.8	7.9	0.25	17.8	0.00	80.7
TEA+NIT	79.8	0.81	80.6	51.6	8.6	0.25	17.5	0.00	77.9
Experiment 3 (L. cows)									
CON	105.3	1.27	106.6	108.8	18.6	0.39	0.0	0.00	127.7
TEA	99.9	1.14	101.1	110.6	14.5	0.34	0.0	0.00	125.4

N.L. cows: non-lactating cows; L. cows: lactating cows.

References

- Abdouli, H., and D. M. Schaefer. 1986. Effects of two dietary niacin concentrations on ruminal fluid free niacin concentration, and of supplemental niacin and source of inoculum on in vitro microbial growth, fermentative activity and nicotinamide adenine dinucleotide pool size. *J. Anim. Sci.* 62: 254-262.
- Abecia, L., P. G. Toral, A. I. Martín-García, G. Martínez, N. W. Tomkins, E. Molina-Alcaide, C. J. Newbold, and D. R. Yanez-Ruiz. 2012. Effect of bromochloromethane on methane emission, rumen fermentation pattern, milk yield, and fatty acid profile in lactating dairy goats. *J. Dairy Sci.* 95: 2027-2036.
- Akhmanova, A., F. Voncken, T. van Alen, A. van Hoek, B. Boxma, G. Vogels, M. Veenhuis, and J. H. P. Hackstein. 1998. A hydrogenosome with a genome. *Nature* 396: 527-528.
- Alemu, A. W., J. Dijkstra, A. Bannink, J. France, and E. Kebreab. 2011. Rumen stoichiometric models and their contribution and challenges in predicting enteric methane production. *Anim. Feed Sci. Technol.* 166-167: 761-778.
- Anderson, R. C., G. E. Carstens, R. K. Miller, T. R. Callaway, C. L. Schultz, T. S. Edrington, R. B. Harvey, and D. J. Nisbet. 2006. Effect of oral nitroethane and 2-nitropropanol administration on methane-producing activity and volatile fatty acid production in the ovine rumen. *Bioresour. Technol.* 97: 2421-2426.
- Animut, G., R. Puchala, A. L. Goetsch, A. K. Patra, T. Sahl, V. H. Varel, and J. Wells. 2008. Methane emission by goats consuming different sources of condensed tannins. *Anim. Feed Sci. Technol.* 144: 228-241.
- Appuhamy, J. A., A. B. Strathe, S. Jayasundara, C. Wagner-Riddle, J. Dijkstra, J. France, and E. Kebreab. 2013. Anti-methanogenic effects of monensin in dairy and beef cattle: a meta-analysis. *J. Dairy Sci.* 96: 5161-5173.
- Barry, T. N., A. Thompson, and D. G. Armstrong. 1977. Rumen fermentation studies on two contrasting diets. 1. Some characteristics of the in vivo fermentation, with special reference to the composition of the gas phase, oxidation/reduction state and volatile fatty acid proportions. *J. Agric. Sci.* 89: 183-195.
- Barz, M., C. Beimgraben, T. Staller, F. Germer, F. Opitz, C. Marquardt, C. Schwarz, K. Gutekunst, K. H. Vanselow, R. Schmitz, J. LaRoche, R. Schulz, and J. Appel. 2010. Distribution analysis of hydrogenases in surface waters of marine and freshwater environments. *PLoS ONE* 5: 1-17.
- Bayaru, E., S. Kanda, T. Kamada, H. Itabashi, S. Andoh, T. Nishida, M. Ishida, T. Itoh, K. Nagara, and Y. Isobe. 2001. Effect of fumaric acid on methane production, rumen fermentation and digestibility of cattle fed roughage alone. *Anim. Sci. J.* 72: 139-146.
- Beauchemin, K. A., M. Kreuzer, F. O'Mara, and T. A. McAllister. 2008. Nutritional management for enteric methane abatement: a review. *Anim. Prod. Sci.* 48: 21-27.
- Beauchemin, K. A., and S. M. McGinn. 2006. Methane emissions from beef cattle: Effects of fumaric acid, essential oil, and canola oil. *J. Anim. Sci.* 84: 1489-1496.
- Beauchemin, K. A., S. M. McGinn, C. Benchaar, and L. Holtshausen. 2009. Crushed sunflower, flax, or canola seeds in lactating dairy cow diets: Effects on methane production, rumen fermentation, and milk production. *J. Dairy Sci.* 92: 2118-2127.
- Beinert, H., R. H. Holm, and E. Münck. 1997. Iron-Sulfur clusters: Nature's modular, multipurpose structures. *Science* 277: 653-659.
- Benchaar, C., and H. Greathead. 2011. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. *Anim. Feed Sci. Technol.* 166-167: 338-355.
- Benchaar, C., J. Rivest, C. Pomar, and J. Chiquette. 1998. Prediction of methane production from dairy cows using existing mechanistic models and regression equations. *J. Anim. Sci.* 76: 617-627.
- Blaxter, K. L., and J. L. Clapperton. 1965. Prediction of the amount of methane produced by ruminants. *Br. J. Nutr.* 19: 511-522.
- Boone, D. R., R. L. Johnson, and Y. Liu. 1989. Diffusion of the interspecies electron carriers H₂ and formate in methanogenic ecosystems and its Implications in the measurement of K_m for H₂ or formate uptake. *Appl. Environ. Microbiol.* 55: 1735-1741.
- Boxma, B., G. Ricard, A. HAM van Hoek, E. Severing, S. Y. Moon-van der Staay, G. WM van der Staay, T. A. van Alen, R. M. de Graaf, G. Cremers, M. Kwantes, N. R. McEwan, C. J. Newbold, J. P. Jouany, T. Michalowski, P. Pristas, M. A. Huynen, and J. H. P. Hackstein. 2007. The [FeFe] hydrogenase of *Nyctotherus ovalis* has a chimeric origin. *BMC Evol. Biol.* 7.
- Broderick, J. B. 2001. Coenzymes and cofactors. *Encyclopedia of life sciences*, Nature Publishing Group.
- Brown, E. G., R. C. Anderson, G. E. Carstens, H. Gutierrez-Bañuelos, J. L. McReynolds, L. J. Slay, T. R. Callaway, and D. J. Nisbet. 2011. Effects of oral nitroethane administration on enteric methane emissions and ruminal fermentation in cattle. *Anim. Feed Sci. Technol.* 166-167: 275-281.
- Bruning-Fann, C. S., and J. B. Kaneene. 1993. The effects of nitrate, nitrite, and N-nitroso compounds on animal health. *Vet. Hum. Toxicol.* 35: 237-253.
- Callaghan, M. J., N. W. Tomkins, I. Benu, and A. J. Parker. 2014. How feasible is it to replace urea with nitrates to mitigate greenhouse gas emissions from extensively managed beef cattle ? *Anim. Prod. Sci.* 54: 1300-1304.

- Calsamiglia, S., M. Busquet, P. W. Cardozo, L. Castillejos, and A. Ferret. 2007. Invited review: essential oils as modifiers of rumen microbial fermentation. *J. Dairy Sci.* 90: 2580-2595.
- Calsamiglia, S., A. Ferret, C. K. Reynolds, N. B. Kristensen, and A. M. van Vuuren. 2010. Strategies for optimizing nitrogen use by ruminants. *Animal* 4: 1184-1196.
- Chilliard, Y., C. Martin, J. Rouel, and M. Doreau. 2009. Milk fatty acids in dairy cows fed whole crude linseed, extruded linseed, or linseed oil, and their relationship with methane output. *J. Dairy Sci.* 92: 5199-5211.
- Chung, Y. H., M. L. He, S. M. McGinn, T. A. McAllister, and K. A. Beauchemin. 2011. Linseed suppresses enteric methane emissions from cattle fed barley silage, but not from those fed grass hay. *Anim. Feed Sci. Technol.* 166-167: 321-329.
- Czerkawski, J. W. 1986. An introduction to rumen studies. Pergamon Press, Oxfordshire and New York.
- Czerkawski, J. W., and G. Breckenridge. 1971. Determination of concentration of hydrogen and some other gases dissolved in biological fluids. *Lab Pract* 20: 403-405.
- Czerkawski, J. W., C. G. Harfoot, and G. Breckenridge. 1972. The relationship between methane production and concentrations of hydrogen in the aqueous and gaseous phases during rumen fermentation in vitro. *J Appl Bacteriol* 35: 537-551.
- Davison, K. L., W. M. Hansel, L. Krook, K. McEntee, and M. J. Wright. 1964. Nitrate toxicity in dairy heifers I. Effects on reproduction, growth, lactation, and vitamin A nutrition. *J. Dairy Sci.* 47: 1065-1073.
- de Raphélis-Soissan, V., L. Li, I. R. Godwin, M. C. Barnett, H. B. Perdok, and R. S. Hegarty. 2014. Use of nitrate and *Propionibacterium acidipropionici* to reduce methane emissions and increase wool growth of Merino sheep. *Anim. Prod. Sci.* 54: 1860-1866.
- Derwent, R. G., P. G. Simmonds, S. O'Doherty, A. Manning, W. Collins, and D. Stevenson. 2006. Global environmental impacts of the hydrogen economy. *International Journal of Nuclear Hydrogen Production and Applications* 1: 57-67.
- Di Bella, J. M., Y. Bao, G. B. Gloor, J. P. Burton, and G. Reid. 2013. High throughput sequencing methods and analysis for microbiome research. *J. Microbiol. Methods* 95: 401-414.
- Dijkstra, J., H. D. Neal, D. E. Beever, and J. France. 1992. Simulation of nutrient digestion, absorption and outflow in the rumen: model description. *The Journal of Nutrition* 122: 2239-2256.
- Dolla, A., F. Guerlesquin, M. Bruschi, and R. Haser. 1990. Interaction Studies Between Redox Proteins, Cytochrome C3, Ferredoxin and Hydrogenase from Sulfate Reducing Bacteria. In: J.-P. Bélaich, M. Bruschi and J.-L. Garcia (eds.) *Microbiology and Biochemistry of Strict Anaerobes Involved in Interspecies Hydrogen Transfer*. Federation of European Microbiological Societies Symposium Series No. 54 Springer US. p 249-258.
- Doreau, M., E. Aurousseau, and C. Martin. 2009. Effects of linseed lipids fed as rolled seeds, extruded seeds or oil on organic matter and crude protein digestion in cows. *Anim. Feed Sci. Technol.* 150: 187-196.
- Doreau, M., L. Bamière, C. Pellerin, M. Lherm, and M. Benoit. 2014a. Mitigation of enteric methane for French cattle: Potential extent and cost of selected actions. *Anim. Prod. Sci.* 54: 1417-1422.
- Doreau, M., and M. Benoit. 2013. Substituer des glucides par des lipides insaturés et utiliser un additif à base de nitrate dans les rations des ruminants pour réduire les émissions de méthane entérique. In: S. Pellerin et al. (eds.) *Quelle contribution de l'agriculture française à la réduction des émissions de gaz à effet de serre ? Potentiel d'atténuation et coût de dix actions techniques*. Synthèse de rapport d'étude, INRA (France). p 92.
- Doreau, M., and A. Ferlay. 1995. Effect of dietary lipids on nitrogen metabolism in the rumen: a review. *Livest. Prod. Sci.* 43: 97-110.
- Doreau, M., A. Ferlay, Y. Rochette, and C. Martin. 2014b. Effects of dehydrated lucerne and soya bean meal on milk production and composition, nutrient digestion, and methane and nitrogen losses in dairy cows receiving two different forages. *Animal* 8: 420-430.
- Doreau, M., C. Martin, M. Eugène, M. Popova, and D. P. Morgavi. 2011. Leviers d'action pour réduire la production de méthane entérique par les ruminants. *INRA Prod. Anim.* 24: 461-474.
- El-Zaiat, H. M., R. C. Araujo, Y. A. Soltan, A. S. Morsy, H. Louvandini, A. V. Pires, H. O. Patino, P. S. Correa, and A. L. Abdalla. 2014. Encapsulated nitrate and cashew nut shell liquid on blood and rumen constituents, methane emission, and growth performance of lambs. *J. Anim. Sci.* 92: 2214-2224.
- Ellis, J. L., E. Kebreab, N. E. Odongo, B. W. McBride, E. K. Okine, and J. France. 2007. Prediction of methane production from dairy and beef cattle. *J. Dairy Sci.* 90: 3456-3467.
- Finlay, B. J., G. Esteban, K. J. Clarke, A. G. Williams, T. M. Embley, and R. P. Hirt. 1994. Some rumen ciliates have endosymbiotic methanogens. *FEMS Microbiol. Lett.* 117: 157-161.
- Foley, P. A., D. A. Kenny, J. J. Callan, T. M. Boland, and F. P. O'Mara. 2009. Effect of dl-malic acid supplementation on feed intake, methane emission, and rumen fermentation in beef cattle. *J. Anim. Sci.* 87: 1048-1057.

- Fonty, G., J. P. Jouany, E. Forano, and P. Gouet. 1995. L'écosystème microbien du réticulo-rumen. In: R. Jarrige, Y. Ruckebusch, C. Demarquilly, M. H. Farce and M. Journet (eds.) Nutrition des ruminants domestiques INRA, Paris, France. p 299-347.
- Fourmond, V., C. Baffert, K. Sybirna, S. Dementin, A. Abou-Hamdan, I. Meynial-Salles, P. Soucaille, H. Bottin, and C. Léger. 2013. The mechanism of inhibition by H₂ of H₂-evolution by hydrogenases. *Chem. Commun.* 49: 6840-6842.
- Gerber, P., N. Key, F. Portet, and H. Steinfeld. 2010. Policy options in addressing livestock's contribution to climate change. *Animal* 4: 393-406.
- Gerber, P. J., B. Henderson, and H. P. S. Makkar. 2013a. Mitigation of greenhouse gas emissions in livestock production: a review of technical options for non-CO₂ emissions. Food and Agriculture Organization of the United Nations, Rome, Italy.
- Gerber, P. J., H. Steinfeld, B. Henderson, A. Mottet, C. Opio, J. Dijkman, A. Falcucci, and G. Tempio. 2013b. Tackling climate change through livestock – A global assessment of emissions and mitigation opportunities. Food and Agriculture Organization of the United Nations, Rome, Italy.
- Gill, M., P. Smith, and J. M. Wilkinson. 2010. Mitigating climate change: the role of domestic livestock. *Animal* 4: 323-333.
- Glass, T. L., M. P. Bryant, and M. J. Wolin. 1977. Partial purification of ferredoxin from *Ruminococcus albus* and its role in pyruvate metabolism and reduction of nicotinamide adenine dinucleotide by H₂. *J. Bacteriol.* 131: 463-472.
- Godwin, I. R. 2014. Are sheep with HK erythrocytes less susceptible to nitrite poisoning ? *Proc Aust Soc Anim Prod* 30 283.
- Goel, G., and H. P. S. Makkar. 2012. Methane mitigation from ruminants using tannins and saponins. *Trop. Anim. Health Prod.* 44: 729-739.
- Gottschalk, G. 1986. *Bacterial Metabolism* (2nd edition). Springer Verlag, New York Inc.
- Gould, D. H. 1998. Polioencephalomalacia. *J. Anim. Sci.* 76: 30-314.
- Grainger, C., and K. A. Beauchemin. 2011. Can enteric methane emissions from ruminants be lowered without lowering their production ? *Anim. Feed Sci. Technol.* 166-167: 308-320.
- Grainger, C., T. Clarke, S. McGinn, M. Auldist, K. Beauchemin, M. Hannah, G. Waghorn, H. Clark, and R. Eckard. 2007. Methane emissions from dairy cows measured using the sulfur hexafluoride (SF₆) tracer and chamber techniques. *J. Dairy Sci.* 90: 2755-2766.
- Grainger, C., Clarke, T., Auldist, M. J., Beauchemin, K. A., McGinn, S. M., Waghorn, G. C., Eckard, R. J. 2009. Potential use of *Acacia mearnsii* condensed tannins to reduce methane emissions and nitrogen excretion from grazing dairy cows. *Can J Anim Sci* 89: 241-251.
- Guan, H., Wittenberg, K. M., Ominski, K. H., Krause, D. O. 2006. Efficacy of ionophores in cattle diets for mitigation of enteric methane. *J. Anim. Sci.* 84: 1896-1906.
- Guyader, J., M. Eugène, P. Nozière, D. P. Morgavi, M. Doreau, and C. Martin. 2014. Influence of rumen protozoa on methane emissions in ruminants: A meta-analysis approach. *Animal* 8: 1816-1825.
- Hackstein, J. H. P. 2010. (Endo)symbiotic methanogenic archaea. Springer-Verlag, Berlin, Germany.
- Hammond, K. J., J. L. Burke, J. P. Koolaard, S. Muetzel, C. S. Pinares-Patiño, and G. C. Waghorn. 2013. Effects of feed intake on enteric methane emissions from sheep fed fresh white clover (*Trifolium repens*) and perennial ryegrass (*Lolium perenne*) forages. *Anim. Feed Sci. Technol.* 179: 121-132.
- Harrison, P. A., M. Vandewalle, M. T. Sykes, P. M. Berry, R. Bugter, F. de Bello, C. K. Feld, U. Grandin, R. Harrington, J. R. Haslett, R. H. G. Jongman, G. W. Luck, P. M. da Silva, M. Moora, J. Settele, J. P. Sousa, and M. Zobel. 2010. Identifying and prioritising services in European terrestrial and freshwater ecosystems. *Biodivers. Conserv.* 19: 2791-2821.
- Hattori, K., and H. Matsui. 2008. Diversity of fumarate reducing bacteria in the bovine rumen revealed by culture dependent and independent approaches. *Anaerobe* 14: 87-93.
- Hegarty, R. S., and R. Gerdes. 1999. Hydrogen production and transfer in the rumen. *Recent Advances in Animal Nutrition* 12: 37-44.
- Henderson, C. 1980. The influence of extracellular hydrogen on the metabolism of *Bacteroides ruminicola*, *Anaerovibrio lipolytica* and *Selenomonas ruminantium*. *J Gen Microbiol* 119: 485-491.
- Hillman, K., D. Lloyd, and A. G. Williams. 1985. Use of a portable quadrupole mass spectrometer for the measurement of dissolved gas concentrations in ovine rumen liquor in situ. *Curr. Microbiol.* 12: 335-340.
- Hulshof, R. B. A., A. Berndt, W. J. J. Gerrits, J. Dijkstra, S. M. Van Zijderveld, J. R. Newbold, and H. B. Perdok. 2012. Dietary nitrate supplementation reduces methane emission in beef cattle fed sugarcane based diets. *J. Anim. Sci.* 90: 2317-2323.
- Hungate, R. E. 1967. Hydrogen as an intermediate in the rumen fermentation. *Archiv für Mikrobiologie* 59: 158-164.
- Illumina. 2010. Illumina sequencing technology. <http://www.illumina.com/> October 14th, 2014.

- INRA. 2010. Alimentation des bovins, ovins et caprins. INRA Editions, Paris, France.
- IPCC. 2007. Climate Change 2007: Synthesis Report. Contribution of Working Groups I, II and III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. IPCC, Geneva, Switzerland.
- ISO. 2004. ISO 14673-1:2004 (IDF 189-1: 2004) : Lait et produits laitiers -- Détermination des teneurs en nitrates et en nitrites -- Partie 1: Méthode par réduction au cadmium et spectrométrie. http://www.iso.org/iso/fr/home/store/catalogue_tc/catalogue_detail.htm?csnumber=38702 Accessed June 19 2014.
- Iwamoto, M., N. Asanuma, and T. Hino. 2001. Effects of energy substrates on nitrate reduction and nitrate reductase activity in a ruminal bacterium, *Selenomonas ruminantium*. *Anaerobe* 7: 315-321.
- Janssen, P. H. 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. *Anim. Feed Sci. Technol.* 160: 1-22.
- Jarrige, R., Y. Ruckebusch, C. Demarquilly, M. H. Farce, and M. Journet. 1995. Nutrition des ruminants domestiques: Ingestion et digestion. INRA, Paris.
- Johnson, D. E. 1972. Effects of a hemiacetal of chloral and starch on methane production and energy balance of sheep fed a pelleted diet. *J. Anim. Sci.* 35: 1064-1068.
- Johnson, D. E. 1974. Adaptational responses in nitrogen and energy balance of lambs fed a methane inhibitor. *J. Anim. Sci.* 38: 154-157.
- Johnson, K., M. Huyler, H. Westberg, B. Lamb, and P. Zimmerman. 1994. Measurement of methane emissions from ruminant livestock using a sulfur hexafluoride tracer technique. *Environ. Sci. Technol.* 28: 359-362.
- Johnson, K. A., and D. E. Johnson. 1995. Methane emissions from cattle. *J. Anim. Sci.* 73: 2483-2492.
- Jouany, J. P., and J. Senaud. 1979. Description d'une technique permettant d'effectuer des prélèvements répétés de gaz dans le rumen. *Reprod. Nutr. Dev.* 19: 1007-1010.
- Joyner, A. E., W. T. Winter, and D. M. Godbout. 1977. Studies on some characteristics of hydrogen production by cell-free extracts of rumen anaerobic bacteria. *Can J Microbiol* 23: 346-353.
- Kern, M., and J. Simon. 2009. Electron transport chains and bioenergetics of respiratory nitrogen metabolism in *Wolinella succinogenes* and other Epsilonproteobacteria. *Biochim Biophys Acta* 1787: 646-656.
- Kittelman, S., H. Seedorf, W. A. Walters, J. C. Clemente, R. Knight, J. I. Gordon, and P. H. Janssen. 2013. Simultaneous amplicon sequencing to explore co-occurrence patterns of bacterial, archaeal and eukaryotic microorganisms in rumen microbial communities. *PLoS ONE* 8: e47879.
- Klevenhusen, F., J. O. Zeitz, S. Duval, M. Kreuzer, and C. R. Soliva. 2011. Garlic oil and its principal component diallyl disulfide fail to mitigate methane, but improve digestibility in sheep. *Anim. Feed Sci. Technol.* 166-167: 356-363.
- Klindworth, A., E. Pruesse, T. Schweer, J. Peplies, C. Quast, M. Horn, and F. O. Glöckner. 2013. Evaluation of general 16S ribosomal RNA gene PCR primers for classical and next-generation sequencing-based diversity studies. *Nucleic Acids Res.* 41: e1.
- Knapp, J. R., G. L. Laur, P. A. Vadas, W. P. Weiss, and J. M. Tricarico. 2014. Invited review: Enteric methane in dairy cattle production: quantifying the opportunities and impact of reducing emissions. *J. Dairy Sci.* 97: 1-31.
- Knight, T., R. S. Ronimus, D. Dey, C. Tootill, G. Naylor, P. Evans, G. Molano, A. Smith, M. Tavendale, C. S. Pinares-Patino, and H. Clark. 2011. Chloroform decreases rumen methanogenesis and methanogen populations without altering rumen function in cattle. *Anim. Feed Sci. Technol.* 166-167: 101-112.
- Kozich, J. J., S. L. Westcott, N. T. Baxter, S. K. Highlander, and P. D. Schloss. 2013. Development of a dual-index sequencing strategy and curation pipeline for analyzing amplicon sequence data on the MiSeq Illumina sequencing platform. *Appl. Environ. Microbiol.* 79: 5112-5120.
- Kröger, A., S. Biel, J. Simon, R. Gross, G. Uden, and C. R. D. Lancaster. 2002. Fumarate respiration of *Wolinella succinogenes*: enzymology, energetics and coupling mechanism. *Biochim Biophys Acta* 1553: 23-38.
- La Penna, G. 2010. The inhibition of hydrogenase by dioxygen. In: *Proceedings of the NIC Symposium 2010*, Jülich, Germany. p 79.
- Laverman, A. M., C. Pallud, J. Abell, and P. van Cappellen. 2012. Comparative survey of potential nitrate and sulfate reduction rates in aquatic sediments. *Geochim. Cosmochim. Acta* 77: 474-488.
- Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2014a. Effects of encapsulated nitrate on nitrogen utilization and enteric methane emissions in beef cattle. In: *Linking animal science and animal agriculture: Meeting the global demands of 2050*, Kansas city, USA. p 323.
- Lee, C., R. C. Araujo, K. M. Koenig, and K. A. Beauchemin. 2014b. Effects of encapsulated nitrate on toxicity, feed intake and feed consumption rates in beef cattle. In: *Linking animal science and animal agriculture: Meeting the global demands of 2050*, Kansas city, USA. p 846.

- Lee, C., and K. A. Beauchemin. 2014a. A meta-analysis of effects of feeding nitrate on toxicity, production, and enteric methane emissions in ruminants. In: *Linking animal science and animal agriculture: Meeting the global demands of 2050*, Kansas City, USA. p 845-846.
- Lee, C., and K. A. Beauchemin. 2014b. A review of feeding supplementary nitrate to ruminant animals: nitrate toxicity, methane emissions, and production performance. *Can J Anim Sci* 94.
- Lemos, V. P., M. L. da Costa, R. L. Lemos, and M. S. G. de Faria. 2007. Vivianite and siderite in lateritic iron crust: an example of bioreduction. *Quim. Nova* 30: 36-40.
- Leng, R. A. 2008. The potential of feeding nitrate to reduce enteric methane production in ruminants. Department of Climate Change, Commonwealth Government of Australia, Canberra, ACT, Australia.
- Leng, R. A. 2014. Interactions between microbial consortia in biofilms: a paradigm shift in rumen microbial ecology and enteric methane mitigation. *Anim. Prod. Sci.* 54: 519-543.
- Lettat, A. 2012. Efficacité et mode d'action des bactéries propioniques et/ou lactiques pour prévenir l'acidose latente chez le ruminant, Thesis PhD, Université Blaise Pascal, Clermont Ferrand, France.
- Lewis, D. 1951. The Metabolism of Nitrate and Nitrite in the Sheep. 1 The reduction of nitrate in the rumen of the sheep. *Biochem. J.* 48: 175-180.
- Li, L., J. Davis, J. V. Nolan, and R. S. Hegarty. 2012. An initial investigation on rumen fermentation pattern and methane emission of sheep offered diets containing urea or nitrate as the nitrogen source. *Anim. Prod. Sci.* 52: 653-658.
- Li, W., and W. Powers. 2012. Effects of saponin extracts on air emissions from steers. *J. Anim. Sci.* 90: 4001-4013.
- Lovley, D. R. 1991. Dissimilatory Fe(III) and Mn(IV) reduction. *Microbiol Rev* 55: 259-287.
- Lund, P., R. Dahl, H. J. Yang, A. L. F. Hellwing, B. B. Cao, and M. R. Weisbjerg. 2014. The acute effect of addition of nitrate on in vitro and in vivo methane emission in dairy cows. *Anim. Prod. Sci.* 54: 1432-1435.
- Machmüller, A., D. A. Ossowski, and M. Kreuzer. 2000. Comparative evaluation of the effects of coconut oil, oilseeds and crystalline fat on methane release, digestion and energy balance in lambs. *Anim. Feed Sci. Technol.* 85: 41-60.
- Mao, H. L., J. K. Wang, Y. Y. Zhou, and J. X. Liu. 2010. Effects of addition of tea saponins and soybean oil on methane production, fermentation and microbial population in the rumen of growing lambs. *Livest. Sci.* 129: 56-62.
- Marden, J. P. 2007. Contribution à l'étude du mode d'action de la levure *Saccharomyces cerevisiae* Sc47 chez le ruminant: Approche thermodynamique chez la vache laitière., Thesis PhD, Institut National Polytechnique, Toulouse, France
- Martin, C., D. P. Morgavi, and M. Doreau. 2010. Methane mitigation in ruminants: from microbe to the farm scale. *Animal* 4: 351-365.
- Martin, C., D. Pomiès, A. Ferlay, Y. Rochette, B. Martin, Y. Chilliard, D. P. Morgavi, and M. Doreau. 2011. Methane output and rumen microbiota in dairy cows in response to long-term supplementation with linseed or rapeseed of grass silage- or pasture-based diets. *Proc. N.Z. Soc. Anim. Prod.* 71: 243-247.
- Martin, C., J. Rouel, J. P. Jouany, M. Doreau, and Y. Chilliard. 2008. Methane output and diet digestibility in response to feeding dairy cows crude linseed, extruded linseed, or linseed oil. *J. Anim. Sci.* 86: 2642-2650.
- Martin, W. 2005. The missing link between hydrogenosomes and mitochondria. *Trends Microbiol.* 13: 457-459.
- Martin, W. F., and M. Müller. 2007. *Origin of mitochondria and hydrogenosomes*. Springer-Verlag, Berlin, Germany.
- McArthur, J. M., and J. E. Miltimore. 1961. Rumen gas analysis by gas-solid chromatography. *Can J Anim Sci* 41: 187-196.
- McGinn, S. M., K. A. Beauchemin, T. Coates, and D. Colombatto. 2004. Methane emissions from beef cattle: Effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. *J. Anim. Sci.* 82: 3346-3356.
- Michel, T. A., and J. M. Macy. 1990. Ferredoxin from *Selenomonas ruminantium*. *Arch. Microbiol.* 153: 518-520.
- Mills, J. A., J. Dijkstra, A. Bannink, S. B. Cammell, E. Kebreab, and J. France. 2001. A mechanistic model of whole-tract digestion and methanogenesis in the lactating dairy cow: model development, evaluation, and application. *J. Anim. Sci.* 79: 1584-1597.
- Mills, J. A. N., E. Kebreab, C. M. Yates, L. A. Crompton, S. B. Cammell, M. S. Dhanoa, R. E. Agnew, and J. France. 2003. Alternative approaches to predicting methane emissions from dairy cows. *J. Anim. Sci.* 81: 3141-3150.
- Mitra, S., and S. R. Dungan. 1997. Micellar properties of Quillaja saponin. 1. Effects of temperature, salt, and pH on solution properties. *J. Agric. Food Chem.* 45: 1587-1595.
- Moate, P. J., T. Clarke, L. H. Davis, and R. H. Laby. 1997. Rumen gases and bloat in grazing dairy cows. *J. Agric. Sci.* 129: 459-469.

- Moate, P. J., S. R. O. Williams, M. H. Deighton, J. L. Jacobs, and W. J. Wales. 2013. Influence of rumen cannulation on feed intake, milk production, enteric methane production and composition of rumen headspace gas. *Advances in Animal Biosciences* 4: 423.
- Moate, P. J., S. R. O. Williams, V. A. Torok, M. C. Hannah, B. E. Ribaux, M. H. Tavendale, R. J. Eckard, J. L. Jacobs, Auld M.J., and W. J. Wales. 2014. Grape marc reduces methane emissions when fed to dairy cows. *J. Dairy Sci.* 97: 5073-5087.
- Mohammed, N., Z. A. Lila, N. Tatsuoka, K. Hara, K. Mikuni, K. Hara, S. Kanda, and H. Itabashi. 2004. Effects of cyclodextrin-iodopropane complex on methane production, ruminal fermentation and microbes, digestibility and blood metabolites in steers. *Anim Sci J* 75: 131-137.
- Morgavi, D. P., E. Forano, C. Martin, and C. J. Newbold. 2010. Microbial ecosystem and methanogenesis in ruminants. *Animal* 4: 1024-1036.
- Morgavi, D. P., C. Martin, and H. Boudra. 2013. Fungal secondary metabolites from *Monascus* spp. reduce rumen methane production in vitro and in vivo. *J. Anim. Sci.* 91: 848-860.
- Muetzel, S., C. Hunt, and M. H. Tavendale. 2014. A fully automated incubation system for the measurement of gas production and gas composition. *Anim. Feed Sci. Technol.* In press.
- Müller, M. 1993. The hydrogenosome: Review article. *J Gen Microbiol* 139: 2879-2889.
- Müller, M., M. Mentel, J. J. van Hellemond, K. Henze, C. Woehle, S. B. Gould, R. Y. Yu, M. van der Giezen, A. G. M. Tielens, and W. F. Martin. 2012. Biochemistry and evolution of anaerobic energy metabolism in eukaryotes. *Microbiol. Mol. Biol. Rev.* 76: 444-495.
- Murray, R. M., A. M. Bryant, and R. A. Leng. 1976. Rates of production of methane in the rumen and large intestine of sheep. *Br. J. Nutr.* 36: 1-14.
- Nealson, K. H., and D. Saffarini. 1994. Iron and manganese in anaerobic respiration: Environmental significance, physiology, and regulation. *Annu. Rev. Microbiol.* 48: 311-343.
- Neumeier, C. J., Q. Wang, A. R. Castillo, Y. Zhao, Y. Pan, and F. M. Mitloehner. 2014. Effects of dietary nitrate supplementation on enteric methane and nitrous oxide emissions from beef cattle. In: *Linking animal science and animal agriculture: Meeting the global demands of 2050*, Kansas city, USA. p 548.
- Newbold, C. J., S. M. El Hassan, J. Wang, M. E. Ortega, and R. J. Wallace. 1997. Influence of foliage from African multipurpose trees on activity of rumen protozoa and bacteria. *Br. J. Nutr.* 78: 237-249.
- Newbold, C. J., B. Lassalas, and J. P. Jouany. 1995. The importance of methanogens associated with ciliate protozoa in ruminal methane production in vitro. *Lett. Appl. Microbiol.* 21: 230-234.
- Newbold, C. J., S. Lopez, N. Nelson, J. O. Ouda, R. J. Wallace, and A. R. Moss. 2005. Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation in vitro. *Br. J. Nutr.* 94: 27-35.
- Noblet, J., and J. van Milgen. 2004. Energy value of pig feeds: Effect of pig body weight and energy evaluation system. *J. Anim. Sci.* 82: E229-E238.
- Nolan, J. V., R. S. Hegarty, J. Hegarty, I. R. Godwin, and R. Woodgate. 2010. Effects of dietary nitrate on fermentation, methane production and digesta kinetics in sheep. *Anim. Prod. Sci.* 50: 801-806.
- Nolan, J. V., R. S. Hegarty, J. Hegarty, I. R. Godwin, and R. Woodgate. 2012. Use of supplementary nitrate to mitigate methane production and provide rumen degradable N for ruminants.
- Nozière, P., F. Glasser, and D. Sauvant. 2010. In vivo production and molar percentages of volatile fatty acids in the rumen: a quantitative review by an empirical approach. *Animal* 5: 403-414.
- Olson, T. M. 1940. Bloat in dairy cattle. *J. Dairy Sci.* 23: 343-353.
- Paul, R. G., A. G. Williams, and R. D. Butler. 1990. Hydrogenosomes in the rumen entodiniomorphid ciliate *Polyplastron multivesiculatum*. *J Gen Microbiol* 136: 1981-1989.
- Pauss, A., G. André, M. Perrier, and S. R. Guiot. 1990. Liquid-to-gas mass transfer in anaerobic processes: inevitable transfer limitations of methane and hydrogen in the biomethanation process. *Appl. Environ. Microbiol.* 56: 1636-1644.
- Petersen, S. O., A. L. F. Hellwing, M. Brask, O. Hojberg, M. Poulsen, Z. Zhu, and P. Lund. 2014. Nitrous oxide emission partly alleviates the methane mitigation effect of nitrate intake by dairy cattle. In: *Livestock, Climate change and food security conference*, Madrid, Spain. 19-20 May 2014
- Pinares-Patiño, C., and G. Waghorn. 2012. Technical manual on respiration chamber designs. Ministry of Agriculture and Forestry Pastoral House, 25 The Terrace PO Box 2526, Wellington 6140, New Zealand.
- Pinares-Patiño, C. S., C. Hunt, R. Martin, J. West, P. Lovejoy, and W. G.C. 2012a. New Zealand ruminant methane measurement centre, AgResearch, Palmerston North. In: C. S. Pinares-Patiño and W. G.C. (eds.) *Technical manual on respiration chamber designs*. p 9-28. Ministry of Agriculture and Forestry, Wellington, New Zealand.
- Pinares-Patiño, C. S., C. Hunt, R. Martin, J. West, P. Lovejoy, and G. Waghorn. 2012b. Chapter 1: New Zealand ruminant methane measurement centre, AgResearch, Palmerston North. In: C. S. Pinares-Patiño and G.

- Waghorn (eds.) Technical manual on respiration chamber designs Ministry of Agriculture and Forestry, Wellington, New Zealand. p.
- Pinares-Patiño, C. S., S. Muetzel, G. Molano, C. Hunt, and H. Clark. 2010. Emissions of hydrogen gas from sheep fed fresh perennial ryegrass and pelleted diets. In: 3rd EAAP International Symposium on energy and protein metabolism and nutrition, Parma, Italy. p 447-448.
- Popova, M. 2011. Structure et activité de la communauté des archaea méthanogènes du rumen en relation avec la production de méthane par les ruminants, Thesis PhD, Université Blaise Pascal, Clermont Ferrand.
- Popova, M., C. Martin, and D. P. Morgavi. 2011. Improved protocol for high-quality co-extraction of DNA and RNA from rumen digesta. *Folia Microbiol.* 55: 368-372.
- Poungchompu, O., M. Wanapat, C. Wachirapakorn, S. Wanapat, and A. Cherdthong. 2009. Manipulation of ruminal fermentation and methane production by dietary saponins and tannins from mangosteen peel and soapberry fruit. *Archives of Animal Nutrition* 63: 389-400.
- Prescott, L. M., J. P. Harley, D. A. Klein, J. M. Willey, L. M. Sherwood, and C. J. Woolverton. 2010. Microbiologie, 3ème édition, Bruxelles, Belgique.
- Ramin, M., and P. Huhtanen. 2013. Development of equations for predicting methane emissions from ruminants. *J. Dairy Sci.* 96: 2476-2493.
- Ramírez-Restrepo, C. A., C. J. O'Neill, N. López-Villalobos, J. Padmanabha, and C. S. McSweeney. 2014. Tropical cattle methane emissions: the role of natural statins supplementation. *Anim. Prod. Sci.* 54: 1294-1299.
- Rasmussen, J., and A. Harrison. 2011. The benefits of supplementary fat in feed rations for ruminants with particular focus on reducing levels of methane production. *ISRN Veterinary Science* 2011, Article ID 613172: 1-10.
- Reddy, C. A., and M. P. Bryant. 1977. Deoxyribonucleic acid base composition of certain species of the genus *Bacteroides*. *Can J Microbiol* 23: 1252-1256.
- Reddy, C. A., and H. D. Peck. 1978. Electron transport phosphorylation coupled to fumarate reduction by H₂ and Mg²⁺ dependent adenosine triphosphatase activity in extracts of the rumen anaerobe *Vibrio succinogenes*. *J. Bacteriol.* 134: 982-991.
- Rees, E. M. R., D. Lloyd, and A. G. Williams. 1998. The effects of differing concentrations of CO₂ and O₂ on the fermentative metabolism of the rumen fungi *Neocallimastix patriciarum* and *Neocallimastix frontalis* L2. *Can J Microbiol* 44: 819-824.
- Reichl, J. R., and R. L. Baldwin. 1975. Rumen modeling: rumen input-output balance models. *J. Dairy Sci.* 58: 879-890.
- Reynolds, C. K., L. A. Crompton, and J. A. N. Mills. 2011. Improving the efficiency of energy utilisation in cattle. *Anim. Prod. Sci.* 51: 6-12.
- Robinson, J. A., R. F. Strayer, and J. M. Tiedje. 1981. Method for measuring dissolved hydrogen in anaerobic ecosystems: Application to the rumen. *Appl. Environ. Microbiol.* 41: 545-548.
- Rooke, J. A., R. J. Wallace, C. A. Duthie, N. McKain, S. Motta de Souza, J. J. Hyslop, D. W. Ross, T. Waterhouse, and R. Roehe. 2014. Hydrogen and methane emissions from beef cattle and their rumen microbial community vary with diet, time after feeding and genotype. *Br. J. Nutr.* 112: 398-407.
- Sander, R. 1999. Compilation of Henry's law constants for inorganic and organic species of potential importance in environmental chemistry. <http://www.mpch-mainz.mpg.de/~sander/res/henry.html> Accessed March 11 2014.
- Sar, C., B. Santoso, B. Mwenya, Y. Gamoa, T. Kobayashi, R. Morikawa, K. Kimura, H. Mizukoshi, and J. Takahashi. 2004. Manipulation of rumen methanogenesis by the combination of nitrate with b1-4 galacto-oligosaccharides or nisin in sheep. *Anim. Feed Sci. Technol.* 115: 129-142.
- Sauvant, D., S. Giger-Reverdin, A. Serment, and L. Broudiscou. 2011. Influences des régimes et de leur fermentation dans le rumen sur la production de méthane par les ruminants. *INRA Prod. Anim.* 24: 433-446.
- Sauvant, D., and P. Nozière. 2013. La quantification des principaux phénomènes digestifs chez les ruminants: les relations utilisées pour rénover les systèmes d'unités d'alimentation énergétique et protéique. *INRA Prod. Anim.* 26: 327-346.
- Schwarz, E., and B. Friedrich. 2003. The H₂-metabolizing prokaryotes. In: M. Dworkin, S. Falkow, E. Rosenberg and K. H. Schleifer (eds.) *The prokaryotes: an evolving electronic resource for the microbiological community* Springer, New-York, USA. p.
- Scollan, N. D., N. J. Choi, E. Kurt, A. V. Fisher, M. Enser, and J. D. Wood. 2001. Manipulating the fatty acid composition of muscle and adipose tissue in beef cattle. *Br. J. Nutr.* 85: 115-124.
- Shinkai, T., O. Enishi, M. Mitsumori, K. Higuchi, Y. Kobayashi, A. Takenaka, K. Nagashima, M. Mochizuki, and Y. Kobayashi. 2012. Mitigation of methane production from cattle by feeding cashew nut shell liquid. *J. Anim. Sci.* 95: 5308-5316.

- Simon, J., M. Sanger, S. C. Schuster, and R. Gross. 2003. Electron transport to periplasmic nitrate reductase (NapA) of *Wolinella succinogenes* is independent of a NapC protein. *Mol. Microbiol.* 49: 69-79.
- Smolenski, W. J., and J. A. Robinson. 1988. In situ rumen hydrogen concentrations in steers fed eight times daily, measured using a mercury reduction detector. *FEMS Microbiol. Ecol.* 53: 95-100.
- Sonderman, J. P., and K. G. Odde. 1993. Effects of nitrates in pregnant beef and dairy cows. OSU Cooperative Extension and OSU Animal Science Department: Management of high nitrate forages for beef and dairy cattle: C1-C14.
- Steinfeld, H., P. Gerber, T. Wassenaar, V. Castel, M. Rosales, and C. de Haan. 2006. Livestock's long shadow environmental issues and options. Food and Agriculture Organization, Rome, Italy.
- Stewart, C. S., H. J. Flint, and M. P. Bryant. 1997. The rumen bacteria. In: P. N. Hobson and C. S. Stewart (eds.) *The rumen microbial ecosystem* Blackie Academic & professional, London, United Kingdom. p 10-72.
- Stiefel, E. I., and G. N. George. 1994. Ferredoxins, hydrogenases, and nitrogenases: metal-sulfide proteins. In: I. Bertini, H. B. Gray, S. J. Lippard and J. S. Valentine (eds.) *Bioinorganic chemistry* University Science Books, Herndon, USA. p 365-455.
- Stripp, S. T., G. Goldet, C. Brandmayr, O. Sanganas, K. A. Vincent, M. Haumann, F. A. Armstrong, and T. Happe. 2009. How oxygen attacks [FeFe] hydrogenases from photosynthetic organisms. *PNAS* 106: 17331-17336.
- Stumm, C. K., H. J. Gijzen, and G. D. Vogels. 1982. Association of methanogenic bacteria with ovine rumen ciliates. *Br. J. Nutr.* 47: 95-99.
- Swainson, N., C. Martin, S. Muetzel, and C. Pinares-Patio. 2011. Hydrogen emissions from sheep: A spill over for methanogenesis ? In: *The 8th International Symposium on the Nutrition of Herbivores*, Aberystwyth University, United Kingdom
- Sylvester, J. T., S. K. R. Karnati, Z. Yu, M. Morrison, and J. L. Firkins. 2004. Development of an assay to quantify rumen ciliate protozoal biomass in cows using real-time PCR. *J. Nutr.* 134: 3378-3384.
- Thauer, R. K., K. Jungermann, and K. Decker. 1977. Energy conservation in chemotrophic anaerobic bacteria. *Bacteriological Reviews* 41: 100-180.
- Tratnyek, P. G., and D. L. Macalady. 2000. Oxidation-reduction reactions in the aquatic environment. In: R. S. Boethling (ed.) *Handbook of property estimation methods for chemicals: Environmental and health sciences* CRC Press, Boca Raton, Florida, USA. p 383-415.
- Unden, G., R. Bocher, J. Knecht, and A. Kroger. 1982. Hydrogenase from *Vibrio succinogenes*, a nickel protein. *FEBS Lett.* 145: 230-234.
- Ungerfeld, E. M., R. A. Kohn, R. J. Wallace, and C. J. Newbold. 2007. A meta-analysis of fumarate effects on methane production in ruminal batch cultures. *J. Anim. Sci.* 85: 2556-2563.
- Ushida, K., and J. P. Jouany. 1996. Methane production associated with rumen-ciliated protozoa and its effect on protozoan activity. *Lett. Appl. Microbiol.* 23: 129-132.
- Valentine, R. C., and R. S. Wolfe. 1963. Role of ferredoxin in the metabolism of molecular hydrogen. *J. Bacteriol.* 85: 1114-1120.
- Van Dijk, C., S. G. Mayhew, H. J. Grande, and C. Veeger. 1979. Purification and properties of hydrogenase from *Megasphaera elsdenii*. *Eur. J. Biochem.* 102: 317-330.
- van Huis, A. 2013. Potential of insects as food and feed in assuring food security. *Annu. Rev. Entomol.* 58: 563-583.
- Van Middelaar, C. E., J. Dijkstra, P. B. M. Berentsen, and I. J. M. De Boer. 2014. Cost-effectiveness of feeding strategies to reduce greenhouse gas emissions from dairy farming. *J. Dairy Sci.* 97: 2427-2439.
- Van Zijderveld, S. M., W. J. J. Gerrits, J. A. Apajalahti, J. R. Newbold, J. Dijkstra, R. A. Leng, and H. B. Perdok. 2010. Nitrate and sulfate: Effective alternative hydrogen sinks for mitigation of ruminal methane production in sheep. *J. Dairy Sci.* 93: 5856-5866.
- Van Zijderveld, S. M., W. J. J. Gerrits, J. Dijkstra, J. R. Newbold, R. B. A. Hulshof, and H. B. Perdok. 2011. Persistency of methane mitigation by dietary nitrate supplementation in dairy cows. *J. Dairy Sci.* 94: 4028-4038.
- Veneman, J. B., S. Muetzel, K. J. Hart, C. L. Faulkner, J. M. Moorby, G. Molano, H. B. Perdok, J. R. Newbold, and C. J. Newbold. 2014. Dietary nitrate but not linseed oil decreases methane emissions in two studies with lactating dairy cows. In: *Livestock, Climate Change and Food Security Conference*, Madrid, Spain.
- Veneman, J. B., S. Muetzel, H. B. Perdok, and C. J. Newbold. 2013. Dietary nitrate but not linseed oil decreases methane emissions in lactating dairy cows fed a maize silage based diet. In: *Proceedings of the 5th Greenhouse Gases and Animal Agriculture Conference*, 23-26 June 2013, Dublin, Ireland
- Vermorel, M., J. P. Jouany, M. Eugene, D. Sauvant, J. Noblet, and J. Y. Dourmad. 2008. Evaluation quantitative des missions de mthane entrique par les animaux d'levage en 2007 en France. *INRA Prod. Anim.* 21: 403-418.

- Veysset, P., M. Lherm, and D. Bébin. 2010. Energy consumption, greenhouse gas emissions and economic performance assessments in French Charolais suckler cattle farms: Model-based analysis and forecasts. *Agricultural Systems* 103: 41-50.
- Vignais, P. M., B. Billoud, and J. Meyer. 2001. Classification and phylogeny of hydrogenases. *FEMS Microbiol. Rev.* 25: 455-501.
- Vignais, P. M., and A. Colbeau. 2004. Molecular biology of microbial hydrogenases. *Curr. Issues Mol. Biol.* 6: 159-188.
- Vogels, G. D., W. F. Hoppe, and C. K. Stumm. 1980. Association of methanogenic bacteria with rumen ciliates. *Appl. Environ. Microbiol.* 40: 608-612.
- Wang, J. K., J. A. Ye, and J. X. Liu. 2012. Effects of tea saponins on rumen microbiota, rumen fermentation, methane production and growth performance - a review. *Trop. Anim. Health Prod.* 44: 697-706.
- Wang, M., X. Z. Sun, P. H. Janssen, S. X. Tang, and Z. L. Tan. 2014. Responses of methane production and fermentation pathways to the increased dissolved hydrogen concentration generated by eight substrates in *in vitro* ruminal cultures. *Anim. Feed Sci. Technol.* 194: 1-11.
- Wei, M. L., L. P. Ren, Z. M. Zhou, and Q. X. Meng. 2012. Effect of addition of three plant extracts on gas production, ruminal fermentation, methane production and ruminal digestibility based on an *in vitro* technique. *J Anim Vet Adv* 11: 4304-4309.
- Weitzberg, E., and J. O. Lundberg. 2013. Novel aspects of dietary nitrate and human health. *Annu. Rev. Nutr.* 33: 129-159.
- White, D. C., M. P. Bryant, and D. R. Caldwell. 1962. Cytochrome-linked fermentation in *Bacteroides ruminicola*. *J. Bacteriol.* 84: 822-828.
- Wiesenburg, D. A., and N. L. Guinasso. 1979. Equilibrium solubilities of methane, carbon monoxide, and hydrogen in water and sea water. *J. Chem. Eng. Data* 24: 356-360.
- Williams, A. G., and G. S. Coleman. 1997. The rumen protozoa. In: P. N. Hobson and C. S. Stewart (eds.) *The rumen microbial ecosystem* Blackie Academic & Professional, London, UK. p 73-139.
- Wolin, M., T. Miller, and C. Stewart. 1997. Microbe-microbe interactions. In: P. N. Hobson and C. S. Stewart (eds.) *The rumen microbial ecosystem* Chapman & Hall, London. p 467-491.
- Wood, T. A., R. J. Wallace, A. Rowe, J. Price, D. R. Yáñez-Ruiz, P. Murray, and C. J. Newbold. 2009. Encapsulated fumaric acid as a feed ingredient to decrease ruminal methane emissions. *Anim. Feed Sci. Technol.* 152: 62-71.
- Wu, G. 2013. *Amino acids: Biochemistry and nutrition*. CRC Press, Taylor & Francis Group, Boca Raton, USA.
- Yarlett, N., G. S. Coleman, A. G. Williams, and D. Lloyd. 1984. Hydrogenosomes in known species of rumen entodiniomorphid protozoa. *FEMS Microbiol. Lett.* 21: 15-19.
- Yarlett, N., Hann A.C., Lloyd D., and A. G. Williams. 1983. Hydrogenosomes in a mixed isolate of *Isotricha prostoma* and *Isotricha intestinalis* from ovine rumen contents. *Comparative Biochemistry and Physiology* 74: 357-364.
- Yarlett, N., A. C. Hann, D. Lloyd, and A. Williams. 1981. Hydrogenosomes in the rumen protozoon *Dasytricha ruminantium* Schuberg. *Biochem. J.* 200: 365-372.
- Yarlett, N., D. Lloyd, and A. G. Williams. 1985. Buyrate formation from glucose by the rumen protozoon *Dasytricha ruminantium*. *Biochem. J.* 228: 187-192.
- Yarlett, N., C. G. Orpin, E. A. Munn, N. C. Yarlett, and C. A. Greenwood. 1986. Hydrogenosomes in the rumen fungus *Neocallimastix patriciarum*. *Biochem. J.* 236: 729-739.
- Yoch, D. C., and R. C. Valentine. 1972. Ferredoxins and flavodoxins of bacteria. *Annu. Rev. Microbiol.* 26: 139-162.
- Yuan, Z. P., C. M. Zhang, L. Zhou, C. X. Zou, Y. Q. Guo, W. T. Li, J. X. Liu, and Y. M. Wu. 2007. Inhibition of methanogenesis by tea saponin and tea saponin plus disodium fumarate in sheep. *Journal of Animal and Feed Sciences* 16: 560-565.
- Zaiß, U., and H. Kaltwasser. 1979. Hydrogenase activity and methanogenesis in anaerobic sewage sludge, in rumen liquid, and in freshwater sediments. *European Journal of Applied Microbiology and Biotechnology* 8.
- Zhou, C. S., W. J. Xiao, Z. L. Tan, A. Z. M. Salem, M. M. Geng, S. X. Tang, M. Wang, X. F. Han, and J. H. Kang. 2012. Effects of dietary supplementation of tea saponins (*Ilex kudingcha* C.J. Tseng) on ruminal fermentation, digestibility and plasma antioxidant parameters in goats. *Anim. Feed Sci. Technol.* 176: 163-169.
- Zhou, Y. Y., H. L. Mao, F. Jiang, J. K. Wang, J. X. Liu, and C. S. McSweeney. 2011. Inhibition of rumen methanogenesis by tea saponins with reference to fermentation pattern and microbial communities in Hu sheep. *Anim. Feed Sci. Technol.* 166-167: 93-100.