Low Emission Feed

Using feed additives to decrease methane production in dairy cows

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SUMMARY

Research into manipulating methane (CH_4) production as a result of enteric fermentation in ruminants currently receives global interest. Using feed additives may be a feasible strategy to mitigate CH_4 as they are supplied in such amounts that the basal diet composition will not be largely affected. The latter is relevant because ruminants have the capacity to convert human inedible feedstuffs into human edible energy and protein. However, the application of CH_4 mitigation feed additives may be hampered by several negative side effects including trade-offs with other environmental impacts, negative effects on animal performance, and lack of persistency of the mitigating effect. The research described in this thesis addresses both the mitigating effect of feed additives as well as its persistency. The main focus was on investigating additivity of the CH_4 mitigating effect of feed additives, on the adaptation of rumen microbes to long term feeding of feed additives, and on exploring the potential of rotational feeding of additives to avoid (or reduce) microbial adaptation.

In an experiment with lactating dairy cows in climate respiration chambers to study potential interactions between the effects of feeding nitrate and docosahexaenoic acid (DHA; C22:6 n-3) on enteric CH_4 production, the effects of nitrate and DHA on CH_4 yield [g/kg dry matter intake (DMI)] and CH_4 intensity [g/kg fat- and protein- corrected milk (FPCM)], were additive (**Chapter 2**). Nitrate decreased CH_4 irrespective of the unit in which it was expressed, and the average decline in CH_4 emission corresponds to 85% of the stoichiometric potential of nitrate to decrease CH_4. Feeding DHA had no effect on CH_4 yield, but resulted in a higher CH_4 intensity, because of milk fat depression. The interaction effect between nitrate and DHA on fiber digestibility indicated that negative effects of nitrate on apparent total tract digestibility of nutrients were alleviated by DHA, probably due to an altered feed intake pattern.

Using an isotope measurement protocol in the same study, it was demonstrated that effects of nitrate as a CH_4 mitigating feed additive on fiber degradation in the rumen can be detected by evaluating diurnal patterns of ^{13}C enrichment of exhaled CO_2 (**Chapter 3**).

Feeding nitrate, but not DHA, resulted in a pronounced increase in ^{13}C enrichment of CO_2 in the first 3 to 4 h after feeding only. Results support the hypothesis that effects of a feed additive on the rate of fiber degradation in the rumen can be detected by evaluating diurnal patterns of ^{13}C enrichment of CO_2. A prerequisite for this detection method is that the main ration components differ in natural ^{13}C enrichment (e.g., C3 and C4 plants), and in content of the nutrients that are expected to be involved in a shift in fermentation (e.g., starch and fiber) or in degradability of a nutrient.

In a combined in vivo and in vitro trial, the adaptation to CH_4 mitigating feed additives, viz. an essential oil blend or lauric acid (C12:0), compared with a control diet was first investigated using the in vitro gas production technique during the period that lactating cows were adapting to certain feed additives (**Chapter 4**). Rumen fluid was collected from each cow at several days relative to the introduction of the additives in the diets and used as inoculum for the gas production experiment with each of the three different substrates that reflected the treatment diets offered to the cows. The feed additives in the donor cow diet had a stronger effect on in vitro gas and CH_4 production than the same additives in the incubation substrate. From day 4 onwards, the C12:0 diet persistently reduced gas and CH_4 production, total volatile fatty acid concentration, acetate molar proportion and in vitro organic matter degradation, and increased propionate molar proportion. In contrast, in vitro CH_4 production was reduced by the essential oils diet on day 8, but not on days 15 and 22. In line with these findings, the molar proportion of propionate in fermentation fluid was higher, and that of acetate smaller, for the essential oils diet than for the control diet on day 8, but not on days 15 and 22. Overall, the data indicate a transient effect of the essential oils on CH_4 production, which may indicate microbial adaptation, whereas the CH_4 mitigating effect of C12:0 persisted. It is recommended that this phenomenon is considered in the planning of future studies on the mitigation potential of feed additives in vitro.

In a follow-up in vivo study, it was investigated whether the alternate feeding of two CH_4 mitigating feed additives with a different mode of action (viz. C12:0 and a blend of essential oils) would result in a persistently lower CH_4 production compared to feeding a

single additive over a period of 10 weeks. The experiment comprised a pre-treatment period and three two-week measurement periods, with two periods of 2 weeks in between in which CH_4 emission was not measured. Cows received either continuously the essential oil blend, or both the essential oil blend and C12:0 following a weekly rotation schedule (**Chapter 5**). Both CH_4 yield and CH_4 intensity changed over time, but were not affected by treatment. Methane yield and intensity were significantly lower (12 and 11%, respectively) in period 1 compared with the pre-treatment period, but no significant difference relative to the pre-treatment period was observed in period 3 (numerically 9 and 7% lower, respectively) and in period 5 (numerically 8 and 4% lower, respectively). These results indicate a transient decrease in CH_4 yield and intensity in time, but no improvement in extent or persistency of CH_4 reduction due to rotational feeding of essential oils and C12:0 in lactating dairy cows. However, there were indications that the concept of rotation may be effective and warrants further investigation.

The additives and concepts tested in this thesis are applied under specific experimental conditions. More mechanistic understanding is required to predict the response of the same additives when supplemented to other basal diets or cows in a different physiological state. Trade-offs in environmental impact, and effects on cow health and performance, and on milk processing parameters and food safety are important aspects to consider in future research on the application of feed additives as CH_4 mitigation strategy.

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Chapter 1

General introduction

GENERAL INTRODUCTION

Methane research

Research into manipulating methane (CH_4) production as a result of enteric fermentation in ruminants currently receives global interest (Hristov et al., 2013b). Approximately 90% of total enteric CH_4 production in ruminants, originates from rumen fermentation of feedstuffs, which implies that nutrition can have a large impact on total CH_4 emissions. For this reason, the topic of nutritional strategies to reduce CH_4 emissions from ruminants has been the subject of several qualitative and quantitative reviews (see Hristov et al., 2013b,c for a recent overview in which more than 900 studies on the mitigation of direct nitrous oxide (N_2O) and CH_4 emissions were reviewed).

Metabolizable energy (ME) and Net energy (NE) systems are widely used in feed evaluation for cattle. The ME is the heat of combustion (gross energy; GE) of feed, minus the energy in faeces, urine and gases. To accurately determine ME, losses of energy in CH_4 have to be measured. Methane represents, on average, a loss of 6.5% of GE, but with a wide range (2-12% of GE; Johnson and Johnson 1995). Initially, research into manipulating CH_4 production was related to the loss of GE represented by CH_4. However, more recently the research focus shifted from enteric CH_4 as an inefficiency in animal production, towards the contribution of CH_4 to global greenhouse gas emissions (see Hristov et al., 2013b,c).

Metrics to express enteric methane production in ruminants

The effect of a mitigation strategy may vary across different units in which enteric CH_4 production can be expressed. As discussed by Hristov et al. (2013b), metrics used to quantify emissions should be standardized. The commonly used CH_4 yield factor that expresses CH_4 production as a percentage of GE intake (GEI) does for example not adequately describe the impact of changes in nutrient composition of the diet. Ellis et al. (2010) explained that using a GEI based calculation cannot distinguish between an increased dry matter intake (DMI) or increased dietary fat content. Both scenarios may result in the same GEI value, but the effect on CH_4 production may differ.

As most of the CH_4 production originates from rumen fermentation, less fermentation will consequently lower the total CH_4 production per day. Less fermentation of feed in the rumen may lower the amount of available nutrients to the animal, and consequently animal productivity. Thus, if a mitigation strategy negatively affects animal performance then CH_4 production rate in g/d may decrease whilst CH_4 production in g/kg DMI and g/kg fat- and protein-corrected milk (FPCM) may actually increase. In the context of global food supply and efficient use of resources, it is important to consider the latter two units, which are often referred to with the terms CH_4 yield and intensity, respectively. The focus in this thesis will also be on lowering CH_4 production per kg DMI and per kg FPCM produced.

Function of methanogenesis in ruminants

Before proposing any CH_4 mitigation strategy, it is important to understand the function of methanogenesis in ruminant animals. For digestion of the fibrous feedstuffs that are typical for ruminant diets, the animals largely depend on the rumen microbial ecosystem. Microbial fermentation in the rumen yields volatile fatty acids (VFA) and microbial protein, which are quantitatively important sources of energy and protein for the animal.

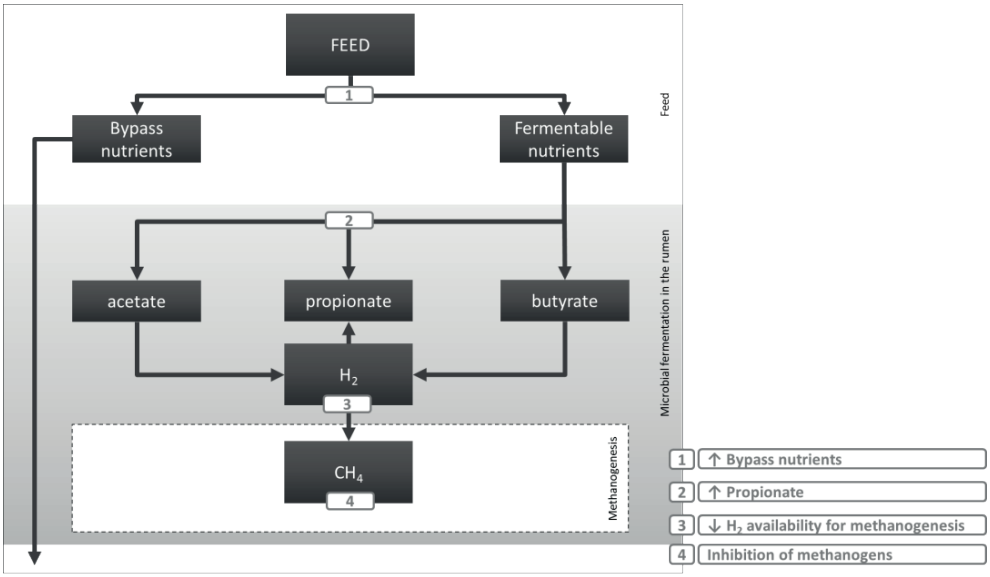

Figure 1.1. Simplified representation of causal factors to decrease methane production in the rumen.

During microbial fermentation of feedstuffs, also hydrogen (H_2) is produced. Methanogens (Archaea) are a specific group of rumen microbes that use carbon sources like carbon dioxide (CO_2), formate and methyl groups together with H_2 to form CH_4. By doing so, a low redox potential is maintained in the rumen. The latter is important to maintain proper rumen fermentation, because an increased H_2 pressure in the rumen would inhibit re-oxidation of reduced enzymatic co-factors (NADH, NADPH and FADH). As a consequence, the rate of rumen fermentation would cease as well (McAllister and Newbold, 2008). Given the crucial role of methanogenesis in supporting adequate conditions for rumen fermentation, any strategy that inhibits the production of CH_4 should provide an alternative H_2 removal pathway (McAllister and Newbold, 2008; Van Zijderveld et al., 2010) or lead to less H_2 being produced.

Mechanisms to decrease ruminal methane production

As most of the CH_4 is produced following fermentation of feed in the rumen, nutrition is the factor with the largest impact on CH_4 production. Several options for lowering the production of CH_4 in the rumen are presented in Figure 1.1. The first option is to increase the proportion of nutrients in the diet that bypass rumen fermentation. An example of this strategy is supplementation of fat (Grainger and Beauchemin, 2011) or increasing the amount of bypass starch or protein. Both fat and bypass starch or protein remain unfermented in the rumen but are enzymatically digested in the small intestine. By feeding more bypass starch and protein, less use is made however of the unique capacity of ruminants to convert human inedible biomass into human edible energy and protein.

An option not indicated in Figure 1.1 is the increase of the formation of microbial mass per unit of organic matter fermented, as this will lower VFA and CH_4 production. Volatile fatty acids are the most important end products of rumen fermentation, as these provide approximately two-third of the required energy for maintenance, production and/or growth. Acetate, propionate and butyrate are quantitatively the most important VFA formed in the rumen. A shift in the profile of VFA formed towards more propionate is the second option indicated in Figure 1.1, as production of acetate and butyrate releases H_2 in the rumen environment, whereas propiogenesis is a H_2 consuming process. Rumen

degradable starch is mainly a propionate precursor, thus increasing the amount of rumen degradable starch could in theory lower CH_4 production. However, both Hassanat et al. (2013) and Van Gastelen et al. (2015) suggested that based on their experimental observations, a minimum starch level is required to achieve a reduction in CH_4 production. Effects of dietary starch on CH_4 emissions in dairy cows were extensively investigated in the PhD work of Hatew (2015) who also concluded that starch contents were too low to obtain a reduced methane yield.

Another way to stimulate propionate formation connects to option number 3 indicated in Figure 1.1, which is lowering the amount of H_2 available for methanogenesis. This can be achieved by directing fermentation processes towards alternative H_2 consuming pathways other than by altering dietary fermentable substrates, such as by propiogenesis, reduction of carboxylic acids, nitrate- or sulfate reduction, and biohydrogenation of fatty acids. However, the quantitative importance of these pathways is variable (Ellis et al., 2008; Martin et al., 2010).

The fourth option indicated in Figure 1.1 is the inhibition of methanogens, not indirectly by lowering substrate availability, but directly upon feeding compounds that are inhibitory to methanogens. Recently, the compound 3-nitrooxypropanol (3NOP) received a lot of attention as a newly developed mitigation strategy. The compound was specifically designed to inhibit methyl coenzyme-M reductase, which is the enzyme that catalyzes the last step of methanogenesis in the rumen. As reviewed by Latham et al. (2016), several in vivo experiments have been conducted to evaluate the effect of 3NOP on CH_4 production in dairy and beef cattle. There seems to be a strong and repeatable mitigating effect, although the size of this effect varies across studies. As this additive was not yet available for research at the start of this PhD project, it could not be considered as mitigation strategy to study in the experiments described here.

Why feed additives?

Feed additives may be a viable mitigation strategy as they are usually only supplied in small amounts to the animal. In this way, the basal diet composition will not be largely

affected. The latter is relevant, because ruminants have the capacity to convert human inedible feedstuffs into human edible energy and protein. According to Regulation (EC) No 1831/2003 on additives for use in animal nutrition, feed additives can be defined as substances, micro-organisms or preparations, other than feed material and premixtures, which are intentionally added to feed or water in order to perform, in particular, one or more of the following functions:

1. favourably affect the characteristics of feed,
2. favourably affect the characteristics of animal products,
3. favourably affect the colour of ornamental fish and birds,
4. satisfy the nutritional needs of animals,
5. favourably affect the environmental consequences of animal production,
6. favourably affect animal production, performance or welfare, particularly by affecting the gastro-intestinal flora or digestibility of feedingstuffs, or
7. have a coccidiostatic or histomonostatic effect.

In the context of this thesis, the fifth characteristic is the target function of the feed additives, but obviously a mitigating feed additive should not negatively affect the characteristics listed under 1, 2, 4, and 6.

Feed additives with potential to decrease methane production

The focus in this thesis will be on three categories of feed additives with potential to decrease CH_4 production:

1. Alternative electron sinks
2. Fat/fatty acids
3. Essential oils

Alternative electron sinks

Chemical reactions, whether carried out by microbes or not, are in general subject to kinetic, or thermodynamic regulation. Kinetic regulation is based on the presence and concentration of the required substrate, whereas thermodynamic regulation can be described as the formation of reaction (end) products based on the ratio between substrate and end product. Kinetic advantage of an alternative H_2 consuming pathway to

methanogenesis in the rumen depends on the H_2 affinity constant (K_m), which should be low (Ellis et al., 2008). Thermodynamic regulation of chemical reactions in the rumen is based on the question whether it is energetically favourable for the reaction to occur. This can be quantified as the change in Gibbs free energy (ΔG). The change in energy under standardized conditions is expressed as ΔG°. A negative ΔG° value indicates that a reaction may occur spontaneously.

Theoretically, the pathway in which carboxylic acids like malate and fumarate are reduced by rumen microbes as precursors of propionate is energetically more favourable (ΔG^0 = -63.6 KJ/mole H_2) than methanogenesis (Ungerfeld et al., 2007; Ellis et al., 2008)[1]. Although the reduction of carboxylic acids is energetically more favourable, the K_m related to these reduction pathways is much higher compared to the K_m of methanogens (Asanuma et al., 1999; Ungerfeld and Kohn, 2006). Moreover, it was demonstrated by Van Zijderveld et al. (2011b) that dietary supplementation of calcium fumarate in concentrations that could be fed in practice did not reduce enteric CH_4 production. It was discussed by the authors that calcium fumarate is not completely converted to propionate but also to acetate, with the latter conversion being a H_2 producing pathway that makes the reduction of fumarate less H_2 consuming. Given the costs and poor palatability of calcium fumarate, it was concluded that the dietary concentrations of fumarate that would be required to achieve a significant CH_4 reduction are too high for practical use. It was also discussed by Van Zijderveld et al. (2011b) that only a few studies had observed a lower CH_4 production upon feeding fumarate (Bayaru et al., 2001; Wallace et al, 2006), but that their results actually were in line with several other studies in which also no effect of fumarate on CH_4 production was found (Beauchemin and McGinn, 2006; Kolver and Aspin, 2006; Molano et al., 2008).

Other pathways with potential of outcompeting methanogenesis are the reduction pathways of sulfate and nitrate. Sulfate-reducing microbes in the rumen have a lower K_m and H_2-threshold compared to methanogens and the sulfate reduction pathway is also

[1] Note: The standardized conditions used to calculate ΔG° differ from the rumen environment, and caution should be taken in drawing firm conclusions related to reaction processes in the rumen based on ΔG°.

energetically slightly more favorable (ΔG^0 = -21.1 KJ/ mole H_2) (Ungerfeld and Kohn, 2006; Ellis et al., 2008). However, the limiting factor in this pathway is the Sulfur (S) concentration of the ration that would be required to substantially reduce CH_4. A potential risk of high S intake is S-associated polioencephalomalacia. This neurological condition is caused by excessive production and absorption of ruminal hydrogen sulphide (H_2S), which is the final product of sulfate reduction. Production and absorption of ruminal H_2S are influenced by S source, total S intake and state of S-reducing ruminal microbes (Dewhurst et al., 2007). Excess H_2S in the rumen head space is released by eructation and subsequent inhalation and systemic absorption can occur (Gould, 1998). The necessary dietary amount of sulfate, required to substantially reduce CH_4 production (Van Zijderveld et al., 2010), exceeds the safety limits set for ruminant diets (NRC, 2001), and, therefore, sulfate is not suitable as a sole H_2 sink.

The use of nitrate as alternative H_2 acceptor was proven to effectively reduce CH_4 production in vivo in sheep (Van Zijderveld et al., 2010), and a persistent effect was also shown in vivo in lactating dairy cows (Van Zijderveld et al., 2011c). However, its use as a mitigating additive may also result in undesirable side effects, which will be discussed later.

Fat and fatty acids

A mitigating effect of fat on CH_4 production has been observed in a large number of studies, but the duration of this effect is not consistent across studies (Grainger and Beauchemin, 2011). Dietary fat is thought to have an influence on CH_4 production by several mechanisms (Martin et al., 2010). Indirect effects of dietary fat on CH_4 production may be found as a result of a reduction in DMI or a dilution of the fermentable organic matter, as fat is not fermented in the rumen but after outflow from the rumen highly digestible in the intestine. Moreover, specific fatty acids may have a direct negative effect on methanogens (medium chain fatty acids) or on cellulolytic bacteria and protozoa (polyunsaturated fatty acids). Utilization of H_2 with biohydrogenation of unsaturated fatty acids reduces the amount of H_2 available for methanogens, but this is quantitatively of minor importance. The meta-analysis of Patra (2013) showed that fat supplementation

also resulted in a linear increase in propionate as proportion of total VFA. As propionate acts as a H_2 sink, this contributes to the mitigating effect of fat supplementation.

A meta-analysis, in which data from in vivo studies in the practical range of dietary fat concentration in ruminant diets (<80 g fat/kg DM) were used to investigate the effects of dietary fat on CH_4 production, showed a strong negative relationship between dietary fat concentration and production of CH_4 (-1 g CH_4/kg DMI per 1% increase of fat in feed DM), but no effect of the fatty acid profile of dietary fat on CH_4 production could be established (Grainger and Beauchemin, 2011). However, the meta-analysis by Patra (2013) showed that CH_4 emissions were not affected by saturated fatty acid concentration in the diet, whereas concentrations of mono- and polyunsaturated fatty acids significantly decreased CH_4 emissions (g/kg DM). It was noted that lauric acid (C12:0) and linolenic acid (C18:3) exerted a strong inhibitory effect on CH_4 production (g/kg DM) compared with other fatty acids. The extent of CH_4 reduction by C12:0 was affected by the non-fiber carbohydrate content of the diet. The dataset of Patra (2013) comprised a larger number of observations than the one of Grainger and Beauchemin (2011), which may explain the contrasting results of both studies. The magnitude of the CH_4 supressing effect of fat supplementation may vary across species and the mitigation effect is likely to be stronger in sheep than in cattle (Grainger and Beauchemin, 2011; Patra, 2014).

Essential oils

Essential oils are plant secondary metabolites that are responsible for specific plant characteristics as flavour and fragrance (Benchaar and Greathead, 2011). The precise mode of action may vary between different essential oils but, generally speaking, they all exhibit some antimicrobial activity. In a recent review by Benchaar and Greathead (2011), it was concluded that some essential oils (derived from garlic and cinnamon) show in vitro a reduction of CH_4 production, but these results have not been confirmed in vivo. Although no CH_4 was measured, Benchaar et al. (2008) observed for example no effect of cinnamaldehyde (1 g/cow/d; 43mg/kg DMI) on pH, total VFA concentration and molar proportions of individual VFA in the rumen of lactating dairy cows. In a recent study by Benchaar (2015), feeding cinnamon oil, cinnamaldehyde, or monensin to dairy cows did

not lower CH_4 production determined with the SF_6 technique. In vitro, promising results have been obtained using other plant extracts and essential oils with potential to be added to a concentrate-based diet (Durmic et al. 2014). Hristov et al. (2013a) observed an in vivo decrease in CH_4 production upon feeding oregano leaves to dairy cattle, but measurements were only taken until 8 h after feeding. Therefore, it is not known if the effect was of the same size on a 24 h basis. If oregano caused a shift in the moment and rate of fermentation after feeding and a more equally divided CH_4 emissions over a 24 h period, the overall CH_4 production may still have remained rather unaffected.

In summary, mixed results have been reported in the scientific literature and mechanisms underlying the (absence of) effects of essential oils on CH_4 production have not been fully elucidated. Therefore, these compounds require further study before deciding if they have potential to be applied in mitigation strategies.

Issues related to the application of additives with potential to mitigate methane production

General issues

Although all three categories of feed additives, as discussed above, show potential for CH_4 mitigation, it is important to consider potential adverse effects and/or trade-offs before applying them in practice. One of the most evident issues is that a decrease in CH_4 production should not be accompanied by a lower DMI, milk production or milk quality. In this respect it is also important to express CH_4 production not only in g/d, but also relative to DMI and milk production (as discussed earlier in the section on metrics to express CH_4 production).

Another issue is that persistency of a mitigating effect of a feed additive often has not been established in vivo (Hristov et al. 2013b). As noted in the general discussion of the PhD thesis of Van Zijderveld (2011), there is a possibility that the effect of feed additives on CH_4 production is amplified in an in vitro test compared to effects obtained with the same level of feed additive applied in vivo (g additive/kg feed or substrate), because of a

higher concentration of additive relative to the microbial density applied in the in vitro test. Moreover, the microbial population used in in vitro systems may have had insufficient time to adapt to the feed additives as occurs in the in vivo situation, or lack adaptive capacity at all, resulting in a larger CH_4 reduction in vitro than observed in the in vivo situation. Recently, Yáñez-Ruiz et al. (2016) published a review on design, implementation and interpretation of in vitro batch culture experiments to assess enteric CH_4 mitigation in ruminants. Aspects like e.g. donor animal species, use of adapted or non-adapted rumen fluid, composition of the buffer, and buffer:medium ratio all have such a strong influence on the results, that these require a well-described protocol. They also argued that in most cases the research question determines the protocol that is adopted for an in vitro study. Therefore, there may not be a standard protocol for evaluating CH_4 production in ruminants using the in vitro gas production technique. Consequently, effects found in in vitro experiments, need to be interpreted with care, as they may differ from the effects observed in vivo.

Besides factors to be considered at the animal level, also factors along the animal production chain should be taken into account when evaluating feed additive-based mitigation strategies. For example, if a mitigating feed additive reduces CH_4 production at the expense of increased nitrogen emissions into the environment, 'pollution swapping' occurs. As shown by Van Middelaar et al. (2013), conclusions on the potential of a mitigation strategy depend on the level of analysis (animal, farm or chain level). This can be explained by trade-offs in environmental pollution between CH_4 production and other emissions along the production chain. Moreover, Van Middelaar et al. (2014) also determined the cost-effectiveness of three mitigating feeding strategies (viz. feeding linseed oil, feeding nitrate, or feeding grass at an earlier stage of maturity) using a chain level approach, and concluded that all these strategies involve additional costs to the farmer. The economic aspects are important factor adopting mitigation strategies in practice.

Issues related to feeding nitrate

Although nitrate persistently reduces CH_4 production, its use as a feed additive also has some disadvantages. Mixed results have been reported regarding the effect of nitrate on DMI (Lee et al., 2014; Newbold et al., 2014), but it may lower voluntary intake. Moreover, nitrite is an intermediate in the process of reduction of nitrate to ammonia. The process of converting nitrate to nitrite in the rumen occurs rapidly whereas the conversion of nitrite to ammonia occurs at a slower rate in non-adapted animals (Allison and Reddy, 1984). Nitrite in the rumen is absorbed through the rumen wall into the bloodstream, where it may cause oxidation of hemoglobin to methemoglobin, thereby inhibiting oxygen transport. However, gradual adaptation to increasing levels of dietary nitrate may prevent the accumulation of nitrite and the occurrence of methemoglobinemia (Van Zijderveld et al., 2010; Van Zijderveld et al., 2011c). As mentioned in the previous paragraph, nitrate is currently not cost-effective as a mitigation strategy, and also pollution swapping is a concern (Van Middelaar et al., 2014). Furthermore, Petersen et al. (2015) found that increasing dietary nitrate, also increases N_2O emission in cows, which is considered to be a more potent greenhouse gas than CH_4.

Issues related to feeding fat and fatty acids

As discussed by Hristov et al. (2013b), expressing the response to dietary fat as CH_4 /kg DMI (Grainger and Beauchemin, 2011) does not account for reduced DMI or milk production upon fat supplementation. In case of negative DMI and milk production responses, more (replacement) animals would be required to produce the same amount of milk which increases emissions, making fat supplementation a less effective mitigation strategy. Increasing dietary fat concentrations above 5-6% of dietary DM increases the risk of negative effects on DMI, fiber digestion, milk production and milk composition (NRC, 2001). If fiber degradation is impaired, both DMI and milk fat concentration might decrease, and such adverse effects upon feeding fat have been reported from quantitative reviews (Grainger and Beauchemin, 2011; Patra, 2013).

Van Middelaar et al. (2014) investigated the cost-effectiveness of feeding linseed oil as a mitigation strategy. It was concluded by the latter authors that the method was the least

cost-effective for current practice compared to the other strategies that were evaluated (feeding grass harvested at a lower stage of maturity, or nitrate), and that the uncertainty range was large.

Issues related to feeding essential oils

The levels of essential oil addition required to effectively reduce CH_4 production in vivo are likely to inhibit overall rumen fermentation as well. Moreover, microbial adaptation to the presence of essential oils may result in a transient effect on CH_4 only (Cardozo et al., 2004).

Another noteworthy aspect is that essential oils may easily be transferred into the animal product. For example, in the study of Van Zijderveld et al. (2011b) feeding diallyl disulfide (a component of garlic oil) at a level of 200 mg/kg DM resulted in a distinctive garlic taint in the milk whereas CH_4 production was not affected. Such effects relate to another important general aspect that needs to be considered before adopting any feed additive-based mitigation strategy, which is consumer acceptance of animal products.

Search for solutions

Negative effects of feed additives on DMI, milk production and/or milk composition are frequently reported in scientific literature. Therefore, a positive interaction of two mitigating additives would be of interest, as it would allow for a similar decrease in CH_4 emissions using lower doses of the separate additives. Subsequently, the risk of negative effects of the additives on cow health and performance will be alleviated too.

Another complication in the search for feed additive-based mitigation strategies is that the rumen microbial ecosystem may adapt to the use of a certain feed additive. In that case, only a transient reduction of CH_4 emissions can be achieved. This process of adaptation is an important aspect that requires further study. Cardozo et al. (2004) reported a transient effect of plant extracts on fermentation characteristics that disappeared after six days. This result indicates that microbial adaptation can occur after short term exposure. The alternating use of two or more CH_4 reducing feed additives with a different mode of action may alleviate the problem of microbial adaptation in the rumen.

This concept is similar to what is used with agronomical applications, where herbicide rotations are applied as a strategy to prevent or to delay the resistance of weeds against herbicides (Beckie, 2006). Similarly, shuttle programmes with two or more anticoccidial compounds, usually with different modes of action, are widely used to reduce resistance of protozoan parasites in broilers (Chapman, 2001).

If the concept of rotational feeding of additives would also be effective in CH_4 mitigation, a persistent lower CH_4 production could be achieved without the need for a persistent CH_4 reduction by a single feed additive. However, several knowledge gaps need to be addressed before this concept can be tested in vivo. First of all, suitable additives need to be selected based on available knowledge from the scientific literature and in vitro screening of their effect. Second, more information is needed on the size and duration of the mitigating effect of these additives to determine the optimal rotation interval for the inclusion of these additives in the diet.

Research objectives

Development of feed additive-based mitigation strategies has been subject of many research efforts, which will likely continue during the next years. To increase our understanding of the CH_4 reducing potential of feed additives, more detailed information regarding the dynamics and effectiveness of these additives to mitigate CH_4 is required than currently available. Moreover, application of feed additives as mitigation strategy may have negative side effects, in particular reduced animal health and performance, and a lack of persistency of the mitigating effect. The overall aim of the work presented in this thesis is, therefore, to investigate possible solutions to those frequently reported problems in relation to feed additive-based mitigation strategies. The effectiveness and side effects of feed additives may vary depending on the mode of action of the additive, the way it is provided to the animal and whether a single additive is fed or additives are fed in combination. Therefore, the specific research objectives of this PhD project are:

1. To investigate if the effects of two different additives, with different modes of action on CH_4 production and dairy cow performance, are additive or not.
2. To study the in vivo adaptation to potential CH_4 reducing feed additives, using the

in vitro gas production technique.

3. To compare CH_4 production and performance of dairy cows, fed either a single feed additive or two different additives following a rotation schedule.

Outline of this thesis

The work described in this thesis was part of the Low Emission Feed project (Dutch project 'EmissieArm Veevoer'). This project comprised research related to the effects of source and quantity of dietary starch, grass silage and grass herbage quality and feed additives on enteric CH_4 production in dairy cows. As outlined above, the research in this thesis focuses on the effect of feed additives on CH_4 production. **Chapter 2** describes an experiment with the aim to determine whether the effects of nitrate and docosahexaenoic acid (DHA) on CH_4 production and animal performance in lactating dairy cows are additive. Methane reducing feed additives, including nitrate, may adversely affect fiber degradation. **Chapter 3** deals with the hypothesis that negative effects of a feed additive on fiber degradation in the rumen can be detected by evaluating diurnal patterns of ^{13}C enrichment of CO_2. The main ration components should then differ in starch and non-fiber carbohydrate content as well as in natural ^{13}C enrichment, as achieved in this trial. In **Chapter 4**, CH_4 production was evaluated at different time points during the course of microbial adaptation to CH_4 reducing feed additives in vivo, using the in vitro gas production technique and inoculum from cows in the in vivo trial. **Chapter 5** outlines an in vivo study that was conducted to compare CH_4 production of dairy cows that were assigned to either continuous feeding of a commercial blend of essential oils or to a weekly rotation in feeding the essential oil blend and C12:0. In **Chapter 6**, the outcomes of the experiments described in the previous chapters are discussed together to derive some overall conclusions and implications of this research project. **Chapter 7** provides an overview of other scientific output, related to the research discussed in this thesis.

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Chapter 2

Feeding nitrate and docosahexaenoic acid affects enteric methane production and milk fatty acid composition in lactating dairy cows

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ABSTRACT

An experiment was conducted to study potential interaction between the effects of feeding nitrate and docosahexaenoic acid (DHA; C22:6 n-3) on enteric CH_4 production and performance of lactating dairy cows. Twenty-eight lactating Holstein dairy cows were grouped into 7 blocks of 4 cows. Within blocks, cows were randomly assigned to 1 of 4 treatments: control (**CON**; urea as alternative nonprotein N source to nitrate), **NO_3** [21 g of nitrate/kg of dry matter (DM)], **DHA** (3 g of DHA/kg of DM and urea as alternative nonprotein N source to nitrate), or **NO_3 + DHA** (21 g of nitrate/kg of DM and 3 g of DHA/kg of DM, respectively). Cows were fed a total mixed ration consisting of 21% grass silage, 49% corn silage, and 30% concentrates on a DM basis. Feed additives were included in the concentrates. Cows assigned to a treatment including nitrate were gradually adapted to the treatment dose of nitrate over a period of 21 d during which no DHA was fed. The experimental period lasted 17 d, and CH_4 production was measured during the last 5 d in climate respiration chambers. Cows produced on average 363, 263, 369, and 298 g of CH_4/d on CON, NO_3, DHA, and NO_3 + DHA treatments, respectively, and a tendency for a nitrate × DHA interaction effect was found where the CH_4-mitigating effect of nitrate decreased when combined with DHA. This tendency was not obtained for CH_4 production relative to dry matter intake (DMI) or to fat- and protein corrected milk (FPCM). The NO_3 treatment decreased CH_4 production irrespective of the unit in which it was expressed, whereas DHA did not affect CH_4 production per kilogram of DMI, but resulted in a higher CH_4 production per kilogram of fat- and protein-corrected milk (**FPCM**) production. The FPCM production (27.9, 24.7, 24.2, and 23.8 kg/d for CON, NO_3, DHA, and NO_3 + DHA, respectively) was lower for DHA-fed cows because of decreased milk fat concentration. The proportion of saturated fatty acids in milk fat was decreased by DHA, and the proportion of polyunsaturated fatty acids was increased by both nitrate and DHA. Milk protein concentration was lower for nitrate-fed cows. In conclusion, nitrate but not DHA decreased enteric CH_4 production and no interaction effects were found on CH_4 production per kilogram of DMI or per kilogram of FPCM.

Key words: methane, nitrate, docosahexaenoic acid, milk fatty acid

INTRODUCTION

Enteric CH_4 production in ruminants has received global interest (Hristov et al., 2013), and various feed additives have been suggested as a nutritional mitigation strategy. Feeding nitrate as alternative electron receptor effectively decreases CH_4 production in sheep (Van Zijderveld et al., 2010), and a persistent effect was shown in lactating dairy cows (Van Zijderveld et al., 2011). A sudden inclusion of high concentrations of nitrate in ruminant diets may result in a condition known as methemoglobinemia, which decreases the oxygen carrying capacity of the blood. Symptoms of nitrate toxicity depend on the level of methemoglobin in the blood and may include reduced intake and performance, brown discoloration of mucosae, and even death (Bruning-Fann and Kaneene, 1993). When animals are gradually adapted to higher concentrations of nitrate in their diets, no signs of (sub)clinical methemoglobinemia were observed (Van Zijderveld et al., 2010, 2011; Lee and Beauchemin, 2014).

Supplementation of fat to ruminant diets also lowers CH_4 production (Grainger and Beauchemin, 2011). Specific fatty acids (**FA**) have been evaluated for their effect on rumen fermentation, and docosahexaenoic acid (**DHA**; an n-3 FA; C22:6 n-3) has been shown to have a particularly marked effect on microbial metabolism in the rumen (Boeckaert et al., 2008a). Micro-algae enriched in DHA have been shown to decrease CH_4 production in vitro (Fievez et al., 2007), but this could not be confirmed in vivo (Moate et al., 2013).

The VFA profile in rumen fluid may shift toward more acetate when nitrate is fed, whereas DHA may cause a shift toward a larger relative proportion of propionate (Boeckaert et al., 2008b; Guyader et al., 2015). Propionate production is an H_2-consuming process and can therefore decrease CH_4 production. Because nitrate and DHA have different mechanisms of affecting ruminal methanogenesis, we hypothesize that their effects on CH_4 production are additive. An additive, or positive, interaction effect of the 2 additives would be of interest because it would allow for a similar decrease in CH_4 emissions using lower doses of the separate additives. The latter would alleviate the risk of negative effects of the additives on cow health and performance. Moreover, feeding DHA to lactating dairy cows

has been reported to increase the proportions of CLA and DHA in milk fat and decrease the SFA proportion (Boeckaert et al., 2008b). From a human health perspective, such an alteration in milk FA composition is of interest (Shingfield et al., 2013). To the best of our knowledge, the effect of feeding nitrate on milk FA profile is unknown.

The main objective of this study was to investigate whether the effects of nitrate and DHA on CH_4 production and animal performance in lactating dairy cows are additive or not. Milk FA profile is a potential indicator of CH_4 production (van Lingen et al., 2014), and, therefore, the effects of nitrate and DHA fed alone or in combination on milk FA composition were also evaluated.

MATERIALS AND METHODS

Experimental design, animals, and housing

All experimental procedures were approved by the Animal Care and Use Committee of Wageningen University (Wageningen, the Netherlands). The experiment was set up as a completely randomized block design with 4 treatments. Eight primiparous and 20 multiparous lactating Holstein cows (125 ± 16 DIM at the start of the experimental period; mean ± SD) were blocked according to parity, lactation stage, milk production and presence or absence of a previously fitted rumen cannula. Within blocks, animals were randomly assigned to 1 of the 4 experimental diets. One of the 8 cows with a rumen cannula had to be culled because of foot injuries and was replaced by a nonfistulated reserve animal already adapted to the same experimental diet (NO_3).

Animals were housed in a freestall barn from which blocks of 4 cows consecutively entered a 17-d experimental period. This 17-d period consisted of 12 d in tie-stalls, and from 1500 h on d 13 until 0900 h on d 17, cows were housed individually in climate respiration chambers (**CRC**).

Diets and feeding

The experimental diets consisted of 49% corn silage, 21% grass silage, and 30% concentrates on a DM basis. Treatments consisted of a control treatment (CON; no nitrate or DHA added), a nitrate treatment (NO_3; 21 g of nitrate/kg of total DM), a DHA treatment (DHA; 3 g of DHA/kg of total DM), and a treatment including both nitrate and DHA in the diet (NO_3 + DHA; 21 g of nitrate/kg of total DM and 3 g of DHA/kg of total DM). Nitrate, DHA, or both were included in the concentrates (Table 2.1). Diets were balanced for N content by isonitrogenous exchange of nitrate and urea. Cellulose and limestone were added to balance DM and Ca content of the concentrate mixtures. DHAgold (DSM Nutritional Products, Columbia, MD) was exchanged against wheat because of the similar CP content. The chemical composition of DHAgold was described by Boeckaert et al. (2007) where the DHA content was 198 g/kg of DM. In the present study, DHA content of DHAgold was 254 g/kg of DM. Chromium oxide (1.7 g/ kg of DM) was included in all concentrates to estimate total-tract diet digestibility of energy and nutrients. Diets were offered to the cows as TMR (Table 2.2). Drinking water was continuously available during the entire experiment.

All animals that were assigned to either the NO_3 or the NO_3 + DHA treatment, including 2 reserve animals, were gradually adapted to the experimental level of dietary nitrate (21 g/kg of DM) over a period of 21 d. Cows were group-fed once daily around 0900 h and received 25% of the experimental dose of dietary nitrate during the first week, followed by incremental steps of 25% per week and thereafter all cows received the full experimental dose of dietary nitrate. No DHA was fed during this period of adaptation to increasing levels of dietary nitrate.

During the experimental periods, cows were fed individually with 2 equal portions offered twice daily (at 0600 and 1600 h). A mixture of grass silage and corn silage was prepared twice weekly and weighed into crates that were stored in a cooling room (±7°C). The concentrates were in meal form and weighed separately into buckets and manually mixed into the roughage mixture at the moment of feeding. Until d 9 of the tie-stall period, each block of cows had free access to feed. Thereafter, DMI within a block was restricted to 95%

Table 2.1. Ingredient composition (g/kg of DM) of the experimental concentrates containing no treatment additive (CON), nitrate (NO_3), docosahexaenoic acid (DHA), or NO_3 + DHA as feed additives

Ingredient	CON	NO_3	DHA	NO_3 + DHA
Wheat	194	194	155	155
Dry, ground corn	145	145	145	145
Beet pulp	165	165	165	165
Formaldehyde-treated soybean meal	321	321	321	321
Molasses	33	33	33	33
Trace mineral and vitamin premix	9	9	9	9
Monocalcium phosphate	17	17	17	17
NaCl	17	17	17	17
$CaCO_3$	57	—	57	—
Nitrate source[1]	—	98	—	98
Urea	39	—	39	—
DHAgold[2]	—	—	39	39
Cellulose	2	—	2	—
Cr_2O_3	1.7	1.7	1.7	1.7

[1] $5Ca(NO_3)2NH_4NO_310H_2O$, containing 75% nitrate.

[2] DHAgold (DSM Nutritional Products, Columbia, MD) = dried, whole cell algae product (seaweed meal), containing 25.4% DHA; trademark of Martek Biosciences Corporation, Royal DSM NV.

of that of the animal with the lowest voluntary DMI between d 5 and 8, while ensuring that none of the animals in the block was restricted to less than 80% of its voluntary DMI.

Measurements, sampling, and laboratory analyses

Methane was measured in CRC with a volume of 35 m^3 (for details of CRC, see van Gastelen et al., 2015). Briefly, temperature in the chambers was set at 16°C and the relative humidity was maintained at 65%. The ventilation rate was 43 m^3/h per chamber, inlet and exhaust air of each compartment was sampled at 10 min intervals, and the light schedule allowed for 16 h of light per d, starting from 0530 h onward. Concentrations of CH_4, O_2, and CO_2 in inlet and exhaust air of each compartment were sampled, and ventilation rates were corrected for air pressure, temperature, and humidity to arrive at standard temperature pressure dew point volumes of inlet and exhaust air. Heat production rates were calculated from gaseous exchange (Brouwer, 1965). Cows were weighed immediately after entering and just before leaving the CRC.

Representative samples of all individual TMR components were collected at the moments of feed preparation for measurement periods in the CRC. Orts were collected during the

period that cows were in the CRC. If the amount composed more than 4% of the estimated DM supply, a representative subsample was analyzed for DM and ash content. If the amount was less than 4% of DM supply, composition of the orts was assumed to be similar to the composition of the offered diet. During CRC periods, the total amount of manure was collected and mixed, and a representative subsample was taken for analysis of DM, gross energy (**GE**), and N content. Fecal grab samples were collected at each milking in the CRC for analysis of DM, GE, N, crude fat, starch, NDF, ash, and chromium content to estimate apparent total-tract digestibility of nutrients. Samples were stored at −20°C pending analysis. After thawing, samples were dried at 60°C until constant weight and ground to pass a 1-mm screen. The N concentrations in manure and of roughages were determined in fresh material. For the determination of NH_3 content, fresh silage samples were deproteinized by the addition of 10% (wt/vol) trichloroacetic acid solution followed by centrifugation. Subsequently, indophenol blue was formed using the Berthelot reaction with phenol and hypochlorite in an alkaline solution, which was determined spectroscopically at 623 nm. The DM content of air dry samples was gravimetrically determined by drying at 103°C until constant weight (ISO 6496; ISO, 1999b). Ash was determined after combustion at 550°C (ISO 5984; ISO, 2002). Crude protein content was calculated as N × 6.25, where N was determined using the Kjeldahl method with $CuSO_4$ as catalyst (ISO 5983; ISO, 2005). Based on findings of Guo et al. (2007), N content of nitrate containing concentrates was corrected assuming a nitrate-N recovery of 53% after Kjeldahl analysis. The nitrate concentrations in all concentrates were analyzed at the Eurofins laboratory (Barendrecht, the Netherlands). Briefly, nitrate was extracted from the feed using Milli-Q water and converted into nitrite using a cadmium/copper column. Subsequently, the reaction product formed after combination of nitrite and sulfanilamide in an acidic environment was combined with N-1-naphtylethylene diamine dihydrochloride into a red/purple color, which was measured spectrophotometrically at 550 nm. Nitrite concentration of the original sample was analyzed separately to correct the result for nitrate. Hydrolysis with HCl and extraction with light petroleum was used to determine crude fat content of samples (ISO 6492; ISO, 1999a). Starch was determined enzymatically (ISO 15914; ISO, 2004). The NDF content of samples was analyzed according to Van Soest et al. (1991) after pretreatment with α-amylase, but without sodium sulfite.

Table 1.2. Average analyzed chemical composition of TMR ingredients (corn silage, grass silage, and concentrates) and calculated composition of complete TMR for the control (CON) diet and diets with nitrate (NO_3), docosahexaenoic acid (DHA), or NO_3 + DHA as feed additives (g/ kg of DM unless otherwise stated)

	Roughages		Concentrates				TMR			
Item	Corn	Grass	CON	NO_3	DHA	NO_3+DHA	CON	NO_3	DHA	NO_3+DHA
Inclusion	490	210	300	300	300	300	-	-	-	-
DM (g/kg)	326	586	884	874	894	881	454	452	455	453
Gross energy	18.6	18.5	16.4	15.7	16.8	16.1	17.9	17.7	18.0	17.8
Crude Ash	36	76	134	135	135	134	74	74	74	74
CP	78	109	347	345	341	349	165	165	163	166
Crude fat	33	30	22	21	40	31	29	29	34	32
NDF	380	561	165	163	155	177	354	356	351	357
ADF	221	327	72	70	70	69	199	198	198	198
ADL	21	25	7	8	7	9	18	18	18	18
Starch	353	NA	239	231	209	209	245	242	236	236
Sugar	6	130	16	13	16	16	35	34	35	35
Nitrate	NA[3]	NA	0	71	0	72	0	21	0	21

[1]NE_L = 6.2 MJ/kg of DM.

[2]NE_L = 6.9 MJ/kg of DM.

[3]NA = not analyzed.

Methods described by Van Soest et al. (1991) were also used for analysis of ADF content and ADL was analyzed using sulfuric acid (Robertson and Van Soest, 1981). An adiabatic bomb calorimeter (IKA-C700, Janke and Kunkel, Heitersheim, Germany) was used for determination of GE content (ISO 9831; ISO, 1998). Chromium contents of concentrates and feces were analyzed using atomic absorption spectrophotometry (Williams et al., 1962).

Milk Production and Milk Composition

Cows were milked twice daily (0600 and 1600 h) throughout the entire experiment. Milk production was recorded at each milking. A subsample of milk from each milking in the CRC was analyzed for fat, protein, lactose, and GE, and N and MUN content were analyzed in a pooled sample from all milkings in the CRC (5 g/kg of milk produced) according to methods described by Hatew et al. (2015a). Average milk composition for each cow was calculated from the weighted average of all samples taken during the 72-h measurement period in the CRC. Fat- and protein-corrected milk yield (**FPCM**) was calculated according to the formula FPCM (kg/d) = (0.337 + 0.116 × fat % + 0.06 × protein %) × milk yield (kg/d) (CVB, 2008). For each cow, an additional milk sample was collected (5 g/kg of milk at each

milking in the chambers) and analyzed for milk FA composition through gas chromatography as described by van Gastelen et al. (2015). Milk FA were expressed in grams per 100 g of total FA.

Blood Samples

During the 21 d of pre-experimental period of adaptation to the final inclusion level of dietary nitrate, a blood sample was collected from all 16 cows fed nitrate after each incremental dose of nitrate in the diet (i.e., d 1, 7, 14, and 21 of this pre-experimental period). Blood was collected from the tail vein in heparinized collection tubes at 3 h post feeding. Blood samples were analyzed for hemoglobin (**Hb**) and methemoglobin (**MetHb**) content within 1.5 h after sampling in the laboratory of Hospital Gelderse Vallei (Ede, the Netherlands) using a blood gas analyzer ABL-825 (Radiometer, Copenhagen, Denmark).

Statistical analysis

Data on DMI, milk production, milk composition, and CH_4 production are based on measurements during the last 72 h of the measurement period when cows were in the CRC. For one cow (DHA treatment) only the last 48 h of the measurement period were used, because this cow had an extremely low DMI and water intake during the first 24 h of the measurement period. Two cows (CON and NO_3 treatment) were excluded from the analyses because of a feeding error in the CRC. Energy and N retention and digestibility values were calculated based on the entire period in the CRC and averaged per day. For milk FA composition, values below the detection limit (<0.02 g/100 g of FA) were considered missing values.

All data were analyzed using PROC MIXED (SAS 9.2, SAS Inst. Inc., Cary, NC). The model contained main and interaction effects of dietary treatment factors (nitrate and DHA) as fixed effects and the effect of period (which is equal to block) as a random factor using a variance components (VC) covariance structure. The effect of chamber was initially included as fixed effect in the model, but was removed because it was not significant. Denominator degrees of freedom were estimated using the Kenward-Roger option. Multiple comparisons between treatments were made using the Tukey-Kramer method.

Results are reported as least squares means, and significance of effects was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Methane production and cow performance

The main objective of this study was to examine if the effects of dietary nitrate and DHA on enteric CH_4 production of lactating dairy cows are additive. For CH_4 production in grams per day, a tendency for a nitrate × DHA interaction was found (Table 2.3), showing that the effect of nitrate and DHA is different when combined. This was most likely a result of the lower DMI of cows receiving the NO_3 treatment, despite the restricted feeding regimen. Nevertheless, if DMI would have been equal across all treatments, the CH_4 production per kilogram of DMI might have been slightly higher for cows on the NO_3 treatment, but not to such an extent that it would have altered the overall conclusions of this experiment because the feed intake of the NO_3 treatment is still ~95% of the intake of the other treatments. Decreased feed intake after feeding dietary nitrate to ruminant animals has been reported previously (Newbold et al., 2014; Lee et al., 2015b).

With CH_4 production expressed in grams per kilogram of DMI or grams per kilogram of FPCM, the nitrate × DHA interaction term was not significant, showing an additive effect between nitrate and DHA. Nitrate decreased CH_4 irrespective of the unit in which it was expressed, whereas DHA had no effect on CH_4 per kilogram of DMI or CH_4 per kilogram of digestible OM intake, but resulted in a higher CH_4 production per kilogram of FPCM (Table 2.3). Moate et al. (2013) reported increased CH_4 emissions per kilogram of DMI and per kilogram of ECM in response to increasing levels of DHA in the diet. Cows in the study of Moate et al. (2013) had unrestricted access to roughage, whereas in the present experiment a restricted feeding regimen was applied. The latter may explain the absence of an effect of DHA on DMI in the present study, whereas in the study of Moate et al. (2013), DMI was significantly reduced at higher doses of DHA (22.1, 22.4, 21.3, and 20.5 kg of DMI/d for the treatments receiving 0, 25, 50, or 75 g of DHA/d, respectively). Previously, DHA has been found to reduce CH_4 production in vitro (Fievez et al., 2007), but this

reduction could not be confirmed in vivo by Moate et al. (2013) and in the present trial. Hatew et al. (2015b) showed that effects of starch source and level on in vitro CH_4 production were not observed in vivo in animals adapted to the various starch sources and levels when CH_4 production was expressed per unit of OM intake. We hypothesize that in the present trial the rumen microbial ecosystem adapted to DHA supply resulting in unchanged CH_4 production compared with the control. If 21 g of nitrate/kg of DMI is completely reduced to ammonia, CH_4 emission should be lowered by 5.4 g/kg of DMI based on stoichiometry. With an average CH_4 production of 17.6 and 22.2 g/kg of DMI for cows receiving a diet with and without nitrate, respectively, the average decline in CH_4 reduction corresponds to 85% of the stoichiometric potential to decrease CH_4. This agrees with findings from previous studies in which similar dietary inclusion levels of nitrate were fed to lactating dairy cows (Lund et al., 2014) or beef cattle (Hulshof et al., 2012) and where CH_4 production was lowered by 86% and 87% of the stoichiometric potential, respectively. The present decrease in CH_4 production is higher compared with the study of Van Zijderveld et al. (2011), who found a decrease of 59% of the theoretical potential. The feed intake of cows in the study of Van Zijderveld et al. (2011) was higher (±19 kg of DMI/d) than the DMI of cows in the current experiment (±16 kg of DMI/d). The lower DMI in the present study may have resulted in a longer retention time of feed or fluid, and of nitrate, in the rumen and thus more time for nitrate to be completely reduced to ammonia. Although this argument seems to be in contrast with findings of Lund et al. (2014), who reported a similar decline in CH_4 yield at DMI values above 19 kg/d when nitrate was fed, this contrast may be partly explained by the differences in experimental setup. In the study of Van Zijderveld et al. (2011) methods of adaptation and feed restriction were similar to the present study, whereas in the study of Lund et al. (2014) no feed restriction was imposed and cows were also not gradually adapted to the experimental level of nitrate in their diet. Such differences in experimental setup may have affected rumen metabolism differently. Moreover, based on visual observations in the tie-stalls, cows receiving any of the additive treatments in the present study also seemed to have a more gradual feed intake pattern than cows on the CON treatment. Based on visual observations of the diurnal patterns of the respiration quotient (RQ; data not included), we noticed that the RQ value showed a sharp increase for the CON

Table 2.3. Dry matter intake, milk production, milk composition, and CH4 production of dairy cattle fed the control (CON) diet or diets with nitrate (NO_3), docosahexaenoic acid (DHA), or NO_3 + DHA as feed additives

	Treatment[1]					*P*-value		
Item	CON	NO_3	DHA	NO_3+DHA	SEM	NO_3	DHA	NO_3×DHA
DMI (kg/d)	16.5	15.7	16.5	16.4	0.81	0.020	0.044	0.060
Milk production (kg/d)	27.8	25.1	28.0	28.0	1.64	0.201	0.180	0.228
FPCM (kg/d)[2]	27.9	24.7	24.2	23.8	1.58	0.128	0.062	0.233
Fat (g/kg)	40.9	39.5	29.8	29.4	2.12	0.602	<0.001	0.744
Fat (g/d)	1147	1008	824	814	76.4	0.231	<0.001	0.296
Protein (g/kg)	31.2	30.4	31.0	29.5	0.68	0.047	0.369	0.561
Protein (g/d)	869	765	869	826	53.0	0.030	0.354	0.338
Lactose (g/kg)	44.8	45.6	46.6	46.2	0.69	0.728	0.043	0.281
MUN (mg/dL)	11.4	11.4	13.1	11.2	0.88	0.288	0.393	0.311
CH_4 (g/d)	363	263	369	298	14.5	<0.001	0.016	0.069
CH_4 (g/kg DMI)	22.0	16.9	22.4	18.2	0.52	<0.001	0.086	0.305
CH_4 (g/kg DOMI[3])	31.4	24.7	31.9	25.5	0.69	<0.001	0.352	0.759
CH_4 (g/kg FPCM)	13.1	10.8	15.4	12.6	0.57	<0.001	0.001	0.629
CH_4 (% of GEI[4])	6.8	5.3	7.0	5.7	0.35	<0.001	0.090	0.487

[1]CON (urea as nonprotein N source), NO_3 (21 g of nitrate/kg of DM), DHA (3 g of DHA/kg of DM and urea as nonprotein N source), NO_3 + DHA (21 g of nitrate/kg of DM and 3 g of DHA/kg of DM). For CON and NO_3 treatments n = 6, for DHA and NO_3 + DHA treatments n = 7.

[2]Fat- and protein-corrected milk (FPCM) = (0.337 + 0.116 × fat % + 0.06 × protein %) × milk yield (kg/d) (CVB, 2008).

[3]DOMI = digestible organic matter intake.

[4]GEI = gross energy intake.

treatment shortly after feeding, whereas the other treatments had lower RQ peak values after feeding. This numerical difference supports the visual observations in tie-stalls and CRC that the feed intake pattern was different across treatments. Alteration of feeding behavior as a result of dietary nitrate supplementation has been reported previously for beef calves (Lichtenwalner et al., 1973). Such a difference in feed intake pattern could not be quantified in the present study, but a more gradual feed intake, with smaller portions per meal, may have contributed as well to a longer retention time of nitrate in the rumen. Guyader et al. (2015) fed nitrate (22.5 g/ kg of DMI) to nonlactating cows with an average DMI of 12.3 kg/d and found a decrease in CH_4 production of 5.6 g/kg of DM compared with the control diet. This corresponds to 96% of the stoichiometric potential of 5.8 g/kg of DM. The difference in physiological state (nonlactating), as well as an increased rumen retention time of nitrate as a result of the lower feed intake in the former study, may explain the larger decrease in CH_4 as compared with the present experiment.

Milk production was not affected by dietary treatment, but FPCM production tended to be decreased by DHA as a result of a significantly lower milk fat production (Table 2.3). Several rumen biohydrogenation intermediates, including *trans*-10 FA, increase upon feeding DHA, and after absorption such intermediates may decrease de novo FA synthesis in the mammary gland (Boeckaert et al., 2008b). Feeding DHA decreased SFA concentrations (expressed as g/100 g of total FA) in milk and increased concentrations of PUFA (Table 2.4). The latter is comparable to findings of Boeckaert et al. (2008b) and Moate et al. (2013). To our knowledge, the effect of dietary nitrate on milk FA composition has not yet been reported. Nitrate had no effect on SFA proportion and proportion of MUFA, but increased the proportion of PUFA in milk FA. The proportion of C4:0 in milk FA was increased by feeding nitrate. Unlike other saturated short-chain FA, C4:0 in milk fat does not require acetate for its production as it can be produced directly from β-hydroxybutyrate derived from the blood. Nitrate also increased the proportion of C18:0 in milk fat (Table 2.4), which is indicative for more biohydrogenation in the rumen. This may be a consequence of the aforementioned longer retention time of feed in the rumen as compared with cows on the CON treatment. The proportions of C14:0 *iso* and C15:0 *iso* were also increased by nitrate, whereas CH_4 was decreased. This is in contrast with findings of Castro-Montoya et al. (2011), who reported a positive relationship between *iso*-FA and CH_4 yield. This relationship was associated with the higher abundance of *iso*-FA in fibrolytic microbes (Vlaeminck et al. 2006), which in turn are associated with a higher CH_4 yield. However, feeding nitrate only was observed to decrease total-tract apparent fiber digestion, and the increased levels of C14:0 *iso* and C15:0 *iso* in milk fat, indicative of increased abundance of fibrolytic bacteria, are not in line with the reduced fiber digestion observed when feeding nitrate without DHA. The increase in *trans*-11 FA together with a decline in CH_4 production in cows receiving nitrate is in line with van Lingen et al. (2014). In contrast to feeding nitrate, feeding DHA decreased the proportion of C18:0 in milk fat. This agrees qualitatively with in vitro studies with DHA added to rumen fluid of cows adapted to DHA, where biohydrogenation of C18:2 *trans*-11,cis-15 was hindered and no biohydrogenation of C18:1 *trans*-11 to C18:0 occurred (Vlaeminck et al., 2008). Feeding DHA increased proportions of several MUFA, including C18:1 *trans*-10

Table 2.4. Fatty acid composition of milk from dairy cattle fed the control (CON) diet or diets with nitrate (NO_3), docosahexaenoic acid (DHA), or NO_3 + DHA as feed additives

	Treatment[1]					*P*-value		
Fatty acid				NO_3				NO_3
C4:0	3.98	4.16	3.85	4.42	0.165	0.034	0.709	0.249
C6:0	2.40	2.41	2.35	2.33	0.071	0.962	0.367	0.833
C8:0	1.22	1.22	1.25	1.14	0.070	0.426	0.736	0.426
C10:0	2.59	2.54	2.92	2.35	0.457	0.188	0.754	0.264
C12:0	2.88	2.79	3.37	2.68	0.263	0.157	0.472	0.265
C14:0	10.62	10.72	12.10	10.87	0.532	0.295	0.141	0.222
C14:0 *iso*	0.06	0.08	0.05	0.07	0.006	0.004	0.056	0.587
C14:1 *cis-9*	1.07	1.00	1.19	1.14	0.099	0.532	0.198	0.943
C15:0	0.78	0.83	0.86	0.83	0.050	0.850	0.425	0.344
C15:0 *iso*	0.18	0.23	0.18	0.19	0.014	0.034	0.093	0.109
C15:0 *anteiso*	0.30	0.38	0.39	0.40	0.022	0.027	0.009	0.070
C16:0	35.00	32.03	33.73	32.22	1.354	0.080	0.666	0.553
C16:0 *iso*	0.15	0.16	0.16	0.14	0.011	0.902	0.976	0.079
C16:1 *cis-9*	2.20	1.84	1.69	1.59	0.149	0.133	0.018	0.375
C16:1 *trans-9*	0.19	0.23	0.19	0.21	0.016	0.045	0.485	0.562
C17:0	0.51[b]	0.58[a]	0.53[ab]	0.53[ab]	0.020	0.036	0.411	0.029
C17:0 *iso*	0.36	0.40	0.42	0.39	0.023	0.762	0.332	0.185
C17:0 *anteiso*	0.36	0.39	0.43	0.41	0.019	0.731	0.027	0.218
C17:1 *cis-9*	0.29	0.28	0.21	0.22	0.023	0.886	0.003	0.721
C18:0	7.23	8.68	2.11	3.54	0.423	0.003	<0.001	0.977
C18:1 *cis*-9[2]	19.38	19.46	10.20	14.13	1.397	0.054	<0.001	0.063
C18:1 *cis*-12	0.29	0.31	0.13	0.16	0.017	0.097	<0.001	0.919
C18:1 *cis*-13	0.14	0.16	0.23	0.21	0.021	0.951	0.001	0.367
C18:1 *trans*-9	0.16	0.20	0.79	0.82	0.065	0.583	<0.001	0.966
C18:1 *trans*-10	0.30[c]	0.48[c]	5.60[a]	1.90[b]	0.336	<0.001	<0.001	<0.001
C18:1 *trans*-11	0.72	1.24	3.20	4.40	0.421	0.037	<0.001	0.380
C18:1 *trans*-15 + C18:1 *cis*-11	0.77	0.82	1.08	1.15	0.071	0.345	<0.001	0.878
C18:2 *cis*-9, *trans*-11	0.40	0.55	1.35	2.08	0.187	0.027	<0.001	0.126

Table 2.4. continued.

	Treatment[1]					*P*-value		
Fatty acid				NO_3				NO_3
C18:2n-6	1.72	1.86	1.86	1.88	0.080	0.340	0.327	0.509
C18:3n-3	0.32	0.33	0.31	0.31	0.018	0.509	0.348	0.612
C18:3n-6	0.07	0.08	0.07	0.08	0.003	0.002	0.125	0.207
C20:0	0.10	0.12	0.06	0.08	0.007	0.043	<0.001	0.915
C20:1 *cis-11*	0.06	0.06	0.09	0.11	0.008	0.125	<0.001	0.365
C20:2n-6	0.04	0.04	0.05	0.05	0.003	0.690	<0.001	0.155
C20:3n-6	0.07	0.07	0.10	0.11	0.007	0.943	<0.001	0.567
C20:4n-6	0.12	0.12	0.25	0.24	0.010	0.506	<0.001	0.906
C20:5n-3	0.05	0.05	0.15	0.14	0.008	0.494	<0.001	0.494
C22:0	0.04	0.04	0.03	0.04	0.004	0.039	0.376	0.335
C22:5n-3	0.08	0.08	0.18	0.17	0.007	0.094	<0.001	0.873
C22:6n-3	N.D.[3]	N.D.	0.65	0.70	-	-	-	-
C24:0	0.03	0.03	0.04	0.04	0.003	0.411	0.021	0.411
SFA[4]	68.76	67.75	64.84	62.61	1.578	0.282	0.007	0.680
MUFA[5]	25.62	26.14	24.58	26.04	1.440	0.402	0.636	0.688
PUFA[6]	2.87	3.18	4.97	5.76	0.258	0.046	<0.001	0.352
n-6 : n-3 ratio[7]	4.59	4.76	1.82	1.79	0.010	0.472	<0.001	0.327

[a–c]Means within a row with different superscripts differ ($P < 0.05$).

[1]CON (urea as nonprotein N source), NO_3 (21 g of nitrate/kg of DM), DHA (3 g of DHA/kg of DM and urea as nonprotein N source), NO_3 + DHA (21 g of nitrate/kg of DM and 3 g of DHA/kg of DM). For CON and NO_3 treatments, n = 6; for DHA and NO_3 + DHA treatments, n = 7.

[2]C18:1 *cis*-9 consists of the sum of C18:1 *cis*-9 and C18:1 *trans*-12, because these 2 fatty acids could not be separated in the analysis. The pro- portion of C18:1 *trans*-12 is usually negligible.

[3]ND = not detected (detection limit = 0.02 g/100 g of fatty acids).

[4]Sum of SFA reported in this table.

[5]Sum of MUFA reported in this table.

[6]Sum of PUFA reported in this table.

[7]Ratio between the sum of C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, and C20:4n-6, and the sum of C18:3n-3, C20:5n-3, C22:5n-3, and for treat- ments DHA and NO_3 + DHA also C22:6n-3.

and C18:1 *trans*-11. The alteration in milk FA profile is in line with findings of Boeckaert et al. (2008b). However, contrary to the present study, these FA are often associated with a decrease in CH_4 per unit of feed and per unit of FPCM (van Lingen et al. 2014). If the best performing equations (viz. equations 3 and 4) of van Lingen et al. (2014) are used to predict CH_4/kg of DMI and CH_4/kg of FPCM, respectively, from the present milk FA data, a considerable deviation is present between observed and predicted values. Predicted CH_4 production is 20.8, 19.9, 12.4, and 14.8 g/kg of DMI and 12.1, 11.5, 9.7, and 9.4 g/kg of FPCM for the CON, NO_3, DHA, and NO_3 + DHA treatments, respectively. Based on the prediction equations, CH_4 production should be decreased by DHA and nitrate would have almost no effect, which is in contrast with the present observations. These comparisons indicate that relationships between CH_4 production and milk FA profile, obtained on a wide variety of diets (van Lingen et al. 2014), differ from relationships between CH_4 production and milk FA profile when CH_4-mitigating supplements such as nitrate and DHA are included in the diet, and thus also limit the general potential of milk FA to predict CH_4 production. No DHA was detected in milk from cows receiving the CON or NO_3 treatment. The absence of DHA levels above the detection limit of 0.02 g/100 g of FA in milk of cows that were not supplemented with DHA corresponds to the findings of van Valenberg et al. (2013), who investigated milk FA composition of representative Dutch bovine milk samples that were collected weekly for a period of 1 yr. On average 0.67 g of DHA/100 g of FA was detected in milk from cows receiving DHA. The DHA content of the TMR (3 g/kg of DM) resulted in a daily intake of almost 50 g of DHA/cow. This intake is comparable to the 50 g of DHA/cow (D50) dose fed by Moate et al. (2013) who found a similar amount of 0.60 g of DHA/100 g of FA in milk.

Milk protein content was not affected by DHA, but feeding nitrate resulted in a small, but significant reduction in milk protein content and yield (Table 2.3). Dietary nitrate also resulted in a lower protein concentration in the study of Van Zijderveld et al. (2011). However, protein yield was not affected by nitrate in their study in contrast to the present study where protein yield was 796 and 869 g/d for diets with or without nitrate, respectively (P = 0.030). In the Dutch protein evaluation system (DVE/OEB system), DVE indicates digestible feed and microbial true protein digested in the small intestine

(Tamminga et al., 1994). In the current experiment, the calculated DVE supply based on diet composition exceeded 100% of the calculated DVE requirements, indicating that supply of protein did not limit milk protein synthesis. Incomplete reduction of dietary nitrate may decrease the amount of rumen-available N and consequently impair microbial protein synthesis and result in a lower DVE supply than expected based on standard feed values. The resulting lower DVE supply would then negatively affect milk protein yield. However, the actual decline in CH_4 production in the current study was rather close to stoichiometric potential of nitrate, which implies that most of the nitrate must have been reduced to ammonia and has contributed to rumen available N. Alternatively, the negative effect of nitrate on milk protein yield may be related to a decreased supply of gluconeogenic precursors. Nitrate has been shown to increase the acetate:propionate ratio in the rumen (Guyader et al., 2015), which could also affect milk protein content. Rigout et al. (2003) reported an experiment and bibliographical study showing a positive linear relationship between the supply of glucogenic precursors and milk protein content. Glucose is an important factor in signaling pathways thought to regulate milk protein synthesis (Rius et al., 2010). No treatment effects were found for MUN content of milk (Table 2.3), and values were comparable to those found by Van Zijderveld et al. (2011), who fed a similar diet as in the present study.

Blood methemoglobin

The average Hb content (mmol/L) of blood of the 16 cows that were gradually adapted to increasing levels of dietary nitrate was 5.9 on d 1 and 7, and 5.6 on d 14 and 21 of the adaptation period before the experimental period. Blood MetHb (% of total Hb) was on average 1.3% on both d 1 and d 7, 2.5% on d 14, and 3.4% on d 21. The highest MetHb value measured for an individual animal was 11.8% on d 21. This level is substantially below the level of 30% that is considered to cause subclinical methemoglobinemia (Bruning-Fann and Kaneene, 1993).

Table 2.5. Daily energy and N balance of lactating cows fed the control (CON) diet or diets with nitrate (NO_3), docosahexaenoic acid (DHA), or NO_3 + DHA as feed additives

	Treatment[1]					*P*-value		
Item	CON	NO_3	DHA	NO_3+DHA	SEM	NO_3	DHA	NO_3×DHA
Metabolic BW (kg $BW^{0.75}$)	121	117	120	124	5.3	0.904	0.340	0.235
Gross energy intake (kJ/kg $BW^{0.75}$)	2476	2378	2464	2367	109.4	0.083	0.834	0.995
ME intake[2] (kJ/kg $BW^{0.75}$)	1530	1478	1561	1509	10.2	0.196	0.453	0.995
MEI:GEI ratio[3]	61.9	62.2	63.3	63.9	20.9	0.416	0.025	0.830
Methane production (kJ/kg $BW^{0.75}$)	167	124	172	135	17.9	<0.001	0.134	0.598
Heat production (kJ/kg $BW^{0.75}$)	862	873	897	893	26.3	0.838	0.138	0.657
Energy in milk (kJ/kg $BW^{0.75}$)	715	646	632	602	32.0	0.136	0.060	0.533
Energy retention total[4] (kJ/kg $BW^{0.75}$)	-51	-44	32	13	35.6	0.777	0.005	0.549
Energy retention protein[5] (kJ/kg $BW^{0.75}$)	30	41	27	41	71.2	0.048	0.866	0.829
Calculated energy retention fat[6] (kJ/kg $BW^{0.75}$)	-81	-85	5	-28	29.0	0.315	0.001	0.438
N intake (g/kg $BW^{0.75}$)	3.67	3.57	3.60	3.52	0.171	0.244	0.459	0.852
N manure (g/kg $BW^{0.75}$)	2.29	2.22	2.24	2.18	0.101	0.293	0.448	0.911
N milk (g/kg $BW^{0.75}$)	1.14	1.02	1.14	1.02	0.055	0.019	0.946	0.908
N retention[7](g/kg $BW^{0.75}$)	0.20	0.28	0.19	0.28	0.115	0.048	0.866	0.829
N efficiency[8]	31.7	29.0	31.6	29.2	1.111	0.025	0.967	0.891

[1]CON (urea as nonprotein N source), NO_3 (21 g of nitrate/kg of DM), DHA (3 g of DHA/kg of DM and urea as nonprotein N source), NO_3 + DHA (21 g of nitrate/kg of DM and 3 g of DHA/kg of DM). For CON and NO_3 treatments, n = 6; for DHA and NO_3 + DHA treatments, n = 7.

[2]ME intake = gross energy intake – CH4 production – energy in manure.

[3]MEI:GEI = ratio between ME intake and gross energy intake.

[4]Energy retention total = ME intake – heat production – energy in milk.

[5]Energy retention protein = protein gain × 23.6 kJ/g of protein.

[6]Calculated energy retention fat = energy retention total – energy retention protein.

[7]N retention = N intake – N in manure – N in milk – N from condensate that was collected from heat exchanger – N trapped from the outflowing air.

[8]Fraction of N intake that is incorporated in milk (N milk/N intake; in % of N intake).

Energy and nitrogen retention

No NO_3 × DHA interaction effects on energy and N retention were found (Table 2.5). The MEI:GEI ratio was higher for the diets containing DHA (Table 2.5). The calculated energy retention was positive for cows receiving DHA and negative for cows on the CON or NO_3 treatment. The tendency for decreased energy output in milk may explain the positive energy retention of the cows receiving DHA. The absence of a significant effect of nitrate on FPCM production or milk energy output is in line with results from a recent review by Lee and Beauchemin (2014), who also reported that the consistent decline in CH_4 yield by dietary nitrate appears to be without directing additional energy toward animal production.

Nitrogen retention was positive for all treatments (Table 2.5). The average N retention was 28 g/d, which is in line with the generally small positive N retention reported for dairy cattle N balance trials as reviewed by Spanghero and Kowalski (1997). Intake and excretion of N was similar among treatments. As expected based on results for milk protein, the N output in milk and N efficiency of milk production were lower for cows receiving nitrate (Table 2.5). Nitrogen retention was significantly higher for cows receiving nitrate, whereas N in manure was not affected.

Digestibility of nutrients

Supplementation of DHA generally resulted in higher total-tract digestibility of various nutrients (Table 2.6). The higher fat digestibility on treatments with DHA is probably caused by the slight difference in fat content of the TMR with and without DHA (Table 2.2). If fat supplementation is higher, the calculated digestibility values are less affected by fecal excretion of endogenous fat sources (Kil et al., 2010). This difference in dietary fat content could not be prevented in the experimental set-up as exchanging DHA against another fat source would not allow to distinguish between the effect of fat or a specific FA on CH_4 emissions.

Unlike the results for CH_4 production, effects of nitrate and DHA on apparent total-tract digestibility of nutrients were often not additive (Table 2.6). Digestibility of CP was not

Table 2.6. Apparent total-tract digestibility of nutrients in lactating dairy cows fed the control (CON) diet or diets with nitrate (NO_3), docosahexaenoic acid (DHA), or NO_3 + DHA as feed additives

	Treatment[1]					*P*-value		
Digestibility (%)	CON	NO_3	DHA	NO_3+DHA	SEM	NO_3	DHA	NO_3×DHA
DM	73.9	71.2	75.2	75.6	0.72	0.133	0.001	0.051
OM	75.6[ab]	73.2[b]	76.9[a]	77.3[a]	0.67	0.143	<0.001	0.048
CP	70.8	69.7	68.6	69.8	1.10	0.989	0.313	0.265
NDF	61.0[a]	55.3[b]	63.6[a]	65.1[a]	1.31	0.132	<0.001	0.011
Crude fat	70.2	71.3	74.4	76.0	1.37	0.330	0.004	0.884
Starch	99.0	99.3	99.7	99.6	0.18	0.597	0.014	0.193
Gross energy	73.4[a]	70.3[b]	74.6[a]	75.1[a]	0.75	0.099	<0.001	0.027

[a,b]Means within a row with different superscripts differ ($P < 0.05$).

[1]CON (urea as nonprotein N source), NO_3 (21 g of nitrate/kg of DM), DHA (3 g of DHA/kg of DM and urea as nonprotein N source), NO_3 + DHA (21 g of nitrate/kg of DM and 3 g of DHA/kg of DM). For CON and NO_3 treatments, n = 6; for DHA and NO_3 + DHA treatments, n = 7.

different between treatments and does therefore not provide an explanation for the difference in milk protein yield and N utilization for milk production that was observed between treatments with and without nitrate. Moreover, a reduction in DMI and nutrient digestibility was only found for the NO_3 treatment and not for the NO_3 + DHA treatment (Table 2.6). The effect of DHA on NDF digestibility and significance of the interaction term seems mainly to be the result of the low NDF digestibility value obtained for the NO_3 treatment (Table 2.6). The significantly lower NDF digestion may be related to a decreased functioning of cell wall degrading microorganisms as a result of a temporarily increased ruminal concentrations of H_2. Such increases in H_2 concentration after nitrate supplementation have been reported previously (Van Zijderveld et al., 2011; Lund et al., 2014). Accumulation of H_2 in the rumen may impair regeneration of NAD+ from NADH (McAllister and Newbold, 2008), and this may negatively affect cell wall degradation by rumen microbes. Nitrite, as intermediate in the reduction of nitrate to ammonia, decreased in vitro cell wall digestion and inhibited growth of cellulolytic bacteria (Marais et al., 1988) and may also have negatively affected NDF digestibility. However, the MetHb concentrations in blood of cows receiving nitrate were relatively low in the present study, and it is thus less likely that nitrite accumulated to substantial amounts in the rumen. Nevertheless, a possible negative effect of nitrite on fiber digestion cannot be excluded. The findings of Lee et al. (2015b) suggest that a restricted feeding regimen influences the potential adverse effects of nitrate on animal health and performance. Despite the poor

palatability of nitrate, cows may consume relatively large amounts of nitrate in one meal under restricted feeding, which may exert negative effects in the rumen. Lee et al. (2015a) observed no effect of nitrate on NDF digestibility in beef cattle that had free access to feed. However, ADF digestibility was significantly decreased by nitrate, which indicated that also in their study, fiber degradability did not remain completely unaffected. Better NDF degradation in the rumen and thus more fermentation, probably explains the numerically smaller decrease in CH_4 per kilogram of DMI for cows on the NO_3 + DHA treatment as compared with cows on the NO_3 treatment (Table 2.3).

CONCLUSIONS

Additive CH_4-mitigating effects, or a positive interaction, of nitrate and DHA fed together would have allowed for a significant decrease in CH_4 at lower doses of individual additives. Feeding DHA strongly affected milk FA composition, but did not decrease CH_4 production per kilogram of DMI and increased CH_4 production per kilogram of FPCM, whereas nitrate showed a large and consistent decrease in CH_4 production irrespective of the unit in which it was expressed. No interaction effect was found for the effects of nitrate and DHA on CH_4 in grams per kilogram of DMI and CH_4 in grams per kilogram of FPCM. A significant interaction effect between nitrate and DHA on NDF digestibility indicated that negative effects of nitrate on apparent total-tract digestibility of nutrients were alleviated by DHA. Such an interaction effect between nitrate and DHA could be of interest if nitrate is fed to decrease CH_4 production, because a decrease in CH_4 production should not be accompanied by reduced animal performance. Given the significant reductions in milk fat and protein yield by DHA and nitrate, respectively, the current doses of the additives are not recommended for application in practice.

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Chapter 3

Using diurnal patterns of ^{13}C enrichment of CO_2 to evaluate the effects of nitrate and docosahexaenoic acid on fiber degradation in the rumen of lactating dairy cows

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ABSTRACT

Nitrate decreases enteric methane (CH_4) production in ruminants, but may also negatively affect fiber degradation. In this experiment, twenty-eight lactating Holstein dairy cows were grouped into seven blocks. Within blocks, cows were randomly assigned to 1 of 4 isonitrogenous treatments in a 2 × 2 factorial arrangement: Control (CON); NO_3 [21 g of nitrate/kg dry matter (DM)]; DHA [3 g of docosahexaenoic acid (DHA)/kg of DM]; or NO_3+DHA (21 g of nitrate/kg of DM and 3 g of DHA/kg of DM). Cows were fed a total mixed ration consisting of 21% grass silage, 49% corn silage and 30% concentrates on a DM basis. Based on the difference in natural ^{13}C enrichment and neutral detergent fiber and starch content between grass silage and corn silage, we investigated whether a negative effect on rumen fiber degradation could be detected by evaluating diurnal patterns of ^{13}C enrichment of exhaled carbon dioxide. A significant nitrate × DHA interaction was found for neutral detergent fiber digestibility, which was reduced on the NO_3 treatment to an average of 55%, as compared with 61, 64, and 65% on treatments CON, DHA and NO_3+DHA, respectively. Feeding nitrate, but not DHA, resulted in a pronounced increase in ^{13}C enrichment of CO_2 in the first 3 to 4 h after feeding only. Results support the hypothesis that effects of a feed additive on the rate of fiber degradation in the rumen can be detected by evaluating diurnal patterns of ^{13}C enrichment of CO_2. To be able to detect this, the main ration components have to differ considerably in fiber and nonfiber carbohydrate content as well as in natural ^{13}C enrichment.

Key words: feed additives, methane, fiber degradation, ^{13}C enrichment

SHORT COMMUNICATION

Nitrate is among the relatively few feed additives that have been shown to effectively mitigate enteric CH_4 production in ruminants (reviewed by Hristov et al., 2013). However, CH_4 mitigating additives should not adversely affect animal health and performance or quality of animal products to be applicable in practice. Methemoglobinemia is a health risk of feeding nitrate to ruminants, but a gradual introduction of nitrate in the diet alleviates this risk (Van Zijderveld et al., 2011). A growing body of literature also documents the effects of nitrate feeding on animal performance. As discussed by Newbold et al. (2014), nitrate might reduce feed intake and the magnitude of the decrease seems larger on diets that contain more NDF. At least two factors may be associated with this decrease in feed intake. First, nitrite, as an intermediate in the reduction of nitrate to ammonia, is toxic for fibrolytic bacteria in vitro (Marais et al. 1988). Second, nitrate feeding increases hydrogen production in the rumen (Van Zijderveld et al. 2011; Lund et al. 2014; Guyader et al., 2015), which may indicate increased aqueous hydrogen concentrations (Guyader et al. 2015). Increased hydrogen concentration inhibits the regeneration of NAD^+ from NADH (Hegarty and Gerdes, 1999), which may impair metabolism of fibrolytic bacteria. An increased ratio of NADH to NAD^+ will cause a shift from acetate towards propionate formation, partly via the lactic acid pathway. The latter pathway yields less ATP for the fibrolytic microbes. Impaired fibrolytic activity increases retention time of fiber in the rumen and therefore may reduce feed intake.

The effects of nitrate and docosahexaenoic acid (C22:6n-3; **DHA**) on total enteric CH_4 production and milk fatty acid composition in lactating dairy cows in climate respiration chambers (**CRC**) were recently reported by Klop et al. (2016). A negative effect of nitrate on DMI was observed as well as reduced total tract digestibility of fiber on the NO_3 treatment. However, no adverse effect of DHA on total tract fiber digestion was observed. Digestibility of NDF was not reported in other studies in which DHA was supplemented to dairy cows (Boeckaert et al., 2008; Moate et al., 2013). It was hypothesized that the between-treatment differences in fiber digestion would cause variation in diurnal pattern of ^{13}C enrichment of CO_2. When nitrate is supplemented to a diet of which the main

components differ considerably in starch (or another non-NDF carbohydrate source) and NDF content as well as in natural ^{13}C enrichment, an adverse effect on fiber degradation may be detectable from an increased ^{13}C enrichment of exhaled CO_2. In comparison with corn, grass has a lower natural ^{13}C enrichment (Knobbe et al. 2006) and it does not contain starch. When fed as a TMR, changes in the ^{13}C enrichment of CO_2 after a meal hence likely reflect changes in the degradation rate of starch or fiber by the rumen bacteria. The aim of this study was to investigate if a negative effect on ruminal fiber degradation upon feeding nitrate to dairy cattle can be detected by evaluating diurnal patterns of ^{13}C enrichment of exhaled CO_2.

The experimental procedures were approved by the Animal Care and Use Committee of Wageningen University (Wageningen, The Netherlands). Detailed information regarding experimental design, diets, feeding, and measurements was reported by Klop et al. (2016). Briefly, 8 primiparous and 20 multiparous (125 ± 16 DIM at the start of the experimental period; mean ± SD) lactating Holstein dairy cows were divided over 7 blocks based on parity, lactation stage, milk production, and presence or absence of a rumen cannula. Within blocks, cows were randomly assigned to 1 of 4 experimental diets: control (**CON**; no nitrate or DHA added and urea as NPN source), nitrate (**NO_3**; 21 g of nitrate/kg of DM), DHA (**DHA**; 3 g of DHA/kg of DM and urea as NPN source), or nitrate and DHA (**NO_3+DHA**; 21 g of nitrate/kg of DM and 3 g of DHA/kg of DM). The sources of nitrate and DHA were Bolifor CNF (Yara, Norway) containing 75% nitrate, and DHAgold (DSM Nutritional Products, Columbia, MD), a whole cell algae product containing 25.4% DHA, respectively. Feed additives were included in the concentrates and chromium oxide (Cr_2O_3; 1.7 g/kg of DM) was used as external marker to determine apparent total-tract digestibility of nutrients.

Diets were isonitrogenous, offered as TMR, and consisted of 49% corn silage, 21% grass silage, and 30% concentrate on a DM basis. Dietary DM, CP, NDF and starch contents were on average 454, 165, 355 and 240 g/kg of DM, respectively. Due to lameness, 1 of 8 rumen cannulated cows was replaced by a non-cannulated cow already adapted to the same experimental diet (NO_3).

The experimental period lasted 17 d. During the experimental periods, cows were fed individually with equal portions offered twice daily (at 0600 and 1600h). Until d 9, each block of cows had free access to feed. Thereafter, DMI within a block was restricted to 95% of that of the animal with the lowest voluntary DMI between d 5 and 8, while ensuring that none of the animals in the block was restricted to less than 80% of its voluntary DMI. Cows were housed in tie stalls until the afternoon of day 13 and in CRC for the remainder of the experimental period. In the CRC, gaseous exchange (CH_4, O_2, and CO_2) was measured as described by van Gastelen et al. (2015) and $^{13}CO_2$ production was determined as described by Gerrits et al. (2012). Data relate to the last 72 h in the CRC, except for digestibility values calculated over the complete period the cows were in the CRC. Natural ^{13}C enrichment of the TMR components was determined by means of combustion isotope ratio MS (Gerrits et al., 2012).

Data were analyzed using PROC MIXED (SAS Inst. Inc., Cary, NC). Two cows (receiving the CON or NO_3 treatment) were excluded from analysis because of a feeding error in the CRC. Gaseous exchange data, heat production (HP), respiration quotient (RQ), and ^{13}C enrichment of CO_2 were averaged per hour and analyzed using repeated measures ANOVA. Main and interaction effects of nitrate, DHA, and time were included as fixed effects in the model. Period (which equals block) was included as random factor in the model and average values for each hour of the day were treated as repeated measures per cow × treatment combination using a first order autoregressive covariance structure. Multiple comparisons between treatment least squares means for each hour were made using a SLICE statement in the model. Effects were considered significant if $P \leq 0.05$, and trends if $0.05 < P < 0.10$.

Cows receiving nitrate consumed less DM and a tendency for a NO_3 × DHA interaction was observed, because the NO_3 cows consumed less DM than the NO_3+DHA cows. Daily fat- and protein-corrected milk production was not affected by nitrate, and tended to be reduced by DHA (Klop et al., 2016). Distinct responses to meals were observed for HP, RQ, and CH_4, and for ^{13}C enrichment of the CO_2 produced (Figure 3.1). Both HP and RQ

changed over time within day, but no interactions between treatment factors and time were found. The numerically higher RQ and HP peak values for cows on the CON treatment shortly after feeding (Figure 3.1B) can be explained by our visual observations that cows on the CON treatment consumed their meals faster than cows on the other treatments. In line with Van Zijderveld et al. (2011), the CH_4-mitigating effect of nitrate was largest during the first hours postfeeding (Figure 3.1C). The restricted feeding regimen imposed during the measurement period and the more rapid degradation of starch compared with fiber may explain the overall increase in ^{13}C enrichment shortly after feeding. The shorter interval between morning and afternoon feeding (10 h) as compared with the interval between afternoon and morning feeding (14 h) may explain the numerically lower ^{13}C enrichment of CO_2 in the morning (Figure 3.1D). Feeding nitrate, but not DHA, resulted in a pronounced increase in ^{13}C enrichment of CO_2 in the first 3 to 4 h after feeding only, which resulted in a significant nitrate × time interaction.

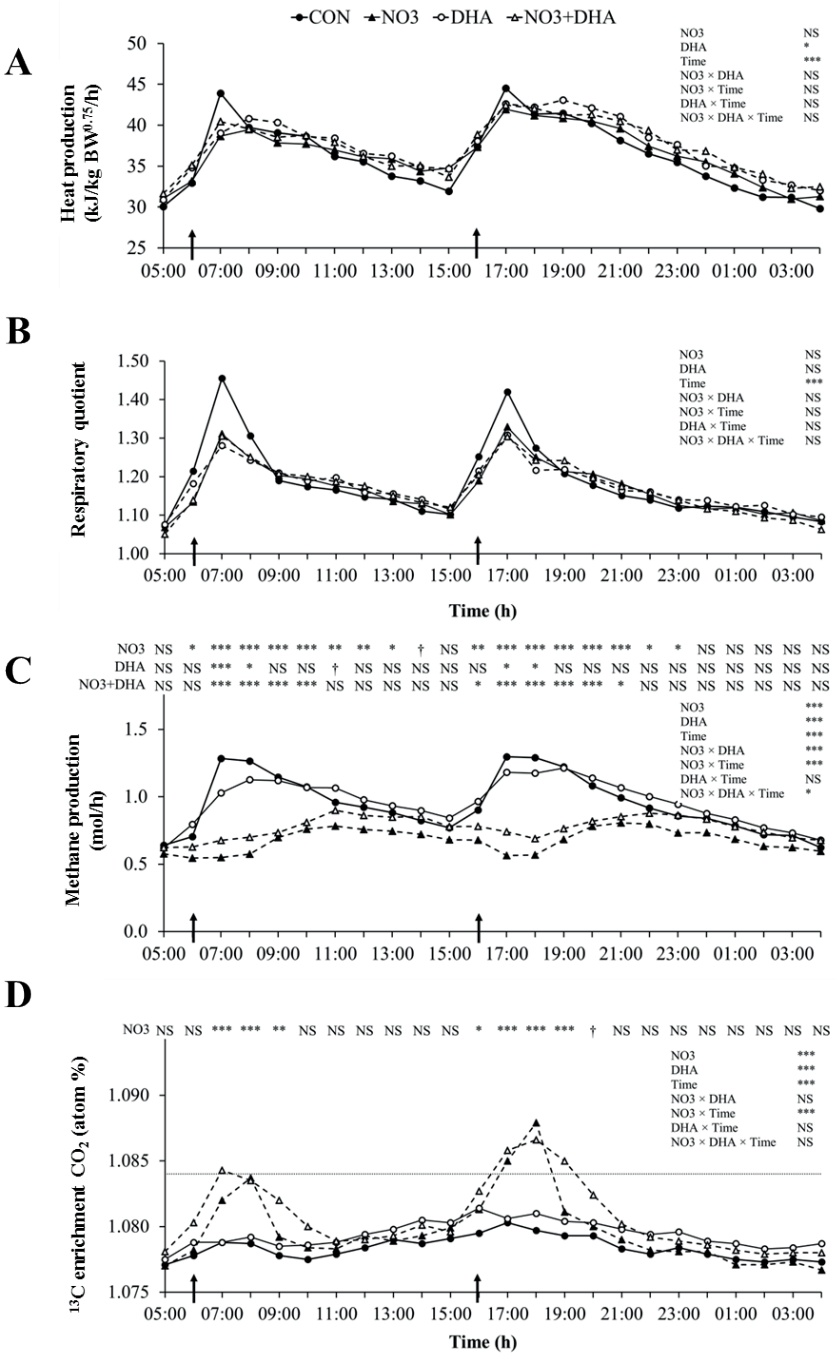
For cows receiving nitrate, the timing of the increased ^{13}C enrichment of CO_2 coincided with that of a marked decrease in CH_4 emission. Based on previous findings (Van Zijderveld et al. 2011; Lund et al. 2014; Guyader et al., 2015), it is likely that the period of decreased CH_4 production coincided with an increased concentration of aqueous H_2 in the rumen, which may have impaired the functioning of fibrolytic bacteria. However, only Guyader et al. (2015) measured aqueous H_2 in the rumen, whereas Lund et al. (2014) and Van Zijderveld et al. 2011, measured in vivo H_2 emissions. An increase in aqueous H_2 is likely associated with increased H_2 emissions (Hegarty and Gerdes, 1999), but it can also be speculated that supersaturation in the liquid phase may occur, which may give rise to poor relationships between concentrations of dissolved H_2 and gaseous H_2.

Neither aqueous H_2 concentrations, nor H_2 emissions were measured in the present experiment. Therefore, it cannot be excluded that other mechanisms were involved in the adverse effect of nitrate on ruminal fiber digestion and CH_4 production. As reviewed by Latham et al. (2016), the reduction intermediate nitrite can be particularly toxic to certain fibrolytic microbes, as well as to methanogens. Also, a changed intraruminal reduction

potential may disturb reactions involved in electron transfer by microorganisms (Latham et al., 2016).

A nitrate × DHA interaction was found for apparent total-tract digestibility of NDF (P = 0.011; Klop et al., 2016), with NDF digestibility only being reduced on the NO_3 treatment where it averaged 55%, compared with 61, 64, and 65% on treatments CON, DHA and NO_3+DHA, respectively. In line with reduced feed intake resulting from nitrate feeding (Newbold et al., 2014), based on visual observations, cows on the NO_3+DHA treatment appeared to have a more gradual intake pattern than cows on the NO_3 treatment. This may explain why values for ^{13}C enrichment of CO_2 of these cows returned to baseline values at a later time point than for the NO_3 treatment (Figure 3.1D). The more gradual feed intake pattern may also have resulted in a higher overall NDF degradation in the rumen of NO_3+DHA cows as compared with NO_3 cows, because of a longer retention time of feed. It would also provide an explanation for the numerically smaller decrease in CH_4 per kg of DMI observed for cows on the NO_3+DHA treatment compared with the NO_3 treatment (CH_4 production was 1.37, 1.05, 1.40, and 1.13 mol/kg of DMI on treatments CON, NO_3, DHA, and NO_3+DHA, respectively; Klop et al., 2016). Total-tract starch digestibility (> 99%) was not affected by feeding nitrate (Klop et al., 2016).

The significantly lower NDF digestibility, absence of changes in starch digestibility, and the significantly higher ^{13}C enrichment of CO_2 for cows on the NO_3 treatment provides a strong lead that effects of feed additives on fiber degradation in the rumen can be detected by evaluating diurnal patterns of ^{13}C enrichment of CO_2. The difference required to detect effects depends on accuracy of the measurements, number of measurements and repeats, frequency of feeding and meal size, and the expected size of the effect. The concept of evaluating ^{13}C enrichment of CO_2 to evaluate dietary effects on fiber degradation in the rumen might have a broader application potential than the study of effects of feed


A
CON NO3 DHA NO3+DHA
Heat production (kJ/kg BW^0.75/h)
NO3 NS
DHA *
Time ***
NO3 × DHA NS
NO3 × Time NS
DHA × Time NS
NO3 × DHA × Time NS
B
Respiratory quotient
Time (h)
C
Methane production (mol/h)
D
13C enrichment CO2 (atom %)
05:00 07:00 09:00 11:00 13:00 15:00 17:00 19:00 21:00 23:00 01:00 03:00

<< Figure 3.1. Diurnal patterns of heat production (**A**), respiration quotient (**B**), methane production (**C**) and ^{13}C enrichment of CO_2 (**D**) of cows receiving different dietary treatments: control (CON); NO_3 (21 g of nitrate/kg of dry matter (DM)); DHA [3 g of docosahexaenoic acid (DHA)/kg of DM]; or NO_3+DHA (21 g of nitrate/kg of DM and 3 g of DHA/kg of DM). For CON and NO_3 treatments n=6, for DHA and NO_3+DHA treatments n=7. Arrows indicate feeding times. Significance of main effects and interaction effects is indicated in the figure. Symbols (NS not significant; † $0.05 < P < 0.10$; * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$) in (C) indicate hourly comparison of each of the additive treatments with the CON treatment. Symbols in (D) indicate effect of nitrate at different time points. The dashed line indicates the overall average background ^{13}C enrichment of the TMR (1.0844). Values for atom% ^{13}C were 1.0924 and 1.0722 for corn silage and grass silage, respectively, and 1.0794, 1.0791, 1.0808 and 1.0806 for concentrates of the CON, NO_3, DHA, and NO_3+DHA treatment, respectively. The pooled SEM values were 1.4, 0.024, 0.05, and 0.0007 for A, B, C, and D respectively.

additives only. For example, fiber degradation may also be impaired in cows with a low rumen pH, and measuring ^{13}C enrichment of CO_2 in repeated spot samples of breath could then be a tool to detect individuals with suboptimal conditions for fiber fermentation.

Results presented here indicate that effects of a CH_4-mitigating feed additive on fiber degradation in the rumen can be detected by evaluating the change in the diurnal pattern of ^{13}C enrichment of CO_2. A prerequisite is that the main ration components differ in natural ^{13}C enrichment (e.g., C3 and C4 plants; Sudekum et al. 1995), and in content of the nutrients that are expected to be involved in a shift in fermentation (e.g., starch and fiber) or in degradability of a nutrient.

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Chapter 4

Changes in in vitro gas and methane production from rumen fluid from dairy cows during adaptation to feed additives in vivo

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ABSTRACT

The adaptation of dairy cows to methane (CH_4) mitigating feed additives was evaluated using the in vitro gas production (GP) technique. Nine rumen-fistulated lactating Holstein cows were grouped into three blocks and within blocks randomly assigned to one of three experimental diets: Control (CON; no feed additive), Agolin Ruminant® (AR; 0.05 g/kg DM) or lauric acid (LA; 30 g/kg DM). Total mixed rations composed of maize silage, grass silage and concentrate were fed in a 40:30:30 ratio on DM basis. Rumen fluid was collected from each cow at days -4, 1, 4, 8, 15 and 22 relative to the introduction of the additives in the diets. On each of these days, a 48 h GP experiment was performed in which rumen fluid from each donor cow was incubated with each of the three substrates that reflected the treatment diets offered to the cows. Dry matter intake was on average 19.8, 20.1, and 16.2 kg/d with an average fat- and protein-corrected milk production of 30.7, 31.7, and 26.2 kg/d with diet CON, AR, and LA, respectively. In general, feed additives in the donor cow diet had a larger effect on gas and CH_4 production than the same additives in the incubation substrate. Incubation substrate affected asymptotic GP, half-time of asymptotic CH_4 production, total volatile fatty acid (VFA) concentration, molar proportions of propionate and butyrate, and degradation of organic matter (OMD), but did not affect CH_4 production. No substrate × day interactions were observed. A significant diet × day interaction was observed for *in vitro* gas and CH_4 production, total volatile fatty acid (VFA) concentration, molar proportions of VFA and OMD. From day 4 onwards, the LA diet persistently reduced gas and CH_4 production, total VFA concentration, acetate molar proportion and OMD, and increased propionate molar proportion. In vitro CH_4 production was reduced by the AR diet on day 8, but not on days 15 and 22. In line with these findings, the molar proportion of propionate in fermentation fluid was higher, and that of acetate smaller, for the AR diet than for the CON diet on day 8, but not on days 15 and 22. Overall, the data indicate a transient effect of AR on CH_4 production, which may indicate microbial adaptation, whereas the CH_4 mitigating effect of LA persisted.

Keywords: rumen fermentation, adaptation, essential oils, lauric acid, methane

IMPLICATIONS

In vitro fermentation characteristics and methane production depend on the composition of the diet fed to donor animal, giving rise to inconsistent effects of additives in vitro. Feed additives in the donor cow diet had stronger effects on in vitro gas and methane production than the same additives in the incubation substrate. Over time, the extent of this effect was affected by the adaptation to a diet with essential oils, but not with lauric acid. These findings help to better understand adaptation to methane mitigating feeding strategies.

INTRODUCTION

Several feed additives may mitigate methane (CH_4) emissions from ruminants (Hristov et al. 2013). However, the rumen microbial ecosystem can adapt to feed additives, which results mostly in a transient decrease of CH_4 production only. For example, promising results on CH_4 reduction using essential oils or their active ingredients have been obtained using in vitro batch cultures, whereas no or only a temporary effect on fermentation characteristics was found in continuous cultures or in vivo (Benchaar and Greathead, 2011; Van Zijderveld et al., 2011). Cardozo et al. (2004) reported a transient effect of plant extracts on fermentation characteristics that disappeared after six days. The latter indicates that microbial adaptation can occur after short term exposure. The response in CH_4 production to plant extracts evaluated in vitro may also vary with composition of the diet consumed by the donor animals (O'Brien et al., 2014), as diet composition affects the microbial activity in rumen inoculum.

In broilers, shuttle programmes with two or more anticoccidial compounds, usually with different modes of action, are widely used to reduce resistance of protozoan parasites (Chapman 2001). Similarly, the alternating use of two or more CH_4 reducing feed additives with a different mode of action may alleviate the problem of microbial adaptation in the rumen. If successful, a persistently lower CH_4 production could be achieved without the requisite for a persistent CH_4 reduction by a single feed additive. Before testing this concept in vivo, more information is needed on the duration and persistency of the CH_4

reducing effect of the selected additives. The present study, therefore, examined the adaptation of dairy cows to CH_4 reducing feed additives that have different modes of action in vivo, using the in vitro gas production (**GP**) technique.

MATERIALS AND METHODS

Animals, diets and feeding

All experimental procedures were approved by the Animal Care and Use Committee of Wageningen University (Wageningen, The Netherlands). Nine rumen cannulated, second parity cows (105 ± 6.5 DIM; mean ± S.D. at the start of the experiment) were assigned to three blocks based on milk yield. Within blocks, cows were randomly assigned to one of three diets: Control (**CON**; no feed additive), Agolin Ruminant® (Agolin SA, Bière, Switzerland; **AR**; 0.05 g/kg DM) or lauric acid (C12:0) (Sigma Aldrich, Zwijndrecht, the Netherlands; **LA**; 30 g/kg DM). Agolin Ruminant® contains 0.2 g essential oils/g product (Castro-Montoya et al., 2015) with eugenol, geranyl acetate and coriander oil being the main components.

Additives were included in the concentrate meals (Table 4.1). The CON concentrate was composed of maize (310 g/kg), maize gluten feed (140 g/kg), rapeseed meal (94 g/kg), soybean meal (90 g/kg), rumen-protected soybean meal (formaldehyde-treated; 86 g/kg), beet pulp (75 g/kg), palm kernel expeller (70 g/kg), rumen-protected rapeseed meal (formaldehyde-treated; 57 g/kg), cane molasses (40 g/kg), limestone (13 g/kg), soybean hulls (10 g/kg), vitamin and mineral premix (8 g/kg), salt (3.6 g/kg), sodium bicarbonate (2.5 g/kg), and magnesium oxide (1.5 g/kg). Given the low inclusion level of Agolin Ruminant® the ingredient composition of AR concentrate was the same as of the CON concentrate. Agolin Ruminant® was first homogenously mixed with other ingredients, before it was included in the large concentrate mixture. In the LA concentrate, ingredients were proportionally exchanged against C12:0, except for the minerals and the vitamin and mineral premix, which were kept at the same level as in the other two concentrates. Before introduction of the additives in the diets, cows were adapted for a period of 19 d to a total mixed ration (TMR) that consisted of grass silage, maize silage and CON concentrate in a 30:40:30 ratio on a DM basis. During the first seven days of this period,

animals were housed in a freestall barn. Thereafter, cows were individually housed in tie-stalls to determine dry matter intake (DMI) of each cow. Feed was supplied in equal portions at 0600 and 1600 h. A mixture of grass silage and maize silage was prepared twice weekly and stored in a cooled room (±7°C). The concentrate was weighed separately into buckets and manually mixed into the roughage mixture at the moment of feeding. After five days in the tie-stalls, feed supply to each cow was restricted to 95% of voluntary to minimize the risk of feed refusals during the experiment. For cows assigned to either the AR or LA diet, the CON concentrate in the TMR was replaced by the respective treatment concentrate from day 12 in the tie-stalls onwards.

Sampling and analyses of TMR, substrate components and milk

Representative samples of all individual TMR components were collected at the moments of feed preparation prior to one of the six rumen fluid collection days. The average DMI per time point was calculated based on the two days prior to rumen fluid collection. Milk samples of four milkings prior to each rumen fluid collection day were collected from all cows and analysed for fat, protein and lactose according to Hatew et al. (2015a). Fat and protein corrected milk yield (**FPCM**; kg/d) was calculated as (0.337 + 0.116 × fat% + 0.06 × protein%) × milk yield (kg/d) (CVB, 2008).

Samples of TMR components, incubation substrate components, and orts were analysed for chemical composition as described by Klop et al. (2016) except for crude fat analysis, which was based on NEN-ISO 1735 (ISO, 2004). A modification to the standard procedure was that samples were hydrolysed with hydrochloric acid at 75°C and subsequently, the solution containing hydrochloric acid and ethanol was extracted with diethyl ether and petroleum ether. Solvents were removed by distillation before the mass of the extracted material was determined.

Table 4.1. Analysed, average chemical composition of maize silage, grass silage and concentrates and calculated average composition of the total mixed ration (TMR) for the different diets fed (g/kg DM unless otherwise stated)

	Roughages[1]		Concentrates[2]			TMR		
Item	Maize silage	Grass silage	CON	AR	LA	CON	AR	LA
Inclusion, % of DM	40	30	30	30	30	-	-	-
DM, g/kg	314	524	890	887	885	460	459	459
Gross energy, MJ/kg DM	18.7	18.9	18.2	18.3	20.2	18.6	18.6	19.2
Crude ash	39	103	84	87	81	72	73	71
CP	82	212	222	231	210	163	166	159
Crude fat	35	41	44	45	134	40	40	67
NDF	359	472	213	225	204	349	353	346
ADF	216	257	100	106	97	194	195	193
ADL	11	9	19	22	19	13	14	13
Starch	329	N.A.	265	246	231	211	205	201
Sugar	N.A.[3]	68	80	81	75	44	45	43

[1]Maize silage: NE_L (MJ/kg DM) = 6.9; DVE (g/kg DM; Tamminga et al. 1994) = 52; pH = 3.8; ammonia-N (% total N) = 7

Grass silage: NE_L (MJ/kg DM) = 6.6; DVE (g/kg DM) = 90 ; pH = 5.8; ammonia-N (% total N) = 8

[2]Control (CON; no additive), Agolin Ruminant® (AR; 0.05 g/kg DM) or lauric acid (C12:0) (LA, 30 g/kg DM).

[3]N.A. = not analyzed.

Rumen fluid, in vitro gas production equipment and methods of incubation

Rumen fluid from each cow was collected just before morning feeding in pre-warmed thermos flasks filled with CO_2 as described by Hatew et al. (2015a) on days -4, 1, 4, 8, 15 and 22 relative to the introduction of the additives in the diets. Volatile fatty acid (VFA) concentrations were determined in a subsample of strained rumen fluid from each cow. The equipment described by Pellikaan et al. (2011) was used to determine in vitro gas and CH_4 production. Rumen fluid from individual cows was used as inoculum and after straining through cheesecloths, mixed with a pre-warmed, semi-defined incubation medium (medium B; Lowe et al., 1985 as modified by Williams et al., 2005). Each bottle contained 84 mL of incubation medium mixed with 5mL of filtrated rumen fluid. Bottles were directly placed into a shaking water bath (Haake SWB25, Clausthal-Zellerfeld, Germany) at 39°C, connected to the automated GP system.

The incubation substrates (0.5 g) were a TMR of grass silage, maize silage and one of the three concentrates at a 30:40:30 ratio on a DM basis. Silages were dried at 60°C and all components were ground to pass a 1-mm screen before incubation. The three different substrate components were weighed separately into each bottle and originated from the same batches that were fed to the animals (Table 4.1). In each of the six runs, 90 bottles were incubated for 48h. Total GP was continuously measured in triplicate and CH_4 concentration was measured in duplicate at 2, 4, 6, 8, 10, 12, 14, 16, 24, 36, and 48 h of incubation for all inoculum × substrate combinations.

Sampling and analyses of fermentation fluid and gas

Methane was determined using gas chromatography (see Ellis et al., 2016 for details). At the end of each 48 h incubation, bottles were placed on ice and a subsample (0.6 mL) of fermentation fluid was mixed with an equal volume of ortho-phosphoric acid, containing isocaproic acid as internal standard, and stored at -20^{o}C pending VFA analysis. The VFA were separated by gas chromatography using a 30 m × 0.53 mm × 1.0 µm Agilent J&W HP-FFAP (Santa Clara, USA) column, hydrogen as mobile phase and a flame ionization detector. The residual incubation substrates were analysed for DM and ash following Williams et al. (2005) to calculate organic matter degradation (OMD).

Calculations and curve fitting

For all analyses, the experimental unit was the average of the replicate bottles for each inoculum × substrate combination. Triplicates for each inoculum × substrate combination were visually explored for outliers. In the second run (day 1 after introduction of additives in the diets), gas data from two out of eight units of the automated GP system had to be excluded because of a technical problem. For three diet × substrate combinations (CON × LA, AR × AR, and AR × LA), the gas and CH_4 results of the second run are therefore based on rumen fluid from two instead of three cows.

Cumulative gas and CH_4 production data were fitted with the following monophasic Michaelis-Menten equation (Groot *et al.*, 1996) using the NLIN procedure in SAS (SAS 9.2, SAS Inst. Inc., Cary, NC):

$$G = A / [1 + (C/t)^B]$$

where G (mL/g organic matter (OM)) is the cumulative amount of gas or CH_4 produced, A is the asymptotic G (mL/g OM), B is the switching characteristic of the curve, C is the time at which half of the asymptotic G has been reached (half-time, h) and t is the time during the in vitro incubation (h). Measured CH_4 concentrations in individual bottles were expressed relative to the maximum concentration in each bottle and fitted with the monophasic Michaelis-Menten model, with further details presented by Pellikaan et al. (2011).

Unlike the model estimated kinetic parameters for gas- and CH_4 production, VFA concentration and OMD are endpoint measurements only. As technical issues with the recording of gas and CH_4 do not affect these parameters, no VFA and OMD data were excluded.

Statistical analysis

All data were analysed using PROC MIXED (SAS 9.2, SAS Inst. Inc., Cary, NC). Two-day averages of individual cow data for DMI, milk production and milk composition at each incubation day (-4, 1, 4, 8, 15 or 22 d relative to the introduction of the additives in the

diets) were analysed using a model containing block, diet, day and diet × day interaction as fixed effects. Repeated measures for each cow × diet combination were accounted for. Data on gas and CH_4 production parameter estimates, VFA and OMD for each cow × diet × substrate combination were averaged per incubation day and analysed using repeated measures ANOVA. Block and the main effects of diet, substrate and day and their interactions were included as fixed effects. In all statistical analyses, a spatial power (SP(POW)) covariance structure was fitted, because of unequal time intervals between incubation days. Denominator degrees of freedom were estimated using the Kenward-Roger method. In case of significant interaction terms, between-treatment comparisons for each incubation day were made using a SLICE statement and *P*-values were corrected using the Tukey-Kramer method. Results are reported as least squares means. Significance of effects was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

The objective of this study was to examine adaptation to CH_4 mitigating feed additives with different modes of action in vivo using the in vitro GP technique. The selection of Agolin Ruminant® and C12:0 as feed additives for the present study was based on a pilot study (data not shown) in which the following substrates were screened for their potential to reduce CH_4 production in vitro: Agolin Ruminant®, C12:0, activated charcoal, L-ascorbic acid, coconut oil, krabok oil, and myristic acid (C14:0). The additive selection for the pilot study was based on recent literature (Benchaar and Greathead, 2011; Hansen et al. 2012; O'Brien et al. 2014; Panyakaew et al. 2013) and unpublished data from our research group.

Dry matter intake and milk yield

Dry matter intake was similar for all treatments on days -4 and 1. From day 4 onwards, DMI of LA cows was significantly lower than for CON and AR, which resulted in a significant treatment × day interaction for DMI (Table 4.2). A DMI depression after supplementation with medium-chain fatty acids (MCFA) is frequently reported in literature. As discussed by Faciola and Broderick (2014), dosage, delivery method and characteristics of the basal diet all affect the DMI response to C12:0, and comparison of

effect of doses among experiments is, therefore, complicated. In their study, 13 g C12:0/kg DM did not affect DMI, but did reduce total tract digestibility of NDF. Dohme et al. (2001) found that MCFA, in particular C10:0 and C12:0, supplemented at 50 g/kg DM, negatively affected NDF degradation using the rumen simulation technique (RUSITEC). A treatment × day interaction was also observed for FPCM yield, which declined from day 8 onwards in cows receiving the LA diet. Based on the extent of the observed milk fat depression, it is likely that not only the lower DMI, but also impaired fibre digestion contributed to the lower FPCM yield of LA cows.

In vitro gas and CH_4 production

The asymptotic GP (mL/g OM incubated) was lower for the LA substrate, but did not differ between AR and CON substrate (Table 4.3). No substrate × day or substrate × diet interactions were found (except for a substrate × diet interaction for GP halftime), indicating that the effect of the LA substrate on GP was largely constant throughout the experiment and independent of the effect of donor cow diets. The halftime of CH_4 production (mL/g OM incubated), but not the asymptotic CH_4 production, was affected by substrate (Table 4.4). The effect of donor cow diet on gas and CH_4 production varied after introduction of an additive in the diet, which resulted in a significant diet × day interaction. Using rumen fluid at day -4 from cows assigned to the AR diet resulted in a lower ($P = 0.003$) gas production compared to cows assigned to the LA diet. This difference on day -4 was unexpected, because all cows received the same basal diet. However, no between diet differences were observed on day 1 (less than 24 h after introduction of the additives in the diets).

From day 4 onwards, the LA diet always resulted in a lower ($P \leq 0.05$) gas and CH_4 production than the CON or AR diet. The findings of Zhou *et al.* (2013) using pure ruminal *Methanobrevibacter ruminantium* cell suspensions support such a delay in the effect of C12:0. A significant effect of C12:0 on survival was observed after 3 h, and after 24 h almost all *M. ruminantium* cells were dead, which indicates a delay in the effect of C12:0 on cell death. Results of the present study indicate that similar effects may be present in a mixed culture environment.

Table 4.2. Average dry matter intake (DMI), milk production and milk composition of dairy cattle fed the control diet (CON) or a diet with Agolin Ruminant® (AR) or lauric acid (LA) as feed additives

Item	Diet[1]				*P*-value		
	CON	AR	LA	SEM	Treatment	Day[2]	Treatment×Day
DMI, kg/d	19.8	20.1	16.2	0.45	0.003	<0.001	<0.001
Milk, kg/d	30.0	30.8	28.9	0.94	0.426	<0.001	<0.001
FPCM, kg/d	30.7	31.7	26.2	1.00	0.019	<0.001	<0.001
Fat, g/kg	41.8	42.6	32.5	2.09	0.025	0.716	0.032
Protein, g/kg	34.5	33.0	31.1	1.38	0.333	0.065	0.079
Lactose, g/kg	46.7	45.6	44.2	0.95	0.292	0.013	0.024

FPCM = fat- and protein-corrected milk; SEM = standard error of the mean.

[1]CON (no additive), Agolin Ruminant® (AR; 0.05 g/kg DM) or lauric acid (C12:0) (LA; 30 g/kg DM).

[2]Relates to each day an in vitro run was conducted (-4, 1, 8, 15 and 22 days relative to the introduction of the additives in the diets).

Asymptotic gas and CH_4 production from rumen fluid of cows on the AR diet differed ($P \leq 0.05$) from that of cows on the CON diet on day 8 but not on days 15 and 22. The latter observation may indicate adaptation to effects of AR in the diet. A tendency for a reduced CH_4 production in g/d and g/kg DMI in dairy cows, but not in beef cattle, fed 1.0 g Agolin Ruminant® per day was reported by Castro Montoya et al. (2015). One of the components of Agolin Ruminant® is eugenol, a phenolic compound that has antimicrobial effects on gram-positive and gram-negative bacteria (Calsamiglia et al., 2007). In a dose response experiment, eugenol did not affect in vivo CH_4 production of dairy cattle (Benchaar et al., 2015). In the latter study, the CH_4 measurement period was preceded by an 18-day adaptation period to the experimental diets. Based on the observations in the present study, the absence of an effect of eugenol in the experiment of Benchaar et al. (2015) may be a result of adaptation to the additive. In an in vitro study of Durmic et al. (2013), Agolin Ruminant® significantly reduced CH_4 production (mL/g DM) by almost 30% when added to rumen fluid from non-adapted sheep at a 10-fold higher dose (0.1 mg/g substrate) than used in the present experiment. In contrast to such observations, Pirondini et al. (2015) found no effect of Agolin Ruminant® on in vitro CH_4 production using rumen fluid from non-adapted cows. The dose of Agolin Ruminant® was similar to the dose used in the present study, but it was dissolved in ethanol before incubation. This was not done in the present experiment, as this would not mimic in vivo conditions.

Effects of additives on gas and CH_4 production may appear at initial stages of in vitro fermentation only, and be absent at the end of the incubation (48-h) (Ellis et al., 2016). Endpoint in vitro measurements may not reflect substrate degradation and gas and CH_4 production in vivo because retention time of feed in the rumen may differ from the end-point retention time in vitro. Therefore, we examined the in vitro CH_4 to total gas ratio after 12 h incubation for all diet × substrate combinations on all six measurement days (Figure 4.1). The numerical difference between the CON and AR diet was larger on day 8 than on days 15 and 22, but variation was also large and therefore the difference was not significant ($P = 0.112$). Compared to days -4 and 1, the CH_4 to total gas ratio with the LA diet was significantly reduced from day 4 onwards, to almost zero at day 8, and to increase again on days 15 and 22. The difference in CH_4 to total gas ratio between day 8 on the one hand and day 15 or day 22 on the other hand is not significant, indicating that for the duration of the present trial (22 days) the CH_4 reducing effect of C12:0 in the diet persisted. The higher half-time for CH_4 production from the LA diet at day 8 compared with day 15, but not when compared with day 22, may indicate that the CH_4 depressing effect is not fully persistent.

Results on CH_4 production in vitro should be interpreted with care and may not reflect the in vivo situation (Flachowsky and Lebzien, 2012). The relationship between in vitro and in vivo CH_4 production may also depend on the units of expression of CH_4 production. In an experiment in which rumen inocula was obtained from dairy cattle adapted to the same experimental diet as incubated in vitro, Hatew et al. (2015b) reported CH_4 production in vitro (mL per g OM incubated) to be moderately related ($R^2 = 0.54$) with in vivo CH_4 production (mL per g of estimated rumen-fermentable OM). However, no association was found when in vivo CH_4 production was expressed per unit of ingested OM ($R^2 = 0.05$). Thus, even inoculum obtained from specifically adapted animals may still lead to large differences between CH_4 production observed in vitro and in vivo.

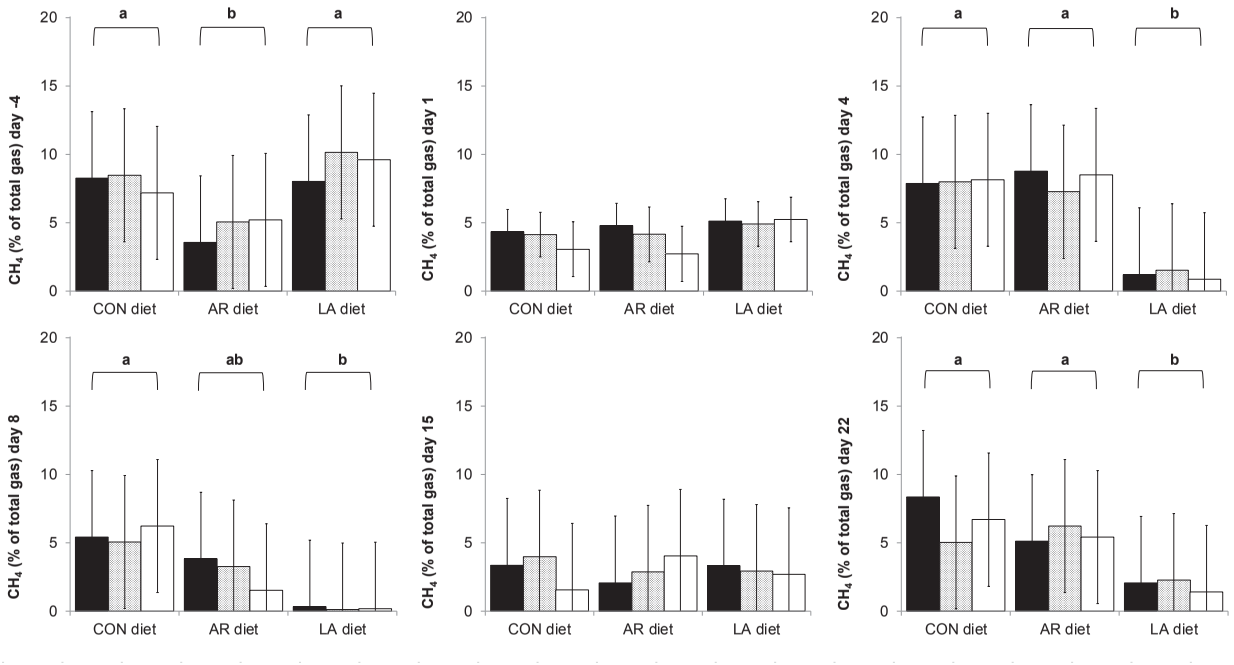

Figure 4.1. In vitro methane (CH_4) production (% of total gas) at 12 h after the start of the incubation on days -4, 1, 4, 8, 15 and 22 relative to the introduction of the additives in the diets of the donor cows. Donor cow diets were: Control (CON; no additive), Agolin Ruminant® (AR; 0.05 g/kg DM) and lauric acid (C12:0) (LA; 30 g/kg DM). Incubation substrates CON (black bars), AR (dotted bars) and LA (white bars) were similar to the three donor cow diets and consisted of the same diet components. Error bars indicate pooled SEM. Statistical analysis resulted in the following *P*-values: diet: $P \leq 0.001$; substrate: $P = 0.825$; day: $P \leq 0.001$; substrate × diet; $P = 0.969$ substrate × day: $P = 0.997$; diet × day: $P \leq 0.001$; substrate × diet × day: $P = 0.997$. Superscripts indicate between diet differences within runs.

Table 4.3. Average gas production curve fit parameters for each combination of donor cow diet (top row) and incubation substrate (second row) during 48 h incubations at -4, 1, 4, 8, 15, and 22 days relative to the introduction of the additives in the diets of the donor cows. Composition of the incubation substrates (g/kg DM) was similar to the experimental diets: Control (CON), Agolin Ruminant® (AR; 0.05 g/kg DM) or lauric acid (LA, 30 g/kg DM)

	Donor cow diet CON			Donor cow diet AR			Donor cow diet LA		
Substrate	CON	AR	LA	CON	AR	LA	CON	AR	LA
A[1], mL/g organic matter									
Day -4	329.1[ab]	335.7[ab]	292.1[ab]	291.1[a]	296.2[a]	281.7[a]	342.2[b]	327.0[b]	330.2[b]
Day 1	310.3	285.4	279.7	327.5	315.6	302.9	334.2	331.4	305.7
Day 4	338.6[b]	324.3[b]	311.7[b]	316.0[b]	316.7[b]	312.0[b]	280.5[a]	267.2[a]	252.1[a]
Day 8	314.3[c]	308.4[c]	285.0[c]	275.5[b]	277.2[b]	221.5[b]	221.2[a]	206.9[a]	195.7[a]
Day 15	296.7[b]	296.5[b]	255.3[b]	292.8[b]	299.5[b]	292.2[b]	249.2[a]	251.7[a]	227.2[a]
Day 22	303.9[b]	303.0[b]	273.2[b]	303.6[b]	308.7[b]	252.5[b]	248.9[a]	246.4[a]	246.5[a]
B[2]									
Day -4	1.92	1.94	1.76	2.13	2.02	1.93	1.77	1.88	1.79
Day 1	1.93[b]	1.98[b]	1.88[b]	1.84[b]	1.85[b]	1.51[b]	1.28[a]	1.28[a]	1.33[a]
Day 4	1.78	1.78	1.62	1.91	1.87	1.79	1.63	1.66	1.70
Day 8	1.77[a]	1.81[a]	1.73[a]	2.15[b]	2.12[b]	2.23[b]	1.96[b]	2.18[b]	2.25[b]
Day 15	1.84	1.67	1.94	1.97	1.98	1.76	1.96	1.86	1.92
Day 22	1.70	1.69	1.56	1.76	1.74	1.70	1.57	1.60	1.42
C[3], h									
Day -4	11.6	11.5	11.4	11.4	11.5	11.6	12.3	11.0	12.1
Day 1	11.9[ab]	11.7[ab]	11.5[ab]	12.2[b]	12.4[b]	13.6[b]	12.1[a]	11.8[a]	10.4[a]
Day 4	10.4[b]	10.3[b]	10.9[b]	10.3[b]	10.7[b]	10.8[b]	9.8[a]	9.6[a]	8.6[a]
Day 8	11.2[b]	11.1[b]	10.9[b]	11.5[b]	12.1[b]	12.0[b]	9.8[a]	9.6[a]	9.1[a]
Day 15	11.3	11.7	10.3	11.1	11.1	11.3	12.0	11.8	10.3
Day 22	10.9	11.3	11.3	10.9	11.3	11.6	10.5	10.3	10.8

	P-Value[4]						
Paramet	Substrate	Donor	Day	Sub[5]×Diet	Sub×Day	Diet×Day	Pooled SEM
A	<0.001	<0.001	<0.001	0.815	0.980	<0.001	15.99
B	0.382	0.003	<0.001	0.671	0.923	<0.001	0.136
C	0.559	<0.001	<0.001	0.009	0.696	<0.001	0.52

[1]A = Asymptotic gas production, mL/g organic matter incubated.

[2]B = Switching characteristic of the curve.

[3]C = Time at which half of the asymptotic gas production has been reached (half-time).

[4]*P*-value for Substrate × Diet × Day interaction non-significant ($P > 0.05$) and not presented.

[5]Sub = substrate.

[ab]Superscripts indicate significance of diet × day interaction term. Diets within rows with different superscripts are significantly ($P \leq 0.05$) different from each other.

Table 4.4. Average gas production curve fit parameters of methane (CH_4) production for each combination of donor cow diet (top row) and incubation substrate (second row) during 48 h incubations at -4, 1, 4, 8, 15, and 22 days relative to the introduction of the additives in the diets of the donor cows. Composition of the incubation substrates (g/kg DM) was similar to the experimental diets: Control (CON), Agolin Ruminant® (AR; 0.05 g/kg DM) or lauric acid (LA, 30 g/kg DM)

	Donor cow diet CON			Donor cow diet AR			Donor cow diet LA		
Substrate	CON	AR	LA	CON	AR	LA	CON	AR	LA
A[1], mL/g organic matter									
Day -4	56.6	53.2	53.7	41.8	46.9	41.9	53.8	55.8	53.3
Day 1	44.5	43.7	44.3	39.9	45.3	42.2	49.7	45.9	39.6
Day 4	47.7[b]	44.9[b]	46.1[b]	48.2[b]	44.4[b]	46.1[b]	25.3[a]	28.0[a]	23.5[a]
Day 8	47.1[c]	46.6[c]	45.7[c]	37.3[b]	34.9[b]	30.8[b]	18.8[a]	14.8[a]	13.6[a]
Day 15	40.7[b]	43.0[b]	28.7[b]	32.7[b]	35.8[b]	40.0[b]	23.7[a]	24.8[a]	22.9[a]
Day 22	50.8[b]	38.5[b]	46.6[b]	40.2[b]	46.8[b]	39.9[b]	23.0[a]	24.0[a]	16.3[a]
B[2]									
Day -4	2.23	2.53	2.63	3.48	2.73	3.08	2.44	2.28	2.40
Day 1	2.95	2.91	2.96	3.27	3.69	3.33	2.36	2.42	2.67
Day 4	2.39	2.46	2.40	2.36	3.12	2.48	3.64	3.35	3.55
Day 8	2.74[a]	2.67[a]	2.70[a]	3.53[a]	3.55[a]	4.30[a]	5.20[b]	6.87[b]	5.71[b]
Day 15	2.89	2.74	5.35	3.81	3.36	2.91	3.29	3.23	3.11
Day 22	2.16	3.13	2.76	3.09	2.67	3.14	3.52	3.50	4.27
C[3],h									
Day -4	20.1	17.5	22.2	23.6	22.7	21.6	19.8	16.3	17.3
Day 1	21.5	23.1	25.9	20.2	22.1	26.4	24.7	23.6	20.9
Day 4	16.6[a]	16.0[a]	17.0[a]	16.9[a]	21.5[a]	16.8[a]	24.0[b]	24.7[b]	26.7[b]
Day 8	21.0[a]	22.0[a]	22.8[a]	23.7[a]	22.2[a]	29.5[a]	28.4[b]	29.2[b]	35.6[b]
Day 15	23.6	22.8	29.9	24.3	24.4	22.2	22.4	23.8	24.2
Day 22	18.6[a]	22.2[a]	23.5[a]	22.7[ab]	24.7[ab]	30.7[ab]	26.7[b]	26.7[b]	28.9[b]

	P-Value[4]						
Parameter	Substrate	Donor	Day	Sub[5]×Diet	Sub×Day	Diet×Day	Pooled SEM
A	0.347	<0.001	<0.001	0.764	1.000	<0.001	5.87
B	0.565	0.013	<0.001	0.832	0.963	<0.001	0.572
C	0.019	0.002	<0.001	0.878	0.672	<0.001	2.61

[1]A = Asymptotic gas production.

[2]B = Switching characteristic of the curve.

[3]C = Time at which half of the asymptotic gas production has been reached (half-time).

[4]*P*-value for Substrate × Diet × Day interaction non-significant ($P > 0.05$) and not presented.

[5]Sub = substrate.

[ab]Superscripts indicate significance of diet × day interaction term. Diets within rows, with different superscripts are significantly ($P \leq 0.05$) different from each other.

Table 4.5. Average concentrations and molar proportions of volatile fatty acids (VFA) in fermentation fluid for each combination of donor cow diet (top row) and incubation substrate (second row) at the end of 48 h incubations at -4, 1, 4, 8, 15, and 22 days relative to the introduction of the additives in the diets of the donor cows. Composition of the incubation substrates (g/kg dry DM) was similar to the experimental diets: Control (CON), Agolin Ruminant® (AR; 0.05 g/kg DM) or lauric acid (LA, 30 g/kg DM)

	Donor cow diet CON			Donor cow diet AR			Donor cow diet LA		
Substrate	CON	AR	LA	CON	AR	LA	CON	AR	LA
Total VFA, mmol/L									
Day -4	55	55	49	53	48	49	54	56	50
Day 1	53	51	46	54	48	46	49	50	42
Day 4	50^{b}	50^{b}	46^{b}	51^{ab}	47^{ab}	45^{ab}	45^{a}	45^{a}	43^{a}
Day 8	53^{b}	50^{b}	48^{b}	49^{b}	51^{b}	43^{b}	43^{a}	41^{a}	37^{a}
Day 15	49^{ab}	47^{ab}	40^{ab}	48^{b}	50^{b}	46^{b}	44^{a}	44^{a}	42^{a}
Day 22	50^{b}	46^{b}	45^{b}	50^{b}	48^{b}	44^{b}	44^{a}	45^{a}	41^{a}
Acetic acid, % of total VFA									
Day -4	62.3	62.7	61.2	60.4	62.6	61.7	61.7	62.2	62.9
Day 1	60.6	59.0	59.8	61.0	61.8	59.3	61.9	61.1	63.6
Day 4	61.6^{b}	61.1^{b}	63.6^{b}	61.3^{b}	59.4^{b}	63.5^{b}	58.3^{a}	57.6^{a}	57.8^{a}
Day 8	61.7^{c}	60.7^{c}	63.0^{c}	58.6^{b}	57.9^{b}	57.2^{b}	54.6^{a}	53.8^{a}	55.4^{a}
Day 15	59.7^{b}	57.8^{b}	58.2^{b}	57.2^{b}	59.7^{b}	60.9^{b}	55.9^{a}	55.3^{a}	56.0^{a}
Day 22	60.1^{b}	58.6^{b}	61.8^{b}	59.7^{b}	58.7^{b}	59.7^{b}	56.3^{a}	56.3^{a}	57.2^{a}
Propionic acid, % of total VFA									
Day -4	21.1	21.2	20.9	24.1	22.8	21.3	21.6	20.8	19.1
Day 1	22.3	22.8	19.8	23.1	23.2	21.9	23.0	22.9	20.6
Day 4	21.9^{a}	22.0^{a}	18.9^{a}	21.3^{a}	22.3^{a}	19.0^{a}	25.1^{b}	25.4^{b}	23.8^{b}
Day 8	20.2^{a}	20.1^{a}	16.7^{a}	23.0^{b}	23.7^{b}	20.3^{b}	25.3^{b}	24.8^{b}	22.8^{b}
Day 15	22.1^{a}	23.3^{a}	20.4^{a}	22.8^{a}	21.9^{a}	18.6^{a}	25.9^{b}	26.0^{b}	25.6^{b}
Day 22	20.8^{a}	21.7^{a}	18.1^{a}	19.8^{a}	19.8^{a}	16.9^{a}	23.9^{b}	24.0^{b}	22.2^{b}
Butyric acid, % of total VFA									
Day -4	9.0	9.0	10.2	8.3	7.0	9.3	10.0	9.7	10.6
Day 1	10.6^{b}	11.2^{b}	12.8^{b}	8.7^{a}	7.6^{a}	9.6^{a}	8.4^{a}	9.1^{a}	8.2^{a}
Day 4	8.0	8.1	9.1	8.6	8.5	8.9	7.6	8.0	8.1
Day 8	11.0	11.8	12.3	9.8	10.0	12.1	10.2	11.3	11.4
Day 15	10.8^{b}	10.9^{b}	12.3^{b}	10.9^{b}	10.2^{b}	11.3^{b}	8.7^{a}	8.8^{a}	9.0^{a}
Day 22	10.7^{b}	11.1^{b}	11.7^{b}	11.4^{b}	11.6^{b}	12.8^{b}	8.8^{a}	8.9^{a}	9.2^{a}

	P-Value[1]						
Parameter	Substrate	Donor	Day	Sub[2]×Diet	Sub×Day	Diet×Day	Pooled SEM
Total VFA	<0.001	<0.001	<0.001	0.412	0.948	<0.001	1.9
Acetic acid	0.253	<0.001	<0.001	0.900	0.837	<0.001	1.38
Propionic	<0.001	<0.001	0.025	0.896	0.997	<0.001	1.38
Butyric acid	0.026	0.005	<0.001	0.579	0.992	<0.001	0.97

[1] *P*-value for Substrate × Diet × Day interaction non-significant ($P > 0.05$) and not presented.

[2] Sub = substrate.

[ab] Superscripts indicate significance of diet × day interaction term. Diets within rows, with different superscripts are significantly ($P \leq 0.05$) different from each other.

Table 4.6. Organic matter degradability (OMD; g/kg OM incubated) for each combination of donor cow diet (top row) and incubation substrate (second row) at the end of 48 h incubations at -4, 1, 4, 8, 15 , and 22 days relative to the introduction of the additives in the diets of the donor cows. Composition of the incubation substrates (g/kg DM) was similar to the experimental diets: Control (CON), Agolin Ruminant® (AR; 0.05 g/kg DM) or lauric acid (LA, 30 g/kg DM)

	Donor cow diet CON			Donor cow diet AR			Donor cow diet LA		
Substrate	CON	AR	LA	CON	AR	LA	CON	AR	LA
OMD									
Day -4	844^{c}	845^{c}	760^{a}	843^{c}	823^{bc}	782^{ab}	835^{c}	845^{c}	786^{ab}
Day 1	837^{b}	841^{b}	771^{a}	839^{b}	823^{b}	767^{a}	842^{b}	817^{b}	739^{a}
Day 4	837^{d}	843^{d}	783^{bc}	836^{d}	824^{cd}	774^{b}	775^{b}	808^{bcd}	731^{a}
Day 8	829^{d}	816^{cd}	775^{bc}	838^{d}	836^{d}	765^{b}	809^{cd}	798^{bcd}	718^{a}
Day 15	825^{cd}	831^{cd}	741^{a}	831^{cd}	834^{d}	779^{ab}	791^{bc}	792^{bcd}	746^{a}
Day 22	832^{d}	821^{cd}	763^{ab}	832^{d}	828^{cd}	769^{ab}	794^{bcd}	788^{abc}	748^{a}

	P-Value							
Parameter	Substrate	Donor diet	Day	Sub[1]×Diet	Sub×Day	Diet×Day	S×D×D[2]	Pooled SEM
OMD	<0.001	<0.001	<0.001	0.330	0.644	<0.001	0.043	9.5

[1]Sub = substrate.

[2]S×D×D = Substrate × Diet × Day

[ab]Superscripts indicate significance of substrate × diet × day interaction term. diet × substrate combinations within rows, with different superscripts are significantly ($P \leq 0.05$) different from each other.

Volatile fatty acids

Overall, significant diet × day interactions indicate that the LA diet reduced VFA concentrations and relative molar proportions of acetate and butyrate in fermentation fluid, and increased molar proportions of propionate, for all three substrates from day 4 onwards (Table 4.5). Such changes in VFA molar proportions correspond to an observed reduction in CH_4 production. Reductions in VFA concentration and acetate molar proportion following C12:0 supplementation have been reported previously (Faciola and Broderick, 2014; Hristov et al. 2011). Similar effects were observed for filtrated rumen fluid before incubation after introduction of C12:0 into the diet of the cows (data not shown). Only 5 mL of rumen fluid was added to 84 mL buffer solution, and thus this similarity in results may indicate that the microbial composition of the rumen fluid was affected by the donor cow diets.

Compared with the CON diet, the acetate and propionate molar proportions for the AR diet were significantly lower and higher, respectively, on day 8 only, whereas total VFA concentration in fermentation fluid was not different. This shift in relative proportions of VFA is in line with the results for CH_4 production on this day. Similar to the absence of differences in CH_4 production between the CON diet and the AR diet at days 15 and 22, the relative proportions of acetate and propionate also did not differ between AR and CON at days 15 and 22. Benchaar et al. (2015) and Pirondini et al. (2015) found no treatment effect of eugenol or Agolin Ruminant® on total VFA concentrations or acetate:propionate ratio, which is in line with the absence of a CH_4 reduction in their studies.

In vitro organic matter degradability

The OMD results (Table 4.6) generally support the data of the in vitro gas and CH_4 production and VFA concentration for the various diet × substrate combinations. A significant substrate × diet × day interaction was observed. The OMD results for the CON and AR diet were not different and consistent over time, whereas OMD was reduced by both the LA diet (from day 4 onwards) and the LA substrate (all days). The latter is in line with earlier findings in vitro (Dohme et al., 2001) and in vivo (Faciola et al., 2014).

CONCLUSIONS

Feed additives in the donor cow diet have a stronger effect on in vitro gas and CH_4 production than the same additives in the incubation substrate. The LA diet persistently reduced in vitro gas and CH_4 production from day 4 onwards, but also decreased DMI and FPCM production of the donor cows. No negative effects on DMI and FPCM production were observed in cows receiving the AR diet. In vitro gas and CH_4 production was reduced by the AR diet on day 8, but not on days 15 and 22, which may indicate a transient effect of AR on CH_4 production and adaptation of the rumen microbial ecosystem to AR.

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Chapter 5

Effects of continuous feeding of essential oils or rotational feeding of essential oils and lauric acid on enteric methane production in lactating dairy cows

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ABSTRACT

The rumen microbes can adapt to feed additives, which may make the decrease in enteric CH_4 production upon feeding an additive a transient response only. This study investigated alternate feeding of two CH_4 mitigating feed additives with a different mode of action on persistency of lowering CH_4 production compared to feeding a single additive over a period of 10 weeks. Four pairs of cows were selected, and within pairs, cows were randomly assigned to either the control (**AR-AR**) or the alternating (**AR-LA**) concentrate treatment. The AR concentrate contained a blend of essential oils (Agolin Ruminant®; 0.17 g/kg of DM) and the LA concentrate contained lauric acid (C12:0; 20 g/kg of DM). A basal concentrate without Agolin Ruminant® and lauric acid was fed during the pre-treatment period (2 weeks). Thereafter, the cows assigned to AR-AR treatment received the AR concentrate during all 10 treatment weeks (5 periods of twee weeks each), whereas cows assigned to the AR-LA treatment received AR and LA concentrates rotated on a weekly basis. Methane emission was measured in climate respiration chambers during periods 1, 3 and 5. From period 3 onwards, DMI and milk protein concentration were reduced in the AR-LA treatment. Milk fat concentration was not affected, but the proportion of C12:0 in milk fat increased upon feeding C12:0. Molar proportions of acetate and propionate in rumen fluid were lower and higher, respectively, in the AR-LA than in the AR-AR treatment. Methane yield (g/kg of DMI) and intensity (g/kg FPCM) were not affected by treatment. Methane yield and intensity were significantly lower (12 and 11%, respectively) in period 1 compared with the pre-treatment period, but no significant difference relative to pre-treatment period was observed in period 3 (numerically 9 and 7% lower, respectively) and in period 5 (numerically 8 and 4% lower, respectively). Results indicate a transient decrease in CH_4 yield and intensity in time, but no improvement in extent or persistency of CH_4 reduction due to rotational feeding of essential oils and C12:0 in lactating dairy cows.

Key words: methane, lauric acid, essential oils, dairy cow

INTRODUCTION

The mitigating effect of feed additives supplemented to dairy cow diets on enteric CH_4 emission may be a transient effect if rumen microbes adapt to these additives. Guan et al. (2006) compared the effect of feeding a single ionophore (monensin) with feeding a rotation of ionophores (monensin and lasalocid) on enteric CH_4 production in Angus steers. Both the size and duration of the decrease in CH_4 production were not different between the two ionophore treatments, and the mitigating effect disappeared after several weeks. The absence of an effect is probably a result of the similar mode of action of both ionophores, which may be overcome if several additives with different mode of action are rotated.

In agronomy, herbicide rotations are applied as tactic to prevent or delay herbicide resistance of weeds (Beckie, 2006). In broilers, shuttle programmes with two or more anticoccidial compounds, usually with different modes of action, are widely used to reduce resistance of these protozoa (Chapman, 2001). Yáñez-Ruiz et al. (2016) reviewed the use of in vitro batch culture technique to assess enteric CH_4 production, and recommended use of inoculum from animals that have been adapted to treatment for at least 2 weeks. In Chapter 4, the adaptation of dairy cows to feed additives with different modes of action in vivo [viz. lauric acid (C12:0) and Agolin Ruminant® (commercial blend of essential oils, with eugenol, geranyl acetate and coriander oil being the main components)] was evaluated using the in vitro gas production technique. Results indicated a transient effect of the essential oil blend on in vitro CH_4 production, with CH_4 production being lowered after 8 d of feeding the additive to the donor cows, whereas after 15 and 22 d, in vitro CH_4 production did not differ anymore from the control treatment. In contrast, a persistent mitigating effect on in vitro CH_4 production was observed when donor cows were fed lauric acid.

Based on these findings, it was hypothesized that continuous feeding of AR would result in a transient decrease of CH_4 emission, whereas weekly rotation of AR and C12:0 would result in a persistent CH_4 decline. Therefore, the aim of the present study was to compare

the extent and duration of changes in CH_4 emission and in performance of dairy cows receiving either AR only or AR and C12:0 using a weekly rotation schedule.

MATERIALS AND METHODS

Experimental design, animals and housing

All experimental procedures were approved by the Animal Care and Use Committee of Wageningen University (Wageningen, The Netherlands). Four pairs of cows (4 primiparous and 4 multiparous; 139 ± 38 DIM at the start of the experimental period; mean ± SD), of which 4 cows were fitted with a permanent rumen cannula (10 cm i.d., Type 1C, Bar Diamond Inc., Parma, ID) were included in the experiment. Cows were paired based on parity, lactation stage, milk production and presence or absence of a rumen fistula. Within pairs, cows were randomly assigned to either the control (**AR-AR**) or the alternating (**AR-LA**) concentrate treatment with a total treatment length of 10 wk (5 periods of 2 wks each). The treatments were preceded by an 11-d pre-treatment period. In the pre-treatment period, cows were housed in tie-stalls and fed the basal diet without experimental feed additives. Thereafter, cows were individually housed in climate respiration chambers (CRC) for a period of 2.5 d to measure CH_4 emissions on the basal diet. Four individual CRC were available at the same time and therefore a staggered approach was taken with the first 2 pairs of cows (block A) starting 3 d earlier with the pre-treatment period than the second 2 pairs of cows (block B). After the initial CH_4 measurement in the CRC, block A cows returned to the tie-stalls and were fed the basal ration without additives for another 17 d. During these 17 d, block B cows were housed in CRC for their initial 2.5-d CH_4 measurements, where after they started a treatment schedule of 2 wks in the CRC (period 1, period 3, period 5) with intermediate 2 wk tie-stall periods (period 2, period 4). In the 2 wk period that the block A cows were housed in the tie-stalls, the cows of block B were housed in the CRC with a similar treatment schedule. For each two-week CRC period, cows entered the CRC at 1500 h and left around 0900 h on d 15. Days 2-7 and 9-14 were used to collect CH_4 data. The CRC were cleaned in the mornings of d 1 (before entrance of the cows) and d 8. Rotation (AR to LA or vice versa) occurred in the mornings of d 2 and d 9.

A detailed description of the CRC design and gas measurements was reported by van Gastelen et al. (2015). Briefly, in each CRC (volume 35 m^3) relative humidity was maintained at 70% and temperature at 16°C, and the ventilation rate in each compartment was 42 m^3/h. Inlet and exhaust air of each compartment was sampled at 10-min intervals. Gas concentrations and ventilation rates were corrected for pressure, temperature and humidity to arrive at standard temperature pressure dew point volumes of inlet and exhaust air. Immediately prior to the experiment, compartments were checked by releasing known amounts of CO_2 in each compartment and the recovery calculated. The recovered amounts of CO_2 were between 98 and 100%. Cows were exposed to 16 h of light per day.

Table 5.1. Ingredient composition (g/kg of DM) of the pre-treatment concentrate (Basal) and the treatment concentrates that contained either Agolin Ruminant® (AR) or lauric acid (LA) as feed additive.

	Concentrates		
Ingredient	Basal	AR	LA
Corn	305	305	285
Corn gluten feed	143	143	133
Soybean meal	99	99	93
Rapeseed meal	93	93	87
Formaldehyde treated soybean meal	85	85	79
Sugar beet pulp	78	78	73
Palm kernel expeller	73	73	68
Formaldehyde treated rapeseed meal	57	57	53
Sugar cane molasses	34	34	32
$CaCO_3$	14	14	14
Trace mineral and vitamin premix	9	9	9
NaCl	4	4	4
$NaHCO_3$	2.8	2.8	2.8
MgO	1.7	1.7	1.7
Cr_2O_3	1.7	1.7	1.7
Agolin Ruminant®	-	0.17	-
Lauric acid (C12:0)	-	-	65

Table 5.2. Average analyzed chemical composition of TMR ingredients (corn silage, grass silage, pre-treatment concentrate (Basal) and the treatment concentrates that contained either Agolin Ruminant® (AR) or lauric acid (LA) as feed additive) and the calculated composition of the complete TMR (g/kg of DM, unless stated otherwise).

	Roughages		Concentrates			TMR		
Item	Corn silage[2]	Grass silage[3]	Basal	AR	LA	Basal	AR	LA
Inclusion, % of DM	40	30	30	30	30	-	-	-
DM, g/kg	309	354	878	879	876	403	403	403
Gross energy, MJ/kg of DM	18.7	19.0	18.2	18.3	19.7	18.6	18.7	19.1
Crude ash	39	100	88	89	86	72	72	71
CP	89	186	221	227	215	158	160	156
Crude fat	37	43	40	43	96	40	41	57
NDF	383	459	245	249	231	364	366	360
ADF	223	277	116	120	111	207	208	206
ADL	13	9	27	29	26	16	17	16
Starch	323	N.A.[1]	247	238	229	203	201	198
Sugar	N.A.	54	78	80	76	40	40	39

[1] N.A. = not analyzed.

[2] NE_L, net energy for lactation, 6.9 MJ/kg of DM; DVE, intestinal digestible protein, 54 g/kg of DM; OEB, rumen-degraded protein balance, -38 g/kg of DM (values based on near-infrared spectrometry; Blgg AgroXpertus, Wageningen, the Netherlands).

[3] NE_L, net energy for lactation, 6.6 MJ/kg of DM; DVE, intestinal digestible protein, 58 g/kg of DM; OEB, rumen-degraded protein balance, 69 g/kg of DM (values based on near-infrared spectrometry; Blgg AgroXpertus, Wageningen, the Netherlands).

Diets and feeding

A TMR with basal concentrate was fed during the pre-treatment period. For the AR-AR treatment, the TMR with AR concentrate was fed during all 10 treatment wks, whereas for the AR-LA treatment AR and LA concentrates were rotated on a weekly basis (AR in wk 1 of each period, LA in wk 2 of each period). The AR concentrate contained Agolin Ruminant® (0.17 g/kg of DM) and the LA concentrate contained lauric acid (C12:0; 20 g/kg of DM) (Table 5.1). During the experimental period in the tie-stalls and CRC, animals were fed twice daily (at 0600 and 1600 h). All cows received their experimental diet as a total mixed ration (TMR), composed of 40% corn silage, 30% grass silage, and 30% concentrate on a DM basis (Table 5.2). Portions of the grass silage and corn silage mixture were weighed in crates twice weekly and stored at 6°C. Concentrates were weighed separately for each cow and these were manually mixed with the roughage at the time of feeding. The external marker Cr_2O_3 (1.7 g/kg of DM) was added to the compound feed (Research Diet Services, Wijk bij Duurstede, the Netherlands) for estimation of apparent total-tract digestibility (ATTD). During the first 8 d of the pre-treatment period, cows received the basal diet ad libitum. From d 9 onwards, cows received their diet in amounts of 95% of the average daily intake of the cow with the lowest intake within a pair. This feed restriction was imposed throughout the remainder of the experiment in an effort to avoid confounding effects of DMI on CH_4 production. Cows had free access to water throughout the experiment.

Measurements, sampling and laboratory analyses

Feed and feces samples

Representative samples of all individual TMR components were collected at the time of feed preparation. Fecal grab samples were collected in the respiration chambers for estimation of ATTD of nutrients. Fecal samples were collected at each milking during the last 4 d before the moment of concentrate switch.

Samples were stored frozen (-20°C) pending analysis. After thawing, samples were air dried at 60°C until constant weight, and ground to pass a 1-mm screen (Wiley mill; Peppink 100AN, Olst, the Netherlands), before analysis. Dried samples were analyzed for

DM, crude ash, N, NDF, ADF, ADL, starch, sugar, GE, and chromium. In fresh silage samples, NH_3 was analyzed according to the methods described by Klop et al. (2016). Crude fat content of dried feed and feces samples was analyzed based on NEN-ISO 1735 (ISO, 2004). A modification to the standard procedure was that samples were hydrolysed with hydrochloric acid at 75°C and subsequently the solution, containing hydrochloric acid and ethanol, was extracted with diethyl ether and petroleum ether. Solvents were removed by distillation before the mass of the extracted material was determined.

Orts were quantitatively collected and weighed daily during the period in the respiration chambers. If the amount comprised more than 4% of DM supply, a representative subsample was analyzed for DM, ash and crude fat content according to the same methods as the feed samples.

Milk production and milk composition

Cows were milked twice daily (at 0600 h and 1600 h) throughout the experiment. Milk production was recorded at each milking. For all cows, a subsample of milk from each milking in the CRC was analyzed for fat, protein, lactose, and urea content according to methods described by Hatew et al. (2015a). Average milk composition for each cow was calculated from the weighted average of all samples taken during the measurement period in the CRC. Separate samples were collected for analysis of milk fatty acid (FA) profile through gas chromatography as detailed by van Gastelen et al. (2015). Fat and protein corrected milk yield (FPCM) was calculated according to the formula: FPCM (kg/d) = (0.337 + 0.116 × fat% + 0.06 × protein%) × milk yield (kg/d) (CVB, 2008).

Rumen content samples

In each of the tie-stall periods (viz. period 2 and 4), rumen fluid samples were collected from the rumen cannulated cows at the day of concentrate switch and the day thereafter (i.e., d 1, 2, 8, and 9 of each of the two 2-wk periods). Samples were collected at 0 h (just before), and at 1, 2, 3, 4, 6, 8 and 10 h after morning feeding on both days. Rumen fluid samples were collected in 3 equal amounts from the front and middle of the ventral sac and from the cranial sac of the rumen. In each sample, pH was measured immediately after sampling using a portable pH meter (HI 99141, Hannah instruments, IJsselstijn, the

Netherlands). A 600 µL aliquot of rumen fluid was mixed with an equal volume of 0.85% *M* ortho-phosphoric acid, containing iso-caproic acid as internal standard and stored at -20°C. After thawing, samples were centrifuged for 10 min at 10,000 × *g* at 4°C. Separation of volatile fatty acids (VFA) was achieved by gas chromatography (Fisons HRGC Mega 2, CE Instruments, Milan, Italy) with H_2 as carrier gas as detailed by Dieho et al. (2016).

Statistical Analysis

All data were analyzed using PROC MIXED (SAS 9.2, SAS Inst. Inc., Cary, NC). For one cow (AR-LA treatment) data from the pre-treatment period were excluded, because the cow had a sudden large drop in milk production, while maintaining feed intake. No clinical signs of disease were observed and milk yield increased again during the following 2 weeks in the tie-stalls. Data for CH_4 emission, intake, milk production, milk composition and ATTD all relate to the CRC periods and were averaged per period and cow. Measurements from d 1 (cows entering the chambers around 1400 h) and d 8 (cleaning of chambers in the morning) were not included in the analyses. Hence, the pre-treatment period included 2 full d of data, and each CRC period comprised 2 weeks of 6 full d of data each.

The 2 pairs of cows that went into the CRC at the same time throughout the experiment were considered as one block. The following time points were included in the analyses: Pre-treatment (background measurement), period 1 (first 2 weeks of dietary treatment), period 3 (weeks 5 and 6 of dietary treatment) and period 5 (weeks 9 and 10 of dietary treatment). The model contained block, treatment, time and treatment × time interaction as fixed effects. Repeated measures over time for each cow × treatment combination were taken into account using a first order autoregressive [AR(1)] covariance structure.

Rumen fluid from two sampling days was pooled before statistical analysis. One cow (AR-LA treatment) had access to other feed than the treatment feed allocated to her on the last rumen sampling day of period 4. Therefore, the values of this day were not used to calculate average values for this cow. Rumen data were analyzed using a model with fixed effects of treatment, time, hour, and treatment × time and treatment × hour. Cow × time

was included as random effect with repeated measurements for each time(cow) combination included, and a spatial power covariance structure was fitted because of unequal time intervals between sampling hours. In all statistical analyses, denominator degrees of freedom were estimated using the Kenward-Roger option. Pairwise comparisons of treatment means were evaluated using the Tukey-Kramer method. In case of significant interaction terms, between-treatment comparisons for each period, or within-treatment comparisons over periods were made using a SLICE statement and *P*-values were corrected using the Tukey-Kramer method. Results are reported as least squares means and significance of effects was declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

RESULTS AND DISCUSSION

Dry matter intake, milk production, and milk composition

The main aim of this study was to evaluate the alternate feeding of two CH_4 mitigating feed additives (Agolin Ruminant® and C12:0; AR-LA treatment) with a different mode of action, compared to feeding a single additive (Agolin Ruminant® only; AR-AR treatment) on CH_4 emission and performance of dairy cows. Dry matter intake of AR-AR cows did not differ between the pre-treatment and treatment periods. In contrast, despite the restricted feeding regimen, DMI of AR-LA was significantly reduced from period 3 onwards (Table 5.3). In comparison to C14:0 and C18:0 supplementation, C12:0 supplementation decreases feed intake (Dohme et al., 2004). In line with the present results, Külling et al. (2002) also observed a reduction in DMI upon supplementation with C12:0. If the palatability of C12:0 is the main reason for the reduced DMI (Külling et al. 2002), encapsulation of the product could provide a solution to avoid reductions in intake.

Milk production was not affected by treatment, but was decreased in period 5 as cows were advancing in lactation. During periods 3 and 5, but not during the pre-treatment period and period 1, milk protein concentration was reduced in the AR-LA treatment which resulted in a treatment × time interaction. Feeding digestible lipid in significant amounts is generally known to reduce the concentration of protein in milk (Walker et al.,

2004), but in the present experiment dietary lipid content only increased by 16 g/kg of DM. The periods of reduced milk protein content correspond to the periods that AR-LA cows had a reduced DMI. Therefore, the effect was most likely caused by a lower intake of metabolizable energy or protein. Milk urea N content was not affected by treatment, time, or their interaction.

Milk fat depression following C12:0 supplementation has been reported previously (Faciola and Broderick, 2014; Hristov et al., 2011; Chapter 4). Although in the present study milk fat concentration was numerically lower in periods 3 and 5 with AR-LA, there was no treatment effect on milk fat concentration (Table 5.3). Santos et al. (2010) reported increased milk fat content and production when cows were fed Agolin Ruminant® (0.85 g/cow/d) and suggested that this could be the result of an increased acetate:propionate (A:P) ratio in the rumen (which was not measured). However, the cows in their study produced 49 kg of milk/d with an average DMI of 26 kg/cow/d, which is higher than in the present experiment. In Chapter 4 a transient shift towards a larger proportion of propionate was observed in vitro using rumen fluid from cows on a diet containing Agolin Ruminant®. As the control cows in the study of Santos et al. (2010) had a numerically higher DMI and a similar milk production level, a plausible explanation for the increased milk fat concentration upon feeding Agolin Ruminant® is increased body fat mobilization rather than a shift in VFA profile. The proportion of C12:0 in milk fat of cows on the AR-LA treatment was higher than for AR-AR (Table 5.4), in particular in period 1. The DMI of these cows was only reduced from period 3 onwards, which may explain that the largest proportion of C12:0 in milk fat was observed in period 1. Dohme et al. (2004) observed a higher proportion of C12:0 in milk fat upon supplementing the diet with C12:0 compared with C14:0 and C18:0. Van Zijderveld et al. (2011) also observed an elevated proportion of C12:0 in milk fat and a lower proportion of C16:0 upon feeding a mixture of additives including C12:0. The increased proportion of C12:0 and the reduced proportion of C16:0 in milk fat of AR-LA cows in period 3 is in line with findings of Hristov et al. (2011), who supplemented dairy cows with 240 g/d of either stearic acid (C18:0; control treatment), C12:0, or myristic acid (C14:0). In their study, but not in the study of Dohme et al. (2004), the proportion of saturated fatty acids (SFA) was lower in cows supplemented

with C12:0 than in cows receiving the C18:0 control treatment. In the present study, a tendency for a treatment × time interaction was found for the proportion of SFA, which was lower in the AR-LA treatment during the treatment periods compared to the pre-treatment period, and is opposite to changes between periods in SFA proportions with the AR-AR treatment.

In comparison with other periods, in period 1, when intake of C12:0 was highest, proportions of several C18:1 fatty acids were increased in the AR-LA treatment but not in the AR-AR treatment, resulting in a significant treatment × time interaction (Table 5.4). Dohme et al. (2004) and Hristov et al. (2011) also reported larger proportions of trans C18:1 and CLA isomers in milk of cows on a C12:0 treatment than in cows on a C18:0 or C14:0 treatment. Apparently, C12:0 causes a larger proportion of biohydrogenation intermediates to escape complete biohydrogenation in the rumen. After intestinal absorption, such intermediates may decrease de novo fatty acid synthesis in the mammary gland (Piperova et al., 2000).

Benchaar et al. (2007) evaluated the effect of a mixture of essential oil compounds (Crina ruminants; includes thymol, eugenol, vanillin, guaiacol, and limonene) in dairy cattle and did not find any effect on milk fatty acid profile. To our knowledge, the effect of Agolin Ruminant® on milk fatty acid profile has not been reported previously. In both AR-AR and AR-LA, the proportions of C15:0 *iso* and C15:0 *anteiso* were reduced in period 1 compared with the pre-treatment period (Table 5.4). Castro Montoya et al. (2011) reported a positive relationship between *iso* FA and calculated CH_4 production (mmol/mol VFA). Fibrolytic bacteria are generally enriched in *iso* FA, whereas amylolytic bacteria contain high amounts of linear odd-chain FA and *anteiso* FA (Vlaeminck et al., 2006). Hence, a positive relation between CH_4 emission and *iso* FA can be assumed, as well as a negative relation between CH_4 emission and linear odd-chain FA and *anteiso* FA (reviewed by Van Gastelen and Dijkstra, 2016). During period 1, in line with the change in *iso*-acid content of milk fat, CH_4 production was indeed lower than during the pre-treatment period, but in contrast C15:0 *anteiso* and C17:0 *anteiso* were reduced in period 3 and C15:0 and C17:0 were not affected by treatment (Table 5.5). Within the AR-AR treatment, also the

Table 5.3. Dry matter intake, milk production and milk composition of cows receiving either the control (AR-AR) or the alternating (AR-LA) concentrate treatment (TRT). The AR concentrate contained Agolin Ruminant® (0.17 g/kg of DM) and the LA concentrate contained lauric acid (C12:0; 20 g/kg of DM). A basal concentrate was fed during the pre-treatment period.

	Pre-treatment		Period 1		Period 3		Period 5		SEM	*P*-values		
Item	AR-AR	AR-LA	AR-AR	AR-LA	AR-AR	AR-LA	AR-AR	AR-LA		TRT	Time	TRT×Time
DMI, kg/d	17.7^{a}	18.0^{a,x}	18.4^{a}	17.1^{a,x}	18.3^{a}	16.1^{b,y}	18.2^{a}	15.6^{b,y}	0.45	0.030	0.071	0.007
Milk production, kg/d	26.6^{X}	27.6^{X}	27.0^{X}	26.4^{X}	26.4^{X}	25.4^{X}	25.0^{Y}	23.8^{Y}	0.96	0.716	0.007	0.404
FPCM, kg/d	28.5^{X}	29.1^{X}	28.5^{XY}	27.5^{XY}	28.2^{YZ}	25.4^{YZ}	27.4^{Z}	24.1^{Z}	0.81	0.156	0.003	0.058
Fat, g/kg	45.4	43.8	44.1	43.6	44.9	40.6	47.2	41.9	2.05	0.308	0.166	0.191
Protein, g/kg	34.8^{a}	34.7^{a,x}	34.7^{a}	33.5^{a,xy}	35.7^{a}	32.5^{b,y}	36.5^{a}	32.6^{b,y}	0.61	0.035	0.217	0.008
Lactose, g/kg	46.2^{X}	48.4^{X}	46.1^{XY}	47.3^{XY}	46.1^{XY}	47.2^{XY}	45.5^{Y}	47.1^{Y}	0.46	0.033	0.040	0.124
MUN, mg/dL	6.4	6.4	6.2	6.7	5.9	6.1	6.5	6.7	0.48	0.642	0.122	0.915

^{ab} Treatment values within period with different lower-case superscript letters differ significantly (P<0.05) from each other.

^{xy} Period values within treatment with different lower-case superscript letters differ significantly (P<0.05) from each other.

^{XYZ} Period values with different upper-case superscript letters differ significantly (P<0.05) from each other.

Table 5.4. Fatty acid composition of milk from cows receiving either the control (AR-AR) or the alternating (AR-LA) concentrate treatment. The AR concentrate contained Agolin Ruminant® (0.17 g/kg of DM) and the LA concentrate contained lauric acid (C12:0; 20 g/kg of DM). A basal concentrate was fed during the pre-treatment period.

	Pre-treatment		Period 1		Period 3		Period 5		SEM	*P*-value		
Fatty acid	AR-AR	AR-LA	AR-AR	AR-LA	AR-AR	AR-LA	AR-AR	AR-LA		TRT	Time	TRT×Time
C4:0	3.33^{X}	3.30^{X}	3.55^{Y}	3.69^{Y}	3.27^{Y}	3.50^{Y}	3.18^{Y}	3.48^{Y}	0.107	0.158	0.004	0.772
C6:0	2.24	2.33	2.26	2.11	2.25	2.05	2.25	2.03	0.080	0.263	0.108	0.053
C8:0	1.18	1.31	1.22	1.18	1.22	1.13	1.23	1.11	0.052	0.641	0.419	0.050
C10:0	2.67^{b}	3.06^{a}	2.88^{a}	2.80^{a}	2.89^{a}	2.70^{a}	2.91^{a}	2.62^{a}	0.110	0.696	0.843	0.042
C12:0	3.20^{a}	$3.57^{a,z}$	3.48^{b}	$7.49^{a,x}$	3.52^{b}	$6.11^{a,y}$	3.53^{b}	$5.19^{a,y}$	0.291	<0.001	<0.001	<0.001
C14:0	11.21	12.38	11.65	11.74	11.68	12.03	11.72	11.67	0.443	0.491	0.779	0.135
C14:0 *iso*	0.09	0.08	0.09	0.07	0.09	0.07	0.09	0.07	0.006	0.021	0.399	0.433
C14:1 *cis-9*	0.84^{Y}	0.91^{Y}	0.93^{X}	1.02^{X}	0.94^{X}	1.12^{X}	0.92^{XY}	1.06^{XY}	0.079	0.281	0.015	0.469
C15:0	1.00	0.97	1.01	0.90	1.01	1.01	1.01	1.04	0.053	0.630	0.488	0.450
C15:0 *iso*	0.23^{X}	0.24^{X}	0.21^{Y}	0.20^{Y}	0.21^{XY}	0.23^{XY}	0.21^{XY}	0.25^{XY}	0.012	0.265	0.023	0.127
C15:0 *anteiso*	0.44^{X}	0.45^{X}	0.40^{Y}	0.35^{Y}	0.38^{Y}	0.40^{Y}	0.38^{XY}	0.40^{XY}	0.018	0.842	0.004	0.123
C16:0	32.81^{a}	$31.08^{a,x}$	33.46^{a}	$28.25^{b,y}$	33.97^{a}	$30.14^{a,x}$	33.87^{a}	$30.20^{a,xy}$	1.172	0.064	0.018	0.007
C16:0 *iso*	0.23	0.21	0.19	0.18	0.19	0.22	0.21	0.18	0.019	0.552	0.275	0.482
C16:1 *cis-9*	1.63	1.21	1.69	1.46	1.67	1.49	1.63	1.50	0.108	0.135	0.051	0.259
C16:1 *trans-9*	0.18^{a}	$0.17^{a,y}$	0.18^{a}	$0.22^{b,x}$	0.17^{a}	$0.21^{b,x}$	0.17^{a}	$0.20^{a,xy}$	0.012	0.139	0.009	0.004
C17:0	0.55	0.55	0.53	0.50	0.51	0.50	0.51	0.54	0.024	0.837	0.140	0.493
C17:0 *iso*	0.33	0.33	0.31	0.34	0.31	0.36	0.30	0.37	0.020	0.147	0.917	0.315
C17:0 *anteiso*	0.42^{X}	0.41^{X}	0.36^{Y}	0.38^{Y}	0.35^{XY}	0.41^{XY}	0.34^{XY}	0.40^{XY}	0.023	0.253	0.022	0.316
C17:1 *cis-9*	0.25	0.19	0.24	0.24	0.22	0.22	0.21	0.24	0.024	0.864	0.522	0.237
C18:0	10.40^{x}	11.53^{x}	9.48^{y}	9.12^{y}	9.42^{xy}	9.25^{y}	9.56^{xy}	9.58^{y}	0.380	0.745	<.0001	0.016
C18:1 *cis*-9[2]	18.48	17.70	17.82	18.07	17.51	18.29	17.49	19.35	0.886	0.568	0.865	0.521
C18:1 *cis*-12	0.26	0.25^{y}	0.27	0.33^{x}	0.26	0.33^{x}	0.26	0.31^{xy}	0.023	0.204	0.005	0.045
C18:1 *cis*-13	0.11^{a}	$0.11^{a,y}$	0.11^{b}	$0.17^{a,x}$	0.10^{b}	$0.16^{a,x}$	0.10^{b}	$0.15^{a,x}$	0.009	0.004	0.001	0.003
C18:1 *trans*-9	0.15	0.14^{y}	0.16	0.18^{x}	0.15	0.17^{xy}	0.16	0.16^{xy}	0.010	0.647	0.002	0.041
C18:1 *trans*-10	0.21	0.17	0.23	0.45	0.24	0.26	0.22	0.23	0.067	0.361	0.087	0.132
C18:1 *trans*-11	0.71^{a}	$0.68^{a,x}$	0.77^{b}	$1.36^{a,y}$	0.74^{a}	$0.91^{a,y}$	0.73^{a}	$0.85^{a,y}$	0.117	0.075	0.006	0.025

Table 5.4. continued.

	Pre-treatment		Period 1		Period 3		Period 5		SEM	*P*-value		
Fatty acid	AR-AR	AR-LA	AR-AR	AR-LA	AR-AR	AR-LA	AR-AR	AR-LA		TRT	Time	TRT×Time
C18:1 *trans*-15 +	0.74^{a}	0.73^{a,y}	0.78^{b}	1.00^{a,x}	0.73^{b}	0.93^{a,x}	0.71^{b}	0.92^{a,x}	0.041	0.018	<0.001	0.007
C18:2 *cis*-9, *trans*-11[1]	0.33^{a}	0.28^{a,y}	0.36^{b}	0.57^{a,x}	0.35^{a}	0.43^{a,xy}	0.33^{a}	0.40^{a,y}	0.052	0.167	0.003	0.021
C18:2n-6	1.38	1.27^{y}	1.42	1.52^{x}	1.39	1.22^{y}	1.35	1.28^{y}	0.107	0.657	0.003	0.011
C18:3n-3	0.41	0.37	0.39	0.36	0.39	0.31	0.37	0.34	0.035	0.382	0.339	0.334
C18:3n-6	0.08	0.09	0.08	0.09	0.08	0.08	0.08	0.09	0.002	0.083	0.224	0.869
C20:0	0.15^{X}	0.16^{X}	0.14^{Y}	0.13^{Y}	0.15^{Y}	0.14^{Y}	0.16^{X}	0.15^{X}	0.005	0.208	<0.001	0.194
C20:1 *cis*-11	0.06^{a}	0.06^{a}	0.05^{b}	0.07^{a}	0.05^{a}	0.05^{a}	0.05^{a}	0.06^{a}	0.005	0.046	0.441	0.035
C20:2n-6[2]	0.04^{X}	0.04^{X}	0.03^{Y}	0.04^{Y}	0.03^{Y}	0.03^{Y}	0.04^{XY}	0.04^{XY}	0.003	0.865	0.007	0.433
C20:3n-6	0.08^{X}	0.08^{X}	0.07^{Y}	0.07^{Y}	0.08^{XY}	0.06^{XY}	0.08^{XY}	0.06^{XY}	0.005	0.206	0.037	0.090
C20:4n-6	0.10	0.10	0.10	0.09	0.10	0.08	0.10	0.09	0.005	0.204	0.265	0.423
C20:5n-3	0.05^{b}	0.06^{a,x}	0.05^{a}	0.05^{a,y}	0.05^{a}	0.05^{a,y}	0.05^{a}	0.05^{a,xy}	0.003	0.386	0.023	0.004
C22:0	0.06^{Y}	0.06^{Y}	0.05^{Y}	0.05^{Y}	0.06^{Y}	0.06^{Y}	0.08^{X}	0.07^{X}	0.003	0.318	<0.001	0.346
C22:5n-3	0.10^{X}	0.09^{X}	0.09^{Y}	0.08^{Y}	0.08^{Y}	0.08^{Y}	0.08^{Y}	0.08^{Y}	0.006	0.450	0.002	0.863
C24:0	0.04	0.04	0.04	0.04	0.04	0.03	0.04	0.04	0.002	0.813	0.077	0.160
SFA[3]	70.93	72.30	71.25	69.08	71.72	70.03	71.83	69.05	1.061	0.315	0.148	0.072
MUFA[4]	23.59	22.30	23.21	24.57	22.78	24.13	22.65	25.01	0.938	0.368	0.446	0.172
PUFA[5]	2.55	2.38^{y}	2.59	2.86^{x}	2.54	2.35^{y}	2.48	2.48^{xy}	0.191	0.871	0.006	0.020
n-6 : n-3 ratio[6]	3.04^{a}	3.03^{a,z}	3.21^{b}	3.70^{a,x}	3.25^{a}	3.41^{a,y}	3.26^{a}	3.30^{a,yz}	0.188	0.145	<0.001	0.005

[1]C18:1 *cis*-9 comprises the sum of C18:1 *cis*-9 and C18:1 *trans*-12, because these 2 fatty acids could not be separated in the analysis. The proportion of C18:1 *trans*-12 is usually negligible.

[2]C20:2n-6 comprises the sum of C20:2n-6 and C21:0, because these 2 fatty acids could not be separated in the analysis.

[3]SFA = sum of saturated fatty acids reported in this table.

[4]MUFA = sum of mono unsaturated fatty acids reported in this table.

[5]PUFA = sum of poly unsaturated fatty acids reported in this table.

[6]Ratio between the sum of C18:2n-6, C18:3n-6, C20:2n-6, C20:3n-6, and C20:4n-6, and the sum of C18:3n-3, C20:5n-3, and C22:5n-3.

[ab] Treatment values within period with different lower-case superscript letters differ significantly (P<0.05) from each other.

[xy] Period values within treatment with different lower-case superscript letters differ significantly (P<0.05) from each other.

[XYZ] Period values with different upper-case superscript letters differ significantly (P<0.05) from each other.

Table 5.5. Methane (CH_4) production and apparent total tract digestibility (ATTD) of nutrients and gross energy in cows receiving either the control (AR-AR) or the alternating (AR-LA) concentrate treatment. The AR concentrate contained Agolin Ruminant® (0.17 g/kg of DM) and the LA concentrate contained lauric acid (C12:0; 20 g/kg of DM). A basal concentrate was fed during the pre-treatment period.

	Pre-treatment		Period 1		Period 3		Period 5		SEM	*P*-value		
Item	AR-AR	AR-LA	AR-AR	AR-LA	AR-AR	AR-LA	AR-AR	AR-LA		TRT	Time	TRT×Time
CH_4, g/d	408^{x}	408^{x}	378^{y}	336^{y}	386^{xy}	328^{y}	386^{xy}	316^{y}	21.9	0.208	<0.001	0.012
CH_4, g/kg	23.1^{W}	22.7^{W}	20.5^{X}	19.7^{X}	21.0^{WX}	20.5^{WX}	21.2^{WX}	20.8^{WX}	1.26	0.769	<0.001	0.947
CH_4, g/kg	14.3^{W}	14.1^{W}	13.2^{X}	12.1^{X}	13.6^{WX}	12.9^{WX}	14.0^{WX}	13.2^{WX}	0.71	0.456	<0.001	0.502
CH_4, %	6.9^{y}	6.8^{Y}	6.1^{X}	5.8^{X}	6.2^{X}	6.0^{X}	6.3^{X}	6.1^{X}	0.38	0.694	<0.001	0.882
ATTD (%)												
DM	71.8^{WX}	75.3^{WX}	74.0^{WX}	71.8^{WX}	73.6^{W}	73.8^{W}	71.4^{X}	72.3^{X}	1.30	0.895	0.004	0.554
OM	73.0^{WX}	74.9^{WX}	74.5^{WX}	74.6^{WX}	73.0^{X}	72.1^{X}	76.4^{W}	76.0^{W}	1.34	0.895	0.001	0.639
CP	63.8^{Y}	64.9^{Y}	67.3^{WX}	68.3^{WX}	65.6^{XY}	66.3^{XY}	70.4^{W}	70.8^{W}	1.23	0.486	<0.001	0.991
NDF	57.8^{WX}	61.1^{WX}	61.1^{WX}	58.9^{WX}	58.9^{X}	54.4^{X}	64.0^{W}	62.6^{W}	2.63	0.719	0.001	0.161
Crude fat	66.3^{YZ}	70.2^{YZ}	68.9^{XZ}	75.3^{XZ}	63.8^{Y}	69.6^{Y}	69.6^{X}	$74.5^{X,}$	1.33	<0.001	0.004	0.849
Starch	97.5	97.7	98.0	98.1	97.6	98.1	98.1	98.3	0.36	0.479	0.315	0.902
Gross	70.3^{YZ}	72.2^{YZ}	72.8^{WY}	73.0^{WY}	70.9^{Z}	70.6^{Z}	74.8^{W}	74.3^{W}	1.27	0.797	0.001	0.715

[1]GEI = Gross energy intake.

^{xy} Period values within treatment with different lower-case superscript differ significantly ($P<0.05$) from each other.

^{WXYZ} Period values with different upper-case superscript letters differ significantly ($P<0.05$) from each other.

proportion of C18:0 in milk fat was reduced in period 1 compared to the pre-treatment period, but not in periods 3 and 5 (Table 5.4). In several studies, milk C18:0 is not related to CH_4 production (Van Gastelen and Dijkstra, 2016), but decreases towards the end of a lactation cycle (Stoop et al., 2009). Overall, Agolin Ruminant® does not seem to have caused major shifts in the milk FA profile.

Methane emission

A significant treatment × time interaction was observed for CH_4 production (g/d) (Table 5.5). Methane production with AR-AR in period 1 was lower than in the pre-treatment period (7% lower), but in period 3 and 5 did not differ with the pre-treatment period (5% lower; numerically only). However, with the AR-LA treatment, methane production in periods 1, 3 and 5 was significantly lower (on average 20%) than in the pre-treatment period. The reduced DMI in period 3 and 5 with the AR-LA treatment but not with the AR-AR treatment offers an explanation for the treatment × time interaction that was observed for CH_4 production. Both CH_4 yield (g/kg of DMI) and CH_4 intensity (g/kg FPCM) changed over time, but were not affected by treatment. Methane yield and intensity were significantly lower (12 and 11%, respectively) in period 1 compared with the pre-treatment period, but no significant difference relative to pre-treatment period was observed in period 3 (numerically 9 and 7% lower, respectively) and in period 5 (numerically 8 and 4% lower, respectively). Similarly, CH_4 energy loss (expressed as a fraction of GE intake) was lower in period 1 compared with pre-treatment period, but in period 3 and 5 the difference with pre-treatment period was not present anymore.

The results suggest that upon continuous feeding of Agolin the CH_4 mitigating effect in the initial 2 weeks is larger than from wk 5 onwards, indicating adaptation to the blend of essential oils used. Furthermore, the absence of a more persistent decrease of CH_4 yield and intensity with rotational feeding implies that this rotation does not prevent or retard adaptation. In a previous experiment (Chapter 4), in which rumen fluid was collected as inoculum from donor cows fed Agolin Ruminant®, in vitro CH_4 production was decreased 8 d after introduction of the additive to the donor cow diet, but no effect was observed after 15 and 21 d. In the same study, feeding C12:0 to donor cows showed a persistent

decrease in CH_4 production in vitro. Based on these in vitro results, in the present experiment we evaluated the hypothesis that in vivo, the AR-AR treatment would result in a transient drop of CH_4 production, whereas AR-LA would decrease CH_4 persistently. In their review of in vitro batch culture systems, Yáñez-Ruiz et al. (2016) made several recommendations related to potential differences in microbial profile and adaptation, including using the same donor animals as the target species, choosing diets and incubation substrates with similar nutrient composition, adapting donor animals to the experimental diet before rumen fluid collection, rumen fluid collection before morning feeding, and applying a restricted feeding regime to obtain a better interpretation of in vitro data for the in vivo situation. In the experiment described in Chapter 4, many of those criteria were met, but nevertheless the hypothesis based on these vitro results could not be confirmed based on results of the present study. Also Hatew et al. (2015b) observed a poor relationship between in vitro and in vivo CH_4 production (expressed in g/kg OM) when using cows in the in vivo trial as donor animals, although a moderate relationship was obtained when CH_4 was expressed per unit rumen fermentable OM. The present results support the conclusion by Yáñez-Ruiz et al. (2016) that results from in vitro incubations have to be interpreted with care, before such mitigation strategies can be translated to the in vivo situation.

Castro-Montoya et al. (2015) supplemented a similar dose of Agolin Ruminant® (1 g/cow/d) as used in the present study to a diet composed of grass silage (460 g/kg of DM), corn silage (370 g/kg of DM), soybean meal (50 g/kg of DM) and concentrates (120 g/kg of DM) for 6 wk. In their experiment, Agolin Ruminant® in wk 2 and 6 after first introduction tended to persistently lessen CH_4 production and CH_4 yield by 15 and 14%, respectively, but methane intensity was not affected. The overall average CH_4 production of 247 g/d and 15.8 g/kg of DMI during the weeks that Agolin Ruminant® was fed was lower than in the present study. Methane expressed per kg milk was similar, because of a higher milk production of cows in the present study. Interestingly, in an experiment with beef cattle of the same authors (Castro-Montoya et al., 2015), daily CH_4 production and CH_4 yield did not change upon Agolin Ruminant® supplementation in wk 2, 4 or 6.

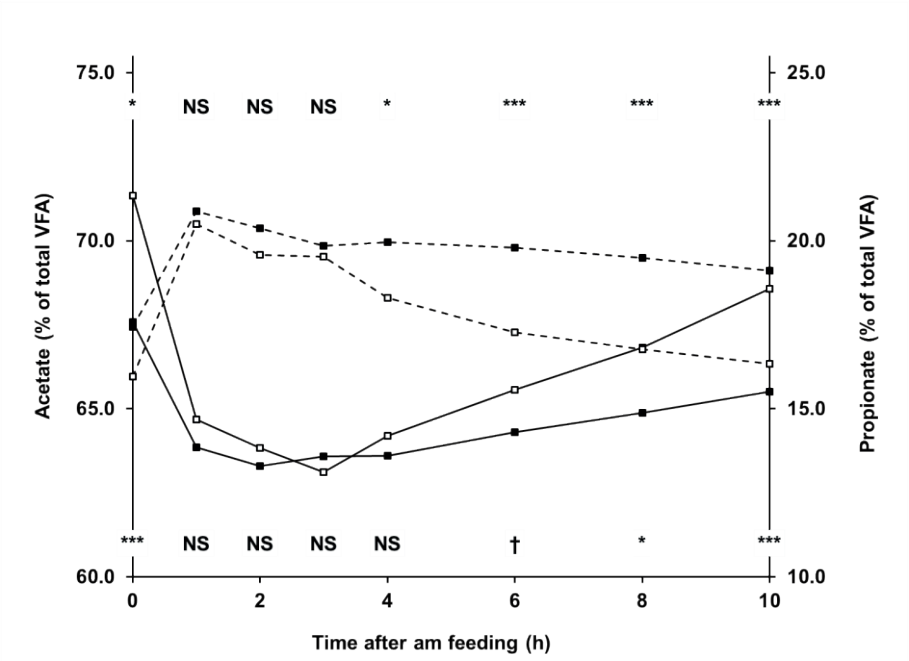

Figure 5.1. Molar percentage of acetate (solid lines) and propionate (dashed lines) as % of total volatile fatty acids (VFA) in rumen fluid from cows after a.m. feeding of either the AR-AR treatment (Agolin Ruminant® (0.17 g/kg of DM; □; n = 2)) or the AR-LA treatment (weekly rotation of Agolin Ruminant® (0.17 g/kg of DM) and lauric acid (20 g/kg of DM; ■; n = 2)). Each data point represents the treatment average of the pre-treatment period, period 2 and period 4 for the hours indicated. Symbols indicate significance of treatment differences at each time point (NS not significant; † 0.05 < *P* < 0.10; * *P* < 0.05; ** *P* < 0.01; *** *P* < 0.001). The top row of symbols relates to propionate and the bottom row to acetate. Pooled SEM values were 0.44 and 0.43 for acetate and propionate, respectively.

Van Zijderveld et al. (2011) found that feeding a mixture of additives (C12:0, C14:0, linseed oil, and calcium fumarate) decreased CH_4 production and CH_4 energy loss as a fraction of gross energy intake, but the additive mixture did not affect CH_4 yield or intensity. In the study of Külling et al. (2002), the addition of C12:0 (40 g/kg of DM) reduced DMI, CH_4 production and CH_4 intensity (expressed in g/kg energy corrected milk) compared with the C18:0 control diet. No effect was observed for CH_4 yield. Martin et al. (2010) concluded that C12:0 and C14:0 have a more depressive effect on CH_4 emission than other fatty acids.

However, Grainger and Beauchemin (2011) did not find an effect of type of fatty acid (C12:0, C14:0, C18:1, C18:2, C18:3) on CH_4 yield when total fat was restricted to < 80 g/kg of DM. The present experiment had a dietary fat content of up to 57 g/kg of DM and is within this range.

In view of the transitory decline in CH_4 yield and intensity with Agolin Ruminant®, and given the negative effects of C12:0 on feed intake, it is worthwhile to investigate rotational feeding of Agolin Ruminant® in combination with another compound than in the present study.

Digestibility of nutrients

Apparent total tract digestibility of nutrients was not affected by time × treatment interaction or treatment, except for crude fat (Table 5.5). The higher fat digestibility in AR-LA cows is most likely caused by the difference in fat content between the AR and LA concentrate (Table 5.2). If fat supplementation is higher, the calculated digestibility values are less affected by fecal excretion of endogenous fat sources (Kil et al., 2010). Faciola and Broderick (2014) reported that both ruminal and total tract fiber digestion were depressed following C12:0 supplementation. In general, milk fat depression caused by intermediates of ruminal biohydrogenation may be associated with factors including low rumen pH and reduced fiber degradation in the rumen (Bauman and Griinari, 2003). The absence of a treatment effect on NDF digestibility in the present study may explain why milk fat content was also not significantly affected with the AR-LA treatment. The period did significantly affect ATTD of most nutrients, with in general a lower digestibility in period 3 than in other periods. The reason for this lower digestibility is unknown.

Rumen pH and VFA

Average rumen pH and total VFA concentration were not affected by treatment (Table 5.6). Molar proportions of acetate and propionate were lower and higher, respectively, in the AR-LA treatment compared with the AR-AR treatment, resulting in a significantly lower A:P ratio with the AR-LA treatment. No treatment × time interaction was found for these parameters, but the numerical differences between AR-AR and AR-LA were larger during

the treatment period than during the pre-treatment period (Table 5.6), and in particular the numerical difference in the A:P ratio became larger with advanced period. Molar proportion of acetate in AR-LA was lower at 0, 8 and 10 h after am feeding, and tended to be lower at 6 h after am feeding compared with that in AR-AR (Figure 5.1). Hristov et al. (2011) and Faciola and Broderick (2014) reported reduced VFA concentrations (123 and 128 m*M* for C12:0 and control, respectively), and in line with the present results reported reduced molar proportion of acetate (63.7 and 65.0% of total VFA for C12:0 and control, respectively) following C12:0 supplementation. In both studies rumen samples were collected at multiple time points relative to feeding, but only averaged values were reported.

In the present study, the molar proportion of propionate was higher in the AR-LA treatment than in the AR-AR treatment from 4 h post feeding onwards at the expense of acetate (Figure 5.1). Feeding C12:0 often reduces protozoa counts in rumen fluid (Hristov et al., 2011; Faciola et al., 2014). In the meta-analysis by Eugène et al. (2008) defaunation resulted in a decreased molar proportion of acetate and butyrate, and an increased molar proportion of propionate in rumen fluid, which might be associated with less CH_4 production. Impaired fiber degradation in the rumen may also cause a relative increase in propionate proportion. Apparent total tract digestibility of NDF was not affected by additive treatment in this study, although numerically values were lower for AR-LA than AR-AR during periods 1, 3 and 5 (Table 5.5). As discussed by Van Zijderveld et al. (2011) a negative effect of a treatment on ruminal fiber degradation may be partly compensated by fermentation in the hindgut. The latter will yield less nutrients to support milk production than rumen degradation of fiber. Probably the lower DMI of the AR-LA treatment in the present study resulted in longer rumen retention time of feed. This might have alleviated treatment effects on NDF digestibility.

Table 5.6. Rumen pH and concentration of volatile fatty acids (VFA) in cows receiving either the control (AR-AR) or the alternating (AR-LA) concentrate treatment. The AR concentrate contained Agolin Ruminant® (0.17 g/kg of DM) and the LA concentrate contained lauric acid (C12:0; 20 g/kg of DM). A basal concentrate was fed during the pre-treatment period.

	Pre-treatment		Period 2		Period 4		SEM	*P*-value				
Item	AR-AR	AR-LA	AR-AR	AR-LA	AR-AR	AR-LA		TRT	Time[1]	Hour[2]	TRT×time	TRT×hour
Rumen pH	6.34	6.31	6.28	6.34	6.28	6.27	0.049	0.936	0.593	<0.001	0.618	0.009
Total VFA (m*M*)	115^{XY}	117^{XY}	109^{XY}	106^{XY}	120^{X}	122^{X}	3.0	0.840	0.014	<0.001	0.612	0.004
VFA (% of Total VFA)												
Acetate (A)	65.7	65.2	65.7	64.3	66.7	64.2	0.51	0.013	0.607	<0.001	0.223	<0.001
Propionate (P)	18.0	18.3	18.1	19.7	18.0	20.8	0.58	0.015	0.174	<0.001	0.176	<0.001
Butyrate	11.5	12.4	12.2	11.7	11.5	11.6	0.86	0.823	0.847	<0.001	0.754	<0.001
Isobutyrate	0.97^{X}	0.90^{X}	0.87^{XY}	0.79^{XY}	0.84^{Y}	0.71^{Y}	0.036	0.019	0.012	<0.001	0.688	<0.001
Valerate	1.61^{X}	1.59^{X}	1.47^{Y}	1.40^{Y}	1.41^{Y}	1.42^{Y}	0.053	0.609	0.021	<0.001	0.723	<0.001
Isovalerate	2.10	1.55	1.75	2.01	1.59	1.33	0.231	0.369	0.220	<0.001	0.282	0.446
A:P	3.66	3.58	3.71	3.35	3.77	3.17	0.107	0.007	0.428	<0.001	0.127	<0.001

[1]Time refers to period (pre-treatment period, period 2, period 4)

[2]Samples were collected at 0, 1, 2, 3, 4, 6, 8 and 10 h after morning feeding.

^{XYZ} Period values with different upper-case superscript letters differ significantly ($P<0.05$) from each other.

CONCLUSIONS

In the present study, continuous feeding of Agolin Ruminant® as well as rotational feeding of Agolin Ruminant® and C12:0 resulted in a transient decline in CH_4 yield and intensity. The rotational feeding of Agolin Ruminant® and C12:0 did not improve the extent and persistency of CH_4 mitigation compared with Agolin Ruminant® only. Dietary levels of C12:0 appeared to be too high for application in practice, as DMI was reduced in the rotation treatment. Future research should clarify if rotational feeding of CH_4 mitigating additives (with a transient effect) can result in a persistent mitigation effect.

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Chapter 6

General discussion

GENERAL DISCUSSION

Background and aims of this thesis

Over the past decades, there have been extensive research efforts aimed at identifying and developing feed additives to mitigate enteric CH_4 production by ruminants. According to Regulation (EC) No 1831/2003 on additives for use in animal nutrition, feed additives can be defined as substances, micro-organisms or preparations, other than feed material and premixtures, which are intentionally added to feed or water in order to perform one of the particular functions listed in the regulation. One of these functions is that the additive shall favorably affect the environmental consequences of animal production, and this aspect is relevant to the research described in this thesis. Methane production is often expressed as CH_4 yield (g/kg of DMI) and CH_4 intensity [g/kg fat- and protein-corrected milk (FPCM)]. At present, the search and development of enteric CH_4 mitigate additive is ongoing and it has become clear that several issues have to be resolved before such feed additives can be applied as a viable mitigation strategy. These issues include for example, long term efficacy, interaction with diet and other additives, safety, environmental trade-offs and adverse effects on animal health and performance.

Feeding nitrate is an example of an effective feed additive-based mitigation strategy that also may have some undesirable side effects. It is commonly agreed that feeding nitrate to ruminant animals decreases CH_4 production (see Van Zijderveld et al., 2011b; Lund et al., 2014; Guyader et al., 2015, 2016; Troy et al., 2015; Olijhoek et al., 2016). However, feeding nitrate imposes several restrictions on the formulation of the total diet. To avoid trade-offs in nitrogen (N) emissions to the environment, the basal diet should be low in rumen degradable N (e.g. corn silage based). This limits the applicability in countries with N-rich pasture based systems, or diets containing relatively large amounts of high quality grass silage.

Besides economic aspects and the possible trade-offs in environmental impact, feeding relatively high doses of nitrate increases the risk of methemoglobinemia. This condition may occur when nitrite, an intermediate in the nitrate reduction process, accumulates in

the rumen and enters the bloodstream. Nitrite causes the conversion of hemoglobin into methemoglobin, with the latter being unable to transport oxygen. Although there is considerable evidence that gradual adaptation and feeding regime (reviewed by Lee and Beauchemin, 2014) successfully alleviates the risk of methemoglobinemia, it remains a potential health risk associated with nitrate supplementation. A third aspect that may be negatively affected is DMI (Newbold et al., 2014; Lee et al., 2015), which lowers reduction in CH_4 intensity due to a reduced animal performance.

Despite aforementioned side effects, an important advantage of nitrate as a CH_4 mitigation strategy is that the effects persist over time (Van Zijderveld et al., 2011b). Persistency is an important criterion in the search for feed additive-based CH_4 mitigation strategies because adaptation of the rumen microbes to the additive may occur, and an initial reduction in CH_4 production may become much smaller or even absent in the longer term. For example, promising results on CH_4 reduction using essential oils or their active ingredients have been obtained using in vitro batch cultures, whereas no or only a temporary effect on fermentation characteristics was found in continuous cultures or in vivo (Benchaar and Greathead, 2011; Van Zijderveld et al., 2011a). Cardozo et al. (2004) reported a transient effect of plant extracts on fermentation characteristics that disappeared after six days. The latter indicates that microbial adaptation can occur after short term exposure.

In summary, the application of CH_4 mitigation feed additives may have several negative side effects including trade-offs on other environmental impacts, negative effects on animal health and performance, and lack of persistency of the mitigating effect. The overall aim of this project was to investigate these aspects of application of feed additives as a CH_4 mitigation strategy, going beyond the evaluation of the effect of single feed additives commonly reported in literature. This thesis had the following objectives, which address aspects of interaction between feed additives, adaptation of the rumen microbiota to feed additives, and consequences of an alternating application of feed additives:

1. To investigate if the effects of two different feed additives, with different modes of action on CH_4 production, are additive.
2. To study adaptation to potential CH_4 mitigating feed additives in vivo, using the in vitro gas production technique.
3. To compare CH_4 production and performance of dairy cows fed either a single feed additive, or two different additives according to a rotation schedule.

Additivity

Nitrate is effective in decreasing CH_4 production (Hristov et al., 2013), but unwanted side effects hamper wide spread adoption. Moreover, the strategy is not cost-effective yet (Van Middelaar et al., 2014). Fat supplementation is also known to have CH_4 mitigating effects (Grainger and Beauchemin, 2011; Patra, 2013), but high inclusion levels of fat or specific fatty acids may adversely affect DMI, fiber digestion and milk fat or protein concentration. Usually the negative effects of feed additives occur at higher inclusion levels (Walker et al., 2004) and, therefore, it is worth investigating if the mitigating effects of two additives in the rumen are additive. If so, a similar decrease in CH_4 production can be achieved by combining a lower dose of both individual additives, to alleviate the risk of these negative side effects.

Van Zijderveld et al. (2010) reported an additive effect of nitrate and sulfate on CH_4 production. However, these additives both act as an alternative hydrogen (H_2) sink and the inclusion level of sulfate in ruminant diets is limited to avoid the occurrence of polioencephalomalacia (Gould, 1998; NRC, 2001). Recently, after completion of the experiment described in Chapter 2, Guyader et al. (2015) reported additive effects of nitrate and linseed oil, additives with different modes of action, on CH_4 emission, with a trend ($P=0.07$) for an interaction effect when CH_4 was expressed per unit digested NDF.

In the experiment described in Chapter 2, the additivity of the effects of nitrate and docosahexaenoic acid (C22:n-6; DHA) on CH_4 production and performance was investigated in lactating dairy cows. These additives have a different mode of action in the rumen, where nitrate acts as an alternative H_2 sink (Van Zijderveld et al., 2010) and DHA

has an effect on microbial metabolism in the rumen (Boeckaert et al., 2008a). In vitro CH_4 production was reduced upon DHA supplementation (Fievez et al., 2007), but these results have not (yet) been confirmed in vivo (Moate et al., 2013). If DHA would decrease CH_4 production, the inclusion level has to be limited as high inclusion levels of DHA were shown to induce severe milk fat depression and reduce DMI (Boeckaert et al., 2008b; Moate et al., 2013).

The results of the experiment described in Chapter 2 indicate that there was no interaction between the additives in affecting CH_4 production (and their effect is hence additive). However, DHA did not reduce CH_4 production in g/kg DMI, and even increased CH_4 production in g/kg FPCM, largely as a result of milk fat depression. Therefore, this particular combination of feed additives does not allow for a lower inclusion level of nitrate. Guyader et al. (2015) tested the additivity of the effects of nitrate and linseed oil on CH_4 production in non-lactating cows. They concluded that effects on CH_4 production were additive, although a trend was observed for CH_4 production per unit digested NDF, and the reduction in CH_4 yield with nitrate and linseed oil combined (-31%) was numerically smaller than the sum of individual reductions (nitrate, -22%; linseed oil, -17%). In a follow-up study the effect of a combination of nitrate and linseed oil on enteric CH_4 production and nitrate and nitrite residuals in milk was compared to a control diet (Guyader et al., 2016). The combination of nitrate and linseed reduced CH_4 yield (-30%), but also reduced DMI (-13%), milk protein yield (-15%), total volatile fatty acid (-12%) and propionate (-31%) concentrations, which indicates that the applied doses (1.8% nitrate and 3.5% fat from linseed on a DM basis) where probably still too high to avoid adverse effects.

Although in the study described in Chapter 2 no interaction effect on CH_4 production was observed, the effects of nitrate and DHA on apparent total tract digestibility of NDF where not additive, as discussed in Chapters 2 and 3. The presence of DHA seemed to alleviate negative effects of nitrate on fiber digestion. Guyader et al. (2015) observed a trend of reduced total tract NDF digestibility with linseed oil, without an effect of nitrate or linseed x nitrate interaction. Numerically, the decline in NDF digestion with linseed oil and nitrate

together was larger (-10%) than the effect of linseed oil (-1%) or nitrate (+1%) only. In the experiment described in Chapter 2, a restricted feeding regime was imposed to avoid a confounding effect of DMI on CH_4 production. Therefore, it is not certain if this interaction effect would also have been present if no feed restriction was applied. The applied treatments in Chapter 2 were: Control (CON); NO_3 [21 g of nitrate/kg dry matter (DM)]; DHA [3 g of docosahexaenoic acid (DHA)/kg of DM]; or NO_3+DHA (21 g of nitrate/kg of DM and 3 g of DHA/kg of DM). Based on visual observations in the tie-stalls, the feed intake pattern of cows receiving NO_3, DHA or NO_3+DHA was more gradual than that of control cows. Cows on the control treatment were most restricted in their voluntary intake level which may explain why they consumed their meals faster. Although in the applied experimental setup it was not possible to quantify the difference in intake pattern, the diurnal pattern of the respiration quotient (Chapter 3) supports the visual observation of differences in the rate of feed intake. Further indications of differences in intake pattern are provided by rumen pH and volatile fatty acids (VFA) data. Table 6.1 and Figure 6.1 contain unpublished data from the experiment described in Chapter 2, in which rumen samples were collected during 2 consecutive days from the rumen-cannulated cows. When cows are fed twice daily, it is typically expected that rumen pH will decrease after a meal, and propionate as a fraction of total VFA will increase. This pattern was indeed observed in the control cows, but not in cows receiving one of the nitrate treatments. In particular, the VFA profile on the NO_3+DHA treatment indicates a gradual, constant rate of fermentation, whereas in the control treatment there seems to be a sharp increase in fermentation shortly after feeding. These observations further support differences in feed intake pattern between treatments.

For molar proportions of propionate, acetate (and [acetate + butyrate]:propionate ratio; data not shown) a significant nitrate × time interaction was found (Table 6.1). The molar proportion of propionate in rumen fluid from cows receiving NO_3+DHA 2 h after a.m. feeding was not different compared to the NO_3 treatment, but was significantly lower than in cows receiving no nitrate (Figure 6.1). The absence of an increase in propionate proportion immediately after feeding nitrate seems to be the main reason for the overall reduction in molar proportion of propionate in the full period in between meals.

Interestingly, Guyader et al. (2015) observed no effects of nitrate × DHA interaction on VFA parameters obtained immediately prior to the morning feeding, but a trend for an interaction was observed for molar proportion of propionate and the [acetate + butyrate]:propionate ratio, 3 h after morning feeding. Overall, some interesting differences were observed, including a non-additive effect of nitrate and DHA on the molar proportion of propionate (Table 6.1).

Aschenbach et al. (2009) reported that nitrate impaired acetate uptake through the rumen wall in vitro. Nolan et al. (2016) suggested this to be a possible explanation for a shift in VFA profile towards acetate that has been reported upon nitrate supplementation, both in vitro and in vivo (Zhou et al., 2012; Guyader et al., 2015, 2016; de Raphélis-Soissan et al., 2016a). However, in the study of Aschenbach et al. (2009) only acetate uptake was measured, and not uptake of propionate and butyrate. As the VFA uptake mechanisms generally are not VFA specific, it is likely that nitrate will also have inhibited the uptake of other VFA, instead of being specific for acetate.

Nolan et al. (2016) showed that methemoglobin (MetHb) levels in sheep receiving a diet containing 2% nitrate remained constant and low (>12% of Hb) until 10 h after the first meal, when the diet was fed in hourly portions of 42 g. The MetHb levels in sheep receiving the same amount of feed (1 kg/d) not in hourly portions, but in either one or two meals per day, peaked during the hours after feeding. The authors discuss that the rate of nitrate reduction is increased when animals eat rapidly and/or when a feed restriction is applied. The MetHb levels determined in the study described in Chapter 2, also remained below the threshold for a subclinical disorder, but these were determined during the period that no feed restriction was yet imposed. The formation of MetHb occurs as a result of nitrite absorption into the bloodstream. It is, therefore, likely that a more gradual intake pattern will alleviate nitrite accumulation in the rumen, and subsequent negative effects on digestion and animal health (Lee and Beauchemin, 2014; Nolan et al., 2016). Such an alteration in feed intake pattern may provide an explanation for the interaction effect between nitrate and DHA on fiber digestion (Chapter 2, and 3).

Table 6.1. Total VFA concentration and VFA molar proportions in cows fed the control (CON; n = 2) diet or diets with nitrate (NO_3; 21 g/kg DM; n=1), docosahexaenoic acid (DHA; 3 g/kg DM; n = 2) or nitrate and DHA (NO_3+DHA; 21 g/kg DM and 3 g/kg DM; n=2) as feed additives

	Treatment					*P*-value		
Item	CON	NO_3	DHA	NO_3+DHA	SEM	NO3	DHA	NO_3×DHA
Rumen pH	6.4[b]	6.6[a]	6.4[b]	6.4[b]	0.03	0.031	0.016	0.001
Total VFA (mM/L)	102[b]	95[b]	101[b]	110[a]	2.0	0.752	0.006	0.001
VFA (mol/100 mol)								
Acetate (A)	66.6	66.5	64.9	66.1	0.43	0.242	0.043	0.152
Propionate	17.3[ab]	16.3[bc]	18.9[a]	14.4[c]	0.41	<0.001	0.730	0.005
Butyrate(B)	12.4	14.1	12.4	15.8	0.69	0.017	0.272	0.268
(A+B):P	4.6[b]	5.0[ab]	4.2[b]	5.7[a]	0.18	0.005	0.544	0.025

[a-c] Means within a row with different superscripts differ ($P < 0.05$).

More recently, 3-nitrooxypropanol (3NOP) received considerable attention as newly developed CH_4 mitigating feed additive. The compound has been specifically designed to inhibit methyl coenzyme-M Reductase, which is the enzyme that catalyzes the last step of methanogenesis in rumen archaea. As reviewed by Latham et al. (2016), several in vivo experiments have been conducted to evaluate the effect of 3NOP on methane production in dairy and beef cattle. Methane production per kg DMI decreased between 6-60% compared to the control treatments (Haisan et al., 2014; Reynolds et al., 2014; Romero-Perez et al., 2014, 2015; Hristov et al., 2015). Although there seems to be an overall mitigating effect after 3NOP supplementation, the variation in the size of the effect is large. Latham et al. (2016) discussed that this is most likely a result from differences in the method of application and methane measurement techniques.

Further research on additivity of the effect of feed additives might focus on the combination of nitrate and 3NOP. It was discussed by Van Zijderveld (2011) that decreasing CH_4 production may rather divert energy losses toward other reducing processes during which more heat is produced than during methanogenesis. The mode of action of 3NOP and nitrate may complement each other in this respect. 3-Nitrooxypropanol inhibits the activity of methanogens and methanogenesis, leading to an increased accumulation of H_2. Nitrate reduction is energetically more favourable than methanogenesis, and may take away the H_2 as a substrate for methanogens. Moreover, the required inclusion level of 3NOP can be very low and, therefore, it likely does not impose a strong restriction directly on the formulation of the basal diet. If the effects are

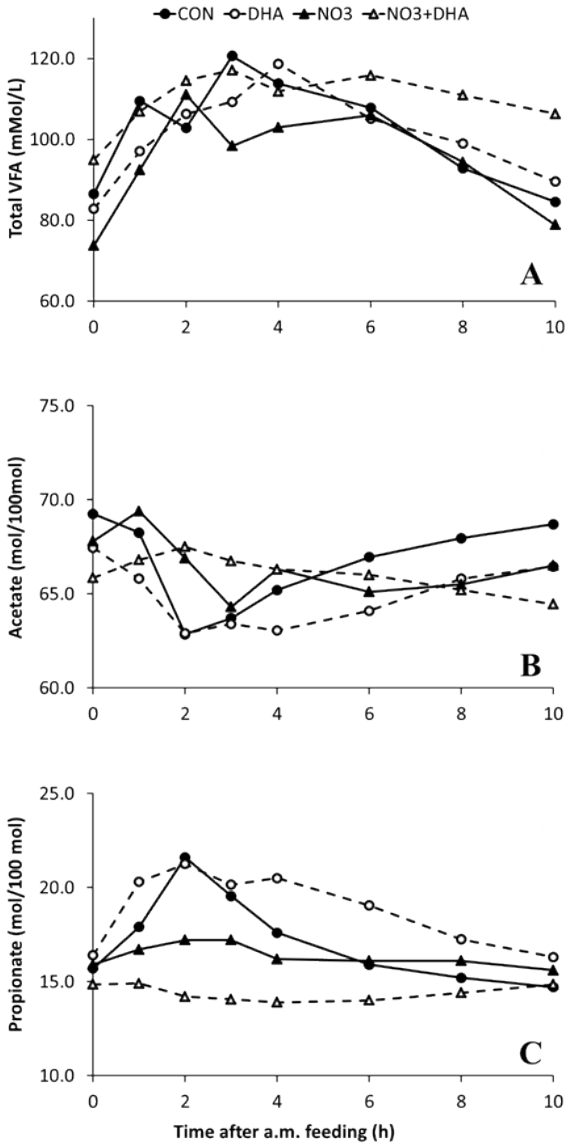

Figure 6.1. Total VFA concentration (mMol/L) (**A**), Molar proportion of acetate (**B**), and propionate (**C**) in rumen fluid from cows after a.m. feeding of one of the following TMR's: Control (CON; ●; urea as alternative NPN source; n = 2), Nitrate (NO_3; ▲; at a level of 21 g per kg DM; n = 1), docosahexaenoic acid (DHA; ○; at a level of 3 g per kg DM and urea as alternative NPN source; n = 2), or both nitrate and DHA (NO_3+DHA; Δ; at the same inclusion levels as in the single additive treatments; n = 2).

additive, a lower dose of nitrate in combination with 3NOP may effectively reduce CH_4 production, without adverse effects of environmental trade-offs, or an impaired animal health and productivity.

Adaptation

As outlined in this thesis, adaptation of the rumen microbes may cause effects of feed additives on CH_4 production to be transient. Adaptation is especially expected to additives that exert an anti-microbial effect. For example, in dual flow continuous culture systems, several effects of essential oils on fermentation disappeared after 6-7 d (reviewed by Benchaar et al., 2008). In Chapter 2, the effect of DHA on CH_4 production was investigated after a 13-day adaptation period. In that experiment, no effect of DHA on CH_4 production was found. The absence of an effect of DHA on CH_4 production in vivo after an adaptation period, does not exclude a transient effect of DHA on CH_4 production during that adaptation period. However, this was not determined in the study described in Chapter 2. Moate et al. (2013) also did not observe a lower CH_4 production of cows fed different levels of DHA after a 2-3 week adaptation period. It is known from in vitro experiments that DHA has a marked effect on microbial metabolism in the rumen (Boeckaert et al., 2008a).

In the scientific literature, the search for mitigating feed additives often focuses on the aim to achieve a persistent decrease in CH_4 production by a single additive. In this type of experiments, CH_4 production is determined after an adaptation period to the experimental diet that includes the feed additive. In this way, with the absence of an effect, a transient effect that may have occurred during the first days of the adaptation period (when CH_4 production was not measured) cannot be ruled out.

Adaptation over time was investigated and described in Chapter 4. Feeding a commercial blend of essential oils decreased in vitro CH_4 production after 8 days of dietary inclusion, but not after 15 and 22 days. No such adaptation was observed for lauric acid (C12:0), which persistently reduced in vitro CH_4 production between 4 and 22 days after dietary inclusion. In vivo observations upon weekly rotation of this blend of essential oils and

C12:0 also did not result in a persistently lower CH_4 production, CH_4 yield and CH_4 intensity compared with feeding the essential oils blend only (Chapter 5).

As recently reviewed by Yáñez-Ruiz et al. (2016), in vitro and in vivo results for the same additives are usually poorly related, and mitigating effects of additives on CH_4 production were usually much more pronounced in vitro compared with in vivo. The authors provided a summary of technical recommendations on the use of in vitro gas production methods for measuring methane production. One of their recommendations that was not met in the experiment described in Chapter 4 was the minimum of 3 independent incubation runs as replications. The effects of time point and incubation run on the gas production measurements were fully confounded, because rumen fluid had to be collected along the course of adaptation to the experimental diets. An important finding of this study was that in general, feed additives in the donor cow diet had a larger effect on gas and CH_4 production than the same additives in the incubation substrate. Incubation substrate affected asymptotic GP, half-time of asymptotic CH_4 production, total volatile fatty acid (VFA) concentration, molar proportions of propionate and butyrate, and degradation of organic matter (OMD), but did not affect the amount of CH_4 produced (mL/g OM). This corresponds to the conclusion of Yáñez-Ruiz et al. (2016), who indicated that using rumen fluid from adapted versus non-adapted animals significantly affects in vitro results, and recommended donor animals to be fed the same diet as incubated or of similar nutrient composition. This should also be considered when translating in vitro results to an in vivo situation.

Rotation

As discussed in the 'adaptation' section, the absence of a mitigating effect of a feed additive (in this case DHA) after an adaptation period does not exclude the possibility that a short-term mitigating effect occurred. However, even if such an effect would have existed the mitigation benefit would probably not have outweighed the negative effects observed on FPCM yield. Nevertheless, short-term mitigating effects of feed additives could still be beneficial if these additives can be applied in a (short term) rotation schedule. Similar to application of rotation schedules in herbicide use (Beckie et al., 2006) or for

anticoccidial compounds in broilers (Chapman, 2001), the rotational application of two or more CH_4 reducing feed additives with a short-term effect and with different modes of action could alleviate the diminishing effect on CH_4 reduction due to microbial adaptation in the rumen.

In e.g. herbicide rotations, effective rotation schedules may also become ineffective in the long run as a result of microbial adaptation. The most important reason for development of herbicide resistance is overreliance on a single herbicide or on a group of herbicides that share the same mode of action (Norsworthy et al., 2012). If a suitable combination of mitigating additives can be found, the search for alternatives should, therefore, be continued. The aim of the study described in Chapter 5 was to compare in vivo CH_4 production and performance of dairy cows receiving either Agolin Ruminant® only and continuously (AR; 0.05 g/kg total DM; AR-AR treatment), or AR and lauric acid (C12:0; 20 g/kg total DM; AR-LA treatment) using a weekly rotation schedule. After introduction of the treatment additives in the diet, the experiment comprised five two-week periods. In periods 1, 3, and 5, cows were housed in respiration chambers for continuous measurement of CH_4 production. A feed restriction was imposed already in the pre-treatment period to avoid confounding effects of DMI on CH_4 production. As the experimental facilities did not allow additional treatment groups, no control group (none of the additives fed) and no group that received only C12:0 could be included. Therefore, the comparison was between continuous feeding of AR and rotation of AR with C12:0 based on two-week averages, and the specific effect of the single additives could not be statistically evaluated within the experimental design. The changes in DMI and CH_4 production on a weekly basis for the AR-LA rotation, though not statistically evaluated, are presented in Figure 6.2.

The DMI in the weeks that the AR diet was fed are similar to those from the pre-treatment period, whereas the numerical differences in DMI between the pre-treatment periods and the C12:0 weeks are substantial. The CH_4 yield (g/kg DMI) in the AR weeks keeps declining over time (from 21.4 to 18.9 g/kg DM in period 1 and 5, respectively; Figure 6.2), but increased from 18.1 (period 1) to 22.7 g/kg DMI (period 5) for the weeks that C12:0 was fed.

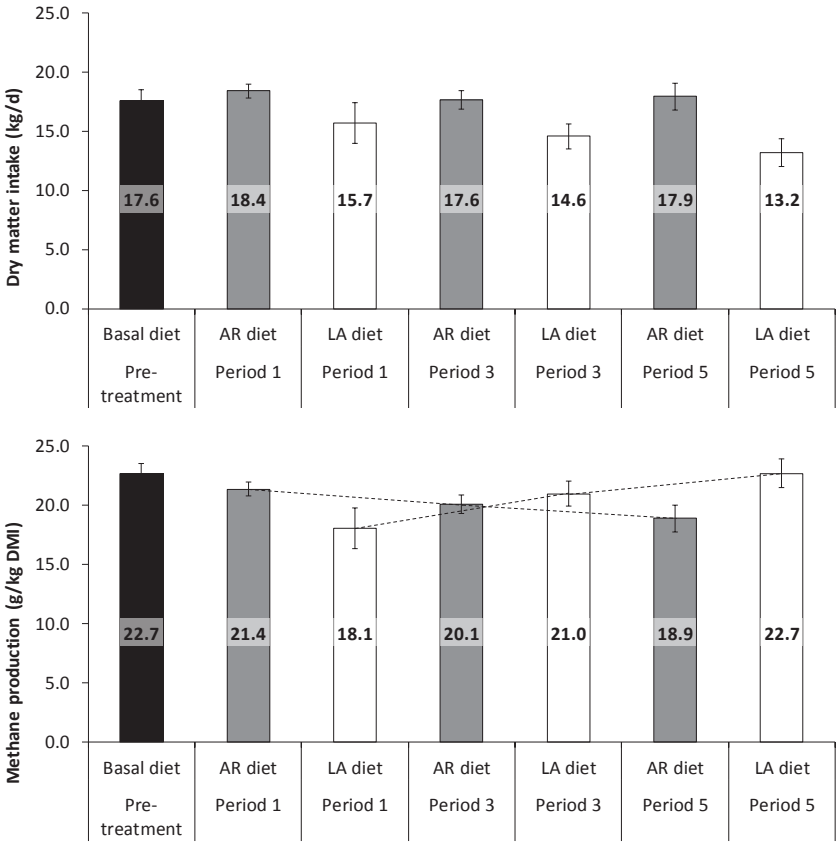

Figure 6.2. Average dry matter intake (DMI; top figure) and methane yield (g/kg DMI; bottom figure) of cows receiving a diet with 30% treatment concentrate on a DM basis following a weekly rotation schedule (first week of each period, AR diet; second week of each period, LA diet). The AR concentrate contained Agolin Ruminant® (0.17 g/kg DM) and the LA concentrate contained lauric acid (C12:0; 65 g/kg DM). A basal concentrate was fed during the pre-treatment period (n = 3 for pre-treatment period, and n = 4 for periods 1, 3, and 5). Error bars represent standard errors.

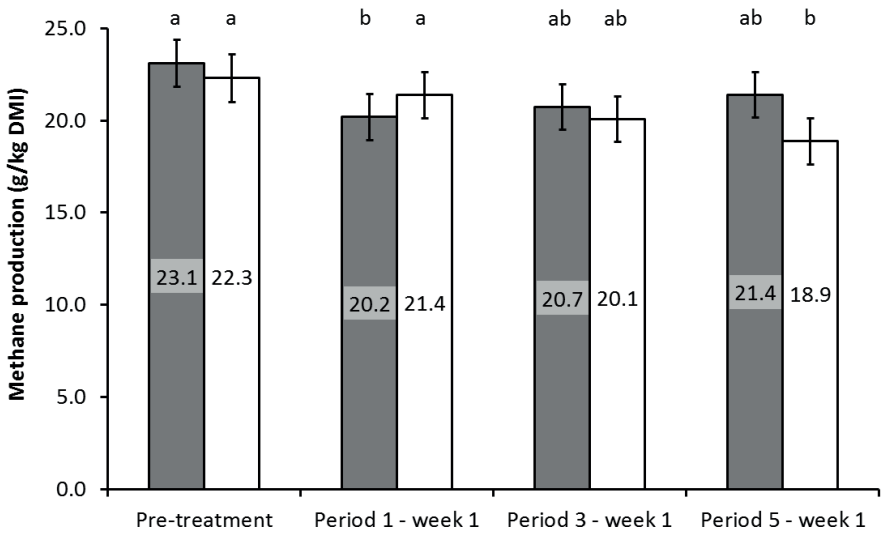

Figure 6.3. Methane production in g/kg dry matter intake (DMI) of cows (n = 4 per treatment) during the weeks that both treatment groups received the AR concentrate, which contained Agolin Ruminant® (0.17 g/kg DM) for periods 1, 3, and 5.). The diet contained 30% concentrate on a DM basis. Grey bars represent the treatment group that received AR for a period of 10 weeks, white bars represent the group that received both AR and lauric acid (C12:0; 65 g/kg concentrate DM) following a weekly rotation schedule (see Chapter 5). Error bars represent SEM. Periods within treatment with different superscript letters differ (P<0.05).

The latter may be explained by an increment in time in the selection of components of the TMR. Based on chemical analysis of feed refusal samples, upon feeding the C12:0 diet, the concentrate proportion in the refusal was larger than in the offered TMR. This increases the relative contribution of fiber to total DMI. A low intake level may also increase rumen retention time during which fiber fermentation by rumen microbes increases. In this scenario it is plausible that CH_4 yield is higher during LA feeding than with AR feeding for which a higher DMI and a relatively larger proportion of concentrate in the diet was achieved.

Weekly rotation of AR and C12:0 did not result in a persistently lower CH_4 production compared to feeding AR only. However, using the same statistical model as for the

complete data (as described in Chapter 5) on only the pre-treatment period and the weeks in which AR was fed to both the AR-LA and AR treatment groups may also provide some further insight in the applicability of the concept of rotation as a mitigation strategy (Figure 6.3). The effect of treatment was not significant, but a significant effect of period and a significant treatment × period interaction was observed. In period 5, but not in period 1 and 3, feeding AR in the AR-LA rotation treatment significantly reduced CH_4 yield compared with the pre-treatment period. This may indicate that alternate feeding of C12:0 and Agolin does result in reduced CH_4 yield in the week that Agolin is fed. However, it cannot be excluded that any carry-over effects of C12:0 in the second week of the previous period have affected the CH_4 yield upon feeding Agolin in the subsequent week, as it is known that C12:0 can also have strong anti-bacterial and anti-methanogenic effects (Hristov et al., 2011; Zhou et al., 2013). Nevertheless, the initial mitigating effect of AR seems to be repeatable after a week of feeding C12:0.

Effects of feed additives on animal performance

Efficient mitigation?

The work described in this thesis focuses on CH_4 yield (expressed per unit of DMI) and intensity (expressed per unit of FPCM produced). These metrics indicate an efficiency, as the emissions are scaled relative to intake or to production. However, one can view efficiency in dairy cow nutrition from different perspectives.

A commonly used approach is to evaluate feed efficiency (defined as kg FPCM yield/kg DMI), which does not directly apply to optimal microbial efficiency or resource use efficiency. In swamps for example, where the concept of passage rate does not apply as in the rumen, human inedible materials are slowly degraded by microbes. In this scenario, a lot of acetate and methane can be formed from fibrous substrate, which could be considered as polluting. However, the extent to which available nutrients are extracted from the substrate is maximized. Feed intake capacity is a factor of interest in selection for economic efficiency of dairy cows (Veerkamp, 1998). Higher feed intake will increase the passage rate of feed, which gives the microbes less time to degrade feedstuffs. It is thus important to realize that the current situation is not inherent to the nature of ruminants,

but has been imposed by humans through breeding and selection. If modern dairy cows are fed in an appropriate manner, more nutrients will be available for milk production, for maintenance energy requirements are diluted. However, present dairy cow diets often include a larger proportion of human edible resources. Hence, increasing feed efficiency is not necessarily the same as maximizing the efficiency of utilization of human inedible resources by rumen microbes, to obtain human edible energy and protein.

The often observed negative effects of additive-based CH_4 mitigation strategies on fiber digestion (Latham et al., 2016; Chapter 2, 3), further add to the less efficient use of human inedible resources to obtain human edible energy and protein. In order to unite the different viewpoints on efficiency, it is important to understand mechanisms underlying a certain response upon a mitigation strategy. This mechanistic understanding is also important to predict the response to an additive under different conditions than the experimental conditions of the research described in this thesis. With the mechanisms unknown, all combinations have to be tested, which is usually not feasible in terms of available time, funds and labour. Before a strategy can be implemented in practice, the response under varying circumstances has to become a predictable one.

Effects of additives on performance in relation to the basal diet

Medium chain fatty acids (MCFA) may exert a stronger effect on CH_4 production when supplemented to a diet that is relatively low in NDF. Machmüller et al. (2001) tested the effect of MCFA on in vitro CH_4 production using incubation substrates with high or low concentrations of fiber. Pure C12:0 strongly (~80%) depressed CH_4 production independent of the basal-diet type used. However, when expressed per unit of NDF fermented, CH_4 production was only significantly reduced when C12:0 was added to the low fiber substrate. Results of Machmüller et al. (2001) may imply that results of the experiment described in Chapter 5 could have been different if the basal diet would have contained relatively more starch and sugars and less NDF. Interactions between CH_4 mitigation additives and basal substrates on CH_4 and VFA production have also been investigated by Castro-Montoya et al. (2012) using an in vitro approach. In their study, both the mitigating effect as well as the fermentation depressing effect of MCFA were

largest when added to a corn silage substrate. However, the strong inhibition of fermentation by MCFA, impaired appropriate evaluation of the most promising substrate × additive combination. Benchaar et al. (2015) investigated the effect of linseed oil supplementation to red clover silage- or corn silage-based diets on CH_4 production in lactating dairy cows. The treatment effect was more pronounced in the corn silage-based diet, which implies that the type of forage included in the basal diet is an important aspect to consider when using fat supplementation as a mitigation strategy. Livingstone et al. (2015) evaluated effects of linseed supplementation on grass silage or maize silage based diets, and concluded that basal diet (fibre rich grass silage vs starch rich maize silage) did not alter the methane emission in response to the linseed supplementation. However, in their experiment, the amount of supplemental lipid provided by linseed was small.

Interaction effects between the feed additive and the composition of the basal diet also have large implications for application of these additives in practice. For example, if an additive only reduces CH_4 emission when it is supplemented to a diet with a large proportion of concentrates, feeding a concentrate rich diet may increase feed costs for the farmer. Moreover, the applicability of such a feeding strategy also depends on lactation stage of the cows, as late lactation or dry cows usually receive no or only small amounts of concentrates. Moreover feeding more concentrates may lead to trade-offs in environmental impacts of ruminant product (Hristov et al., 2013) and may reduce human edible efficiency.

Effects of feed additives on DMI

After introducing the different efficiency perspectives from which the effect of additives on animal performance can be viewed, it is obviously relevant to also compare responses to mitigation strategies observed in the work of this thesis and to speculate about the underlying mechanisms.

As discussed in Chapter 3, accumulation of H_2 in the rumen may impair fiber digestion. If fiber degradation in the rumen is impaired, retention time may increase (Hollman and Beede 2012), which subsequently may lower feed intake. The negative effects of nitrate

and C12:0 (either fed as a single additive or in a rotation schedule) on DMI are likely related to impaired fiber degradation in the rumen. Nitrate and C12:0 may have a direct toxic effect on methanogens in the rumen (Zhou et al., 2013; Latham et al., 2016). Even when an additive acting as an alternative H_2 sink, such as nitrate, is fed, H_2 may still accumulate when methanogens are inhibited at the same time. Increased H_2 levels upon feeding nitrate have been observed previously (e.g., Van Zijderveld et al., 2011b; Lund et al., 2014; Troy et al., 2015; Guyader et al., 2015), and H_2 production increases quadratic with increased nitrate levels in the diet (Olijhoek et al., 2016). Similarly, lauric acid resulted in increased H_2 emissions in vitro (O'Brien et al., 2014).

Petersen et al. (2015) measured a transient increase in ruminal nitrite concentrations in cows receiving the medium (13.6 g nitrate/kg DM) and high (21.1 g nitrate/kg DM) nitrate diets in the study of Olijhoek et al. (2016). As discussed by the latter authors and in Chapter 3 of this thesis, nitrite may exert toxic effects on methanogens. The increased H_2 emissions measured in nitrate fed cows support this hypothesis. Moreover, Latham et al. (2016) discussed that calcium nitrate (often used in animal experiments) is not very soluble in the normal pH range of the rumen. This would imply that not all nitrate will be reduced, especially not at higher fractional passage rates that may occur upon increases in DMI. Therefore, it is likely that nitrate supplementation may reduce CH_4 emission not just by nitrate being an alternative H_2 sink, but also by other (indirect) mechanisms.

In view of the potentially toxic effects of nitrite, Nolan et al. (2016) discussed several control points in nitrate metabolism in the rumen with the goal to alleviate toxic effects of nitrite. Slowing the rate of presentation of nitrate to rumen microbes reduces the risk of nitrite accumulation. Coating of nitrate may result in such a slower release rate of nitrate in the rumen. However this should not lead to increased outflow of nitrate from the rumen before being reduced to ammonia. Frequent feeding will also reduce the peak levels of MetHb in blood, with much lower peak levels in sheep fed once a day compared with sheep fed meals at hourly intervals (de Raphélis-Soissan et al., 2016b). The likelihood of nitrate poisoning is reduced by the inclusion of fermentable energy sources (concentrates) in nitrate-containing diets (Nolan et al., 2016). However, substituting fibre

rich feeds for starch or sugar rich concentrates may not be attractive from a human-edible feed efficiency viewpoint.

Nitric oxide may also induce a DMI response in nitrate supplemented animals (Nolan et al., 2016). The authors explained that nitric oxide can be produced from the reduction intermediate nitrite, and elevated concentrations may reduce rumen primary contractions and digesta turnover rate. The latter could explain the reduction in meal size (Lichtenwalner et al., 1973), or the (tendency for) lower feed intake upon nitrate supplementation (Lund et al., 2014; Newbold et al., 2014; Chapter 2, 3). It can be argued that the latter is also a mechanism to avoid toxicity, as reduced feed intake also reduces nitrate intake which consequently may lower the risk of nitrite formation.

Effects on milk production and milk composition

Except for AR, all other additives tested in in vivo experiments described in this thesis (viz. nitrate, DHA, and C12:0) exerted negative effects on milk production or milk composition (Table 6.2). In some cases the effects were not statistically significant, but the numerical differences between the additive and the control treatment were still considerable. E.g. average FPCM production of cows receiving DHA was around 4 kg lower than the control treatment which would impose an important trade-off in case of practical application.

Impaired fiber degradation upon feeding C12:0 may not only lower voluntary DMI, but may also induce milk fat depression. In Chapter 4, fiber degradation was not determined in vivo, but C12:0 in the diet of the donor cows reduced organic matter degradation in vitro. The observed lower DMI and milk fat concentration in donor cows receiving C12:0 was, therefore, likely related to impaired fiber degradation in the rumen. As discussed in Chapters 4 and 5, others also observed negative effects of C12:0 on fiber digestibility (Dohme et al., 2001, Faciola and Broderick, 2014).

Table 6.2 shows that none of the additives positively affected milk production parameters. In all experiments described in this thesis, a light feed restriction was imposed to avoid confounding effects of DMI on CH_4 production and without detrimental effects on the

Table 6.2. Effects of feed additives investigated in this thesis on dry matter intake (DMI), fat- and protein-corrected milk (FPCM) production and milk composition.

Additive (dose)	Chapter	DMI (kg/d)	FPCM production (kg/d)	Milk fat (g/kg)	Milk protein (g/kg)
Nitrate (21 g/kg DM)	2,3	-	=	=	-
DHA[1] (3 g/kg DM)	2,3	+	= *	-	=
Lauric acid (30 g/kg DM)	4	-	-	-	= *
Agolin ruminant (0.05 g/kg DM) = +/-	4	=	=	=	=
Agolin ruminant (0.05 g/kg DM = +/-)	5	=	=	=	=
Lauric acid / Agolin Ruminant rotation	5	-	=	=	-

[1]DHA: docosahexaenoic acid (C22:6 n-3).
Symbols: = not affected, - decreased, + increased.
** Tendency for decrease.

cows. In view of this feed restriction, an increase in animal performance is unlikely. The seemingly positive effect of DHA on DMI was numerically very small (0.4 kg). As the variation in DMI is strongly reduced with a restricted feeding regimen, this effect is not very likely to occur with ad libitum feeding. Nitrate and C12:0 negatively affected intake even though a feed restriction was imposed. However, although in some cases lower CH_4 production was observed, the lower amount of energy lost in CH_4 was not compensated

by an increased milk production. For nitrate, this is in line with findings by Van Zijderveld et al. (2011b) and Lee and Beauchemin (2014), who reported that the consistent decline in CH_4 yield upon feeding nitrate appears to be without directing additional energy toward animal production.

Implementation of additive-based mitigation strategies

Animal nutrition research into mitigation of enteric CH_4 production usually focuses on the effect of a nutritional strategy on CH_4 yield or intensity. Before effective feeding strategies can be successfully implemented in practice, it should be investigated if there are no trade-offs with other environmental impact factors. This should not only be evaluated at the animal level, but also at the farm and dairy production chain level (e.g., Van Middelaar et al., 2013). Trade-offs may hamper wide spread adoption of a mitigation strategy, but also negative effects related to food safety and food processing may preclude adoption of

a CH_4 reducing additive. Finally, the economic feasibility of a strategy should also be evaluated (Van Middelaar et al., 2014), because strategies with a negative return on investment are unlikely to be adopted by farmers and industry.

Residues in animal products

Nitrate is effective in reducing CH_4 production, but overconsumption of nitrate by humans may impose health risks. Therefore, the European Food Safety Authority has established rules to keep consumption of nitrate and nitrate residuals within the maximum daily allowances (EFSA, 2009). The maximum nitrate concentration allowed in drinking water in Europe is 50 mg/L. Guyader et al. (2016) examined the effect of feeding nitrate and linseed on the presence of nitrate residuals in milk products during a 17-week experiment. The nitrate + linseed diet in their study contained 1.8% nitrate on a DM basis. In curd from the control treatment in week 17 and in cheese from both treatments in week 9, low nitrite concentrations were detected (1.5 mg/kg), but in the vast majority of milk and milk product samples nitrate and nitrite concentrations were below the detection limit. Similarly, El-Zaiat et al. (2013) did not observe a difference in nitrate residuals in meat of lambs fed either a control diet, a nitrate diet with 4.51% of encapsulated calcium nitrate in dietary DM, 4.51% of encapsulated calcium nitrate containing cashew nut shell liquid (2.96% in the product DM). Nitrite was not detected in meat from any of the treatments. Olijhoek et al. (2016) reported a linear increase in milk nitrate concentration (from 0.13 to 1.56 mg/l) with increasing dietary nitrate levels (from 0 to 21 g/kg DM), whereas nitrite concentrations in milk were below the detection limit (< 30 µg/L).

Essential oils and other plant secondary compounds have been studied to examine their mitigating potential, but the main reason for the increasing interest of the feed industry in those compounds relates to the change in legislation on so-called medical feed additives (Greathead, 2003). As outlined by Greathead (2003), these changes are an attempt to prevent development of microbial resistance to antibiotics, but also the increasing pressure from consumers is an important driver. Consumers consider consumption of residues from antibiotics, other drugs, pesticides etc. as a major threat to their health. The advantage of essential oils is that they are of natural origin and, therefore, more likely to

be accepted by consumers. However, the nature of essential oils is that they influence the organoleptic properties of the plant they belong to (Benchaar and Greathead, 2011). Therefore, they may also change organoleptic properties of animal products in a negative way, which will hamper consumer acceptance. For example, in the study of Van Zijderveld et al. (2011a) diallyl disulfide supplementation at a level that did not decrease CH_4 production, already resulted in a clear garlic taint in milk.

Effect on milk processing parameters

Feeding DHA to lactating dairy cows has been reported to increase the proportions of conjugated linoleic acid (CLA) and DHA in milk fat (Boeckaert et al., 2008b, Chapter 2). From a human health perspective, such an alteration in milk composition is of interest (Shingfield et al., 2013). However, alteration of the milk FA profile can also affect milk processing parameters. Tzompa-Sosa et al. (2016a) investigated the association between the ratio of C16:0 and C18:1*cis*-9 and the triacylglycerol (TAG) profile of milk. C16:0 and C18:1*cis*-9 have an opposite effect on physical properties of milk fat (e.g. on solid fat content). In the experiments reported in Chapter 2 and 5 of this thesis, this ratio was affected by some of the dietary treatments applied. The TAG profile also affects solid fat content of milk (Tzompa-Sosa et al., 2016b). From a milk processing perspective, highly unsaturated milk fat with a low C16:0/C18:1*cis*-9 ratio is less desired. In this milk, the type of crystals formed are long and give a sandy taste, whereas in more saturated milk shorter crystals are formed that can form a network of solid fat. Solid fat is an important processing parameter, because it positively influences sensory perception, functionality and structure of fat-rich foods (e.g. muffins, puff pastry, ice cream) (Tzompa-Sosa et al., 2016b). The alteration in milk FA profile towards more unsaturated fatty acids upon feeding DHA is, therefore, desirable from a human health perspective, but not from a processing perspective. Feeding of C12:0 reduced the proportion of C16:0 in milk fat at the expense of C12:0. This fatty acid is associated with increases in low-density-lipoproteins (LDL) cholesterol. This LDL represents the primary source of cholesterol that accumulates in the artery wall, which negatively affects cardiovascular health (Salter, 2013; Siri-Tarino et al., 2015).

As discussed in Chapter 2, milk protein concentration and yield were lowered upon nitrate feeding. As propionate proportion was lowered, this decline may result from a decrease in glucogenic precursors (Rigout et al., 2003), because glucose is an important factor in signaling pathways that regulate milk protein synthesis (Rius et al., 2010). As protein is generally the most valuable milk component, an additive that decreases milk protein production may reduce interest in its adoption in practice. Guyader et al. (2016) also reported reduced milk protein yield when a combination of nitrate and linseed was fed. Van Zijderveld et al. (2011) found reduced milk protein concentration upon nitrate feeding, whereas protein yield remained unaffected. Milk protein yield was not reported by Olijhoek et al. (2016), but milk yield and milk protein concentration were not affected by nitrate feeding although propionate molar proportion in the rumen linearly decreased with increased dietary nitrate levels. As results are not consistent across studies, this aspect as well as options to alleviate the negative effects on milk protein, requires further investigation.

Moate et al. (2016) pointed out that the efficacy of 3NOP in grazing animals has not yet been evaluated. The authors emphasize that the primary focus should be on testing if using the compound does not lead to food safety problems (e.g. residues in animal products). This holds not only for 3NOP, but for any potential mitigating additive. Herrero et al. (2016) recently stressed the importance of issues related to environmental side-effects, as well as consumer acceptance, as such issues may prevent widespread adoption of CH_4 mitigating feed additives.

Conclusions and recommendations

The research described in this thesis addresses issues that are frequently reported to hamper the application of feed additive-based mitigation strategies. The main focus was on investigating additivity of the CH_4 mitigating effect of feed additives, on the adaptation of rumen microbes to long term feeding of feed additives, and on exploring the potential of rotational feeding of additives to avoid adaptation. In summary, the following conclusions and recommendations are drawn:

- The effects of nitrate and DHA on CH_4 yield (g/kg DMI) and CH_4 intensity (g/kg FPCM), were additive. However, the interaction effect between nitrate and DHA on NDF digestibility indicated that negative effects of nitrate on apparent total-tract digestibility of nutrients were alleviated by DHA (Chapter 2, 3), probably due to an altered feed intake pattern.
- The effects of nitrate as a CH_4 mitigating feed additive on fiber degradation in the rumen can be detected by evaluating the change in the diurnal pattern of ^{13}C enrichment of exhaled CO_2. A prerequisite for this detection method is that the main ration components differ in natural ^{13}C enrichment (e.g., C3 and C4 plants), and in content of the nutrients that are expected to be involved in a shift in fermentation (e.g., starch and fiber) or in degradability of a nutrient.
- Feed additives in the donor cow diet have a stronger effect on in vitro gas and CH_4 production than the same additives in the incubation substrate (Chapter 4). This phenomenon should be considered in the planning of future studies on the mitigation potential of feed additives in vitro.
- DHA and nitrate significantly reduced milk fat and protein yield, respectively, and C12:0 reduced DMI (Chapter 4, 5) milk fat content, and FPCM production (Chapter 4). Therefore, the applied doses of these additives are not recommended for application in practice.
- In Chapter 5, rotational feeding of Agolin Ruminant® and C12:0 did not result in a persistent decrease in CH_4. However, there were indications that the concept of rotation may be effective. Future research should clarify if rotational feeding of Agolin Ruminant® with another additive could result in a persistent mitigation effect.
- The additives tested in this thesis are applied under specific circumstances. More mechanistic understanding is required to predict the response of the same additives when supplemented to other basal diets or animals in a different physiological state.
- Trade-offs in environmental impact, and effects of feed additives on animal health and performance, and in milk processing parameters and food safety are important aspects to consider in future research into mitigation strategies.

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Chapter 7

Co-authored peer-reviewed publications related to this thesis

CO-AUTHORED PEER-REVIEWED PUBLICATIONS RELATED TO THIS THESIS

2016

Dijkstra J., S. van Gastelen, E. C. Antunes-Fernandes, D. Warner, B. Hatew, G. Klop, S. C. Podesta, H. J. van Lingen, K. A. Hettinga, and A. Bannink. 2016. Relationships between milk fatty acid profiles and enteric methane production in dairy cattle fed grass- or grass silage-based diets. Anim. Prod. Sci. 56:541–548.

Ellis, J. L., I. K. Hindrichsen, G. Klop, R. D. Kinley, N. Milora, A. Bannink, and J. Dijkstra. 2016. Effects of lactic acid bacteria silage inoculation on methane emission and productivity of Holstein Friesian dairy cattle. J. Dairy Sci. 99:7159–7174.

Warner, D., B. Hatew, S. C. Podesta, G. Klop, S. van Gastelen, H. van Laar, J. Dijkstra, and A. Bannink. 2016. Effects of nitrogen fertilisation rate and maturity of grass silage on methane emission by lactating dairy cows. Animal 10:34–43.

2015

Van Gastelen, S., E. C. Antunes-Fernandes, K. A. Hettinga, G. Klop, S. J. J. Alferink, W. H. Hendriks, and J. Dijkstra. 2015. Enteric methane production, rumen volatile fatty acid concentrations, and milk fatty acid composition in lactating Holstein-Friesian cows fed grass silage- or corn silage-based diets. J. Dairy Sci. 98:1915–1927.

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2014

Heeren, J. A. H., S. C. Podesta, B. Hatew, G. Klop, H. van laar, A. Bannink, D. Warner, L. H. de Jonge, and J. Dijkstra. 2014. Rumen degradation characteristics of ryegrass herbage and ryegrass silage are affected by interactions between stage of maturity and nitrogen fertilisation rate. Anim. Prod. Sci. 54:1263–1267.

CURRICULUM VITAE

Geronda Klop was born in Dordrecht, the Netherlands on the 22nd of May 1987. She followed several years of secondary education (VWO) at Insula College, Dordrecht, the Netherlands. In 2004, she commenced intermediate vocational education (MBO) where she obtained a diploma in animal husbandry and a diploma in veterinary assistance in 2007. In 2010, she completed her BSc in Animal Husbandry at HAS Den Bosch University of applied sciences (HBO), 's-Hertogenbosch, the Netherlands. She followed the MSc programme Animal Sciences at Wageningen University, Wageningen, the Netherlands, where she did a minor thesis in the Soil Quality department and a major thesis in the Animal Nutrition Group. She graduated (Cum Laude) in June 2012, and thereafter she started the PhD project of which the results are described in this thesis.

OVERVIEW OF SCIENTIFIC PUBLICATIONS

Peer-reviewed scientific publications

Klop, G., S. van Laar-van Schuppen, W. F. Pellikaan, W. H. Hendriks, A. Bannink, and J. Dijkstra. Changes in in vitro gas and methane production from rumen fluid from dairy cows during adaptation to feed additives in vivo. Animal (*accepted*).

Klop, G., A. Bannink, K. Dieho, W.J.J. Gerrits, and J. Dijkstra. 2016. Short communication: Using diurnal patterns of ^{13}C enrichment of CO_2 to evaluate the effects of nitrate and docosahexaenoic acid on fiber degradation in the rumen of lactating dairy cows. J. Dairy Sci. 99:7216–7220.

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Klop, G., J.L. Ellis, M. C. Blok, G.G. Brandsma, A. Bannink, and J. Dijkstra. 2014.Variation in phosphorus content of milk from dairy cattle as affected by differences in milk composition. J. Agric. Sci. 152:860 - 869.

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Klop, G., G. L. Velthof, and J. W. van Groenigen. 2012. Application technique affects the potential of mineral concentrates from livestock manure to replace inorganic nitrogen fertilizer. Soil Use Man. 28:468-477.

Contributions to conferences, symposia and other scientific output

Goselink, R.M.A., G. Klop, J. Dijkstra, and A. Bannink. 2015. Phosphorus metabolism in dairy cattle : literature study on recent developments and gaps in knowledge. Wageningen UR Livestock report 910.

Klop, G., B. Hatew, A. Bannink, and J. Dijkstra. 2015. Effects of nitrate and docosahexaenoic acid on methane production in lactating dairy cows. Proceedings Joint Annual Meeting of the American Diary Science Association, Orlando, Fl., USA.

Klop, G., B. Hatew, A. Bannink, and J. Dijkstra. 2015. Nitrate but not docosahexaenoic acid reduces methane production in lactating dairy cows. WIAS Science Day 2015, February 5, Wageningen, the Netherlands.

Dijkstra, J., J. France, A. Bannink, J. L. St-Pierre, E. Kebreab, R. M. A. Goselink, and G. Klop. 2014. A model of phosphorus utilization in lactating dairy cattle. Abstracts of the 8th International Workshop on Modelling Nutrient Digestion and Utilisation in Farm Animals, Cairns, Australia.

Van Gastelen, S., E.C. Antunes Fernandes, K. A. Hettinga, G. Klop, S. J. J. Alferink, and J. Dijkstra. 2014. Replacing grass silage with maize silage affects rumen fermentation

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Podesta, S. C. , B. Hatew, G. Klop, H. van Laar, R. D. Kinley, A. Bannink, and J. Dijkstra. 2013. The effect of nitrogen fertilization level and stage of maturity of grass herbage on methane emission in lactating cows. Proceedings of the 5th Greenhouse Gases and Animal Agriculture Conference (GGAA2013), Dublin, Ireland.

Velthof, G.L., P. Hoeksma, J. J. Schröder, J. C. van Middelkoop, W. C. A. van; Geel, P. A. I. Ehlert, G. Holshof, G. Klop, and J. P. Lesschen. 2012. Agronomic potential of mineral concentrate from processed manure as fertiliser. Proceedings 716 of The International Fertiliser Society, 20th Annual Conference, Leek, United Kingdom.

Van Vuuren, A.M., J. Dijkstra, G. Klop, W. H. Hendriks, and A. A. Dijkhuizen. 2012. Challenges for future dairy nutrition. World Nutrition Forum. Marina Bay, Singapore.

TRAINING AND SUPERVISION PLAN[1]

The Basic Package (3 ECTS)[2]	
WIAS Introduction Course	2012
Course on philosophy of science and/or ethics	2014

Scientific Exposure (**11 ECTS**)	
International conferences	
Greenhouse gases and Animal Agriculture conference, Dublin, Ireland	2013
ADSA-ASAS Joint Annual Meeting, Orlando, Florida, USA	2015
International Symposium on Dairy Cattle Nutrition, Wageningen, the Netherlands	2013, 2014, 2015
Seminars and workshops	
Symposium on phosphorus nutrition in pigs and poultry, Wageningen, the Netherlands	2012
WIAS Science Day, Wageningen, the Netherlands	2014
WIAS Science Day, Wageningen, the Netherlands	2015
Symposium: Solutions for climate change from animal production, Wageningen, the Netherlands	2014
Conference: Nutrition, Health and Welfare of Calves, Wageningen, the Netherlands	2014
Presentations	
'Phosphorus utilization in dairy cattle', Wageningen, the Netherlands, 26 November 2013, oral presentation.	2013
'Fosfor metabolisme in de koe – van het plafond naar de bodem', Hardenberg, the Netherlands, 29 October 2014, oral presentation (in Dutch).	2014
'Nitrate but not docosahexaenoic acid reduces methane production in lactating dairy cows', Wageningen, the Netherlands, 5 February 2015, oral presentation.	2015
'Fosfor in melkvee', Utrecht, the Netherlands, 9 June 2015, oral presentation (in Dutch).	2015
'Nitrate but not docosahexaenoic acid reduces methane production in lactating dairy cows', Orlando, Florida, USA, 16 July 2015, oral presentation.	2015
'Effect of feed additives on methane emission from dairy cows' Wageningen, the Netherlands, 2 March 2016, oral presentation (in Dutch).	2016

In-Depth Studies (7 ECTS)	
Disciplinary and interdisciplinary courses	
Wageningen Business School course: Ruminant Nutrition	2012
Wageningen Business School course: Advances in Feed Evaluation	2013
Environmental Impact Assessment of Livestock Systems	2015
Advanced statistics courses	
Design of Experiments	2012
Statistics of the life sciences	2014

Statutory Courses (3 ECTS)	
Use of Laboratory Animals (Article 9)	2012

Professional Skills Support Courses (3 ECTS)	
Supervising MSc thesis work	2013
Scientific publishing	2014
Data management	2014
Writing Grant Proposals	2015

Research Skills Training (12 ECTS)	
Two research assignments apart from PhD project	
Phosphorus use efficiency in dairy cattle	2012-2013
Isotope study dairy cows	2012-2013
Didactic Skills Training (18 ECTS)	
Supervising practicals and excursions	
Supervising practical 'Introduction to Animal Sciences course'	2012, 2014
Supervising practical 'Applied Animal Biology course'	2013-2015
Supervising theses	
6 MSc theses	2013-2016
1 BSc thesis	2015
1 Internship	2013
Management Skills Training (4 ECTS)	
Membership of boards and committees	
WAPS council – Education Committee member for 2 years	2013-2015
TOTAL: 61 ECTS	

[1] Completed in the fulfilment of the requirements for the education certificate of the Graduate School Wageningen Institute of Animal Sciences (WIAS).

[2] One ECTS equals a study load of 28 hours.

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COLOPHON

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