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Nutrizione e Alimentazione Animale

NUTRITIONAL AND FEEDING STRATEGIES TO REDUCE METHANE EMISSION FROM DAIRY COWS

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SUMMARY

ABSTRACT

NUTRITIONAL AND FEEDING STRATEGIES TO REDUCE METHANE EMISSION FROM DAIRY COWS

The general aim of the present thesis was to evaluate the effects of several nutritional and feeding strategies to reduce methane (CH_4) production from dairy cows. The thesis includes the results of three experiments conducted with the financial support from EU Research Project LIFE 09 ENV/IT/0214 Gas-Off.

The aim of the first study was to measure total gas and CH_4 production from 30 total mixed rations (TMRs) fed to dry and lactating cows in 20 commercial dairy farms of the Po Plain (Italy). Samples were analyzed for chemical composition, *in situ* 48 h fiber digestibility (NDFD) and *in vitro* gas production (GP) and CH_4 concentration at 24 h of incubation. Neutral detergent fiber digestibility of TMRs from dry and lactating cows was identical (52.1%; P=0.995). The TMRs fed to dry and lactating cows differed for GP (43.0 and 54.4 mL/200 mg DM, respectively; P<0.001) and CH_4 (7.24 and 8.85 mL/200 mg DM, respectively; P=0.001), but not for CH_4 as percentage of GP (24.3 and 23.7%, respectively; P=0.286). Data were also analyzed dividing the TMRs into quartiles depending on "starch:ADF" ratio; the average ratios of the groups 1, 2, 3 and 4 were 37, 77, 116 and 138, respectively. Increasing "starch:ADF" ratio determined a higher GP: 42.2, 51.4, 55.1 and 56.2 mL/200 mg DM for groups 1, 2, 3 and 4, respectively (P<0.001), whilst CH_4 (mL/200 mg DM) was lower (P<0.001) for group 1 (7.12) in comparison with the others (8.82 on average). Acetate, expressed as percentage on total volatile fatty acids (VFA) decreased for increasing "starch:ADF" ratio (P=0.009), whereas butyrate tended to increase (from 8.11 to 9.23% on total VFA; P=0.069) and the "acetate:propionate" ratio to decrease (from 3.35 to 3.09; P=0.082). The lack of a higher CH_4 concentration in GP from diets richer in fiber might be attributed mainly to the relatively short time of incubation.

The second trial was aimed to evaluate at first the effects of 18 essential oils, yeast, *Quebracho* tannin and *Quillaja* saponin on ruminal methane and gas production (GP) *in vitro.*

A lactating cow diet was incubated with rumen *inoculum*. Doses of the additives (mg/L) were: essential oils=500; tannin=67; yeast=8.35 and 16.7; saponin=300.

Lemongrass, estragole, eugenol, geraniol, limonen, thyme oil and thymol produced less gas (overall mean 33.8 mL/200 mg DM) than control (43.6 mL/200 mg DM; P<0.001). Methane produced (mL/200 mg DM) by guaiacol (10.7), lemongrass (9.6), limonene (11.4), thyme oil (10.9) and thymol (2.1) was lower than control (12.5) (P<0.001).

Methane percentage on total GP was lower (P<0.001) for cinnamic alcohol (25.4), guaiacol (24.5), thymol (19.7) and vanillin (26.3) than control (28.8).

In the second part of the experiment, thymol, guaiacol and yeast were added to the diet of dry fistulated cows to determine *in situ* NDF digestibility (NDFD) of six forages and *in vivo* dietary NDFD. Thymol and yeast decreased *in situ* NDFD after 24 h (33.9% and 33.5% vs 38.1%; P=0.008). Thymol decreased *in vivo* total tract NDFD (40.8% vs. 51.4%; P=0.02).

Differences in GP and methane levels were registered within classes of additives. A careful selection of additives may allow for the manipulation of ruminal fermentation.

The third experiment was conducted to evaluate the effects of diets with different starch contents and fish oil (FO) supplementation on lactation performance, *in vivo* total-tract nutrient digestibility, N balance and methane (CH_4) production in lactating dairy cows.

The experiment was conducted as a 4×4 Latin square design with a 2×2 factorial arrangement: two levels of dietary starch content (low vs high), the presence or absence of FO supplement (0.80% DM) and their interaction were evaluated. Four Italian Friesian cows were fed one of the following four diets in four consecutive 26-d periods: 1) low starch (LS), 2) low starch plus FO (LSO), 3) high starch (HS) and 4) high starch plus FO (HSO). The diets contained the same amount of forages (corn silage, alfalfa and meadow hay). The starch content was balanced using different proportions of corn meal and soybean hulls. The cows were housed in metabolic stalls inside open-circuit respiration chambers to allow the measurement of CH_4 production and the collection of separate urine and feces samples.

No differences among treatments were observed for dry matter intake: neither FO nor the use of soybean hulls negatively influenced this parameter (22.9 kg/d, on average). A trend was observed for the effect of FO on milk yield (P=0.10), with a higher milk production compared with the diets without FO (29.2 vs 27.5 kg/d on average, respectively). Moreover, FO supplementation favorably decreased the n-6:n-3 ratio of the milk polyunsaturated fatty acids (PUFA). Consistent with the literature, the milk protein percentage was negatively influenced by FO (P<0.01): 3.37% on average for the LSO and HSO diets vs 3.67% for the LS and HS diets. With regard to digestibility, the HS diet negatively influenced all the parameters measured, with starch as the only exception. The percentage of N intake excreted with feces was influenced by both the FO (P=0.09) and starch content (P=0.04): the HS diet showed the highest value, followed by the HSO and LS diets and finally by the LSO diet. With regard to methanogenesis, there was a trend (P=0.08) of lower CH_4 emissions (g/d) with the HS and HSO diets compared with the LS and LSO diets (396 vs 415 on average, respectively). Fish oil supplementation did not reduce methanogenesis. Previous in vitro studies have demonstrated a reduction in CH_4 production as a consequence of FO supplementation; the decrease in CH_4 production is generally accompanied by marked reductions in dry matter and fiber digestibility, which were not observed in the present work.

1. GENERAL INTRODUCTION

1.1 RUMINANTS AND GLOBAL WARMING

Over the past decades concern has arisen over the accumulation of gases in the atmosphere that are capable of trapping heat (Figure 1.1), leading to increased average global temperatures. Since the industrial revolution in the 1750's, it is likely that these so-called greenhouse gases (GHGs) have increased their concentration in the atmosphere due to the increased size of the human population and its concomitant activities (Forster et al., 2007).

Quantitatively, the most important GHG is carbon dioxide (CO_2) and about 77% of global warming is attributed to its increased atmospheric concentration (Forster et al., 2007). Carbon dioxide production mainly results from the energy consumption, burning of fossil fuels, industrial activities and vehicle traffic. However, the atmospheric concentrations of most of the other GHGs (methane, nitrous oxide, chlorofluorocarbons, hydrochlorofluorocarbons, hydrofluorocarbons, perfluorocarbons and sulphur hexafluoride) have also increased over the past decades (Forster et al., 2007).

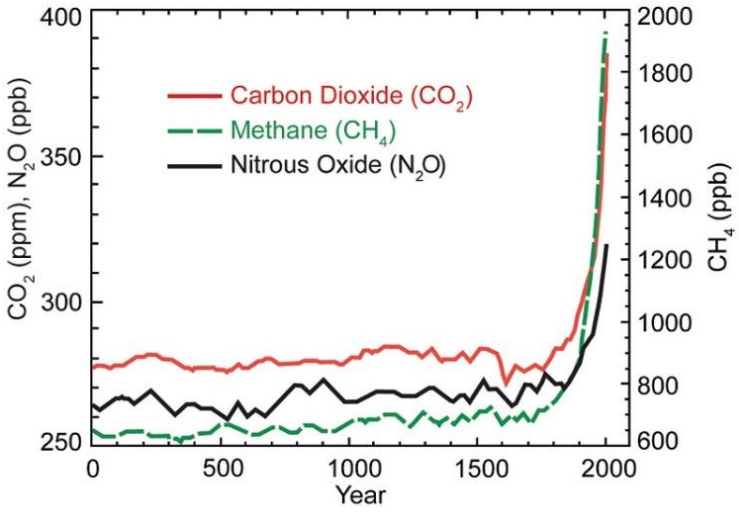

Figure 1.1: Atmospheric concentrations of the three main greenhouse gases (carbon dioxide, methane and nitrous oxide) in the period 0-2005 (Forster et al., 2007)

Methane (CH_4) is one of the three main GHG, together with CO_2 and nitrous oxide (N_2O). CH_4 is the second most important gas involved in global warming and accounts for 14% of the anthropogenic GHG production (Forster et al., 2007) (Figure 1.2 and 1.3). To compare different GHGs, their effect on global warming is usually expressed relative to CO_2. CH_4 and N_2O have global warming potentials that are 25 and 298 times that of CO_2 (on weight basis), respectively; therefore they contain 25 and 298 CO_2-equivalents. These conversion factors are currently used to report emissions under the Kyoto

Protocol, although there is debate over the specific global warming potentials that should be used (Forster et al., 2007). Apart from their contribution to anthropogenic GHGs emissions, energy and N losses (through CH_4 and N_2O) are two of the most significant inefficiencies in ruminant production systems. Therefore, the challenge for research is to develop strategies to improve the efficiency of the energy and N cycles in ruminant production without altering animal performances.

CH_4 emissions are divided into two types: natural emissions (intestinal tract of animals, swamps, landfills, wetlands, gas hydrates, permafrost, termites, oceans, freshwater bodies, non-wetland soils, volcanoes and wildfires) which have always occurred and anthropogenic emissions, which have been induced by the human population. The emission of CH_4 from domesticated ruminants (enteric fermentation and methanogenesis from manure storage) is a consequence of the demand for animal products for human consumption and is thus part of the anthropogenic GHG emissions.

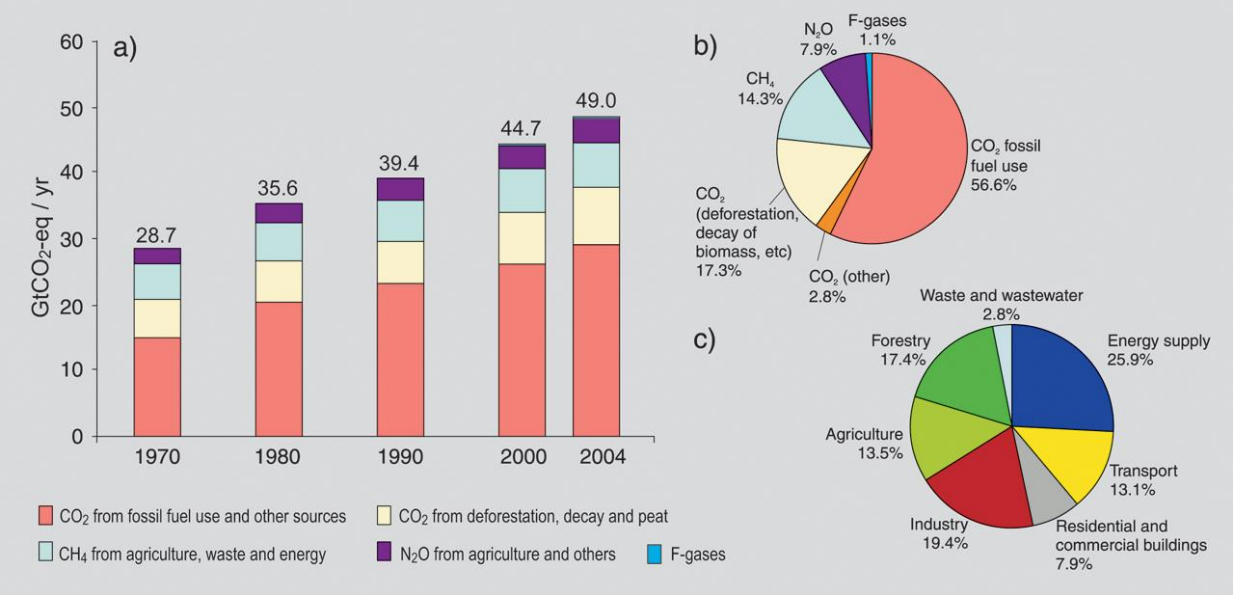

Figure 1.2: Global annual emissions of anthropogenic GHGs from 1970 to 2004 (a). Share of different anthropogenic GHGs in total emissions in 2004 in terms of CO_2-eq (b). Share of different sectors in total anthropogenic GHGs emissions in 2004 in terms of CO_2-eq. (Forestry includes deforestation) (c). (IPCC, 2007).

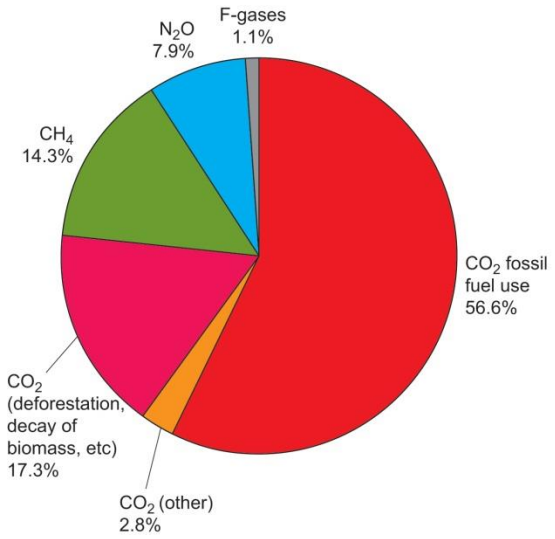

Figure 1.3: Focus on the proportion of different anthropogenic GHGs in total emissions in 2004 in terms of CO_2-eq. (IPCC, 2007)

In particular, agricultural activities are responsible for 10–12% of total global anthropogenic GHGs emissions (Smith et al., 2007), producing about 45% of overall anthropogenic CH_4 emissions, with a wide range of uncertainty in the estimates of both the agricultural contribution and the anthropogenic total (IPCC, 2007). Domestic ruminants are the main responsible of these emissions, ranging from 80 (Beauchemin et al., 2008) to 86 (Steinfeld et al., 2006) million tons annually, which derive primarily from enteric fermentations (Ellis et al., 2007) but also from the fermentation of manure. There is still uncertainty about the real impact of animal husbandry on GHGs: from the first estimates (18%) of Steinfeld et al. (2006), lower impacts (12-13%) were reported by IPCC (2007), even lower (3-8%) by Capper et al. (2009) and eventually to an impact of 3% reported by Pulina et al. (2011) for Italy. Recently, the Food and Agriculture Organization has estimated that the global dairy sector contributes to 4% of the globally produced GHGs, with more than 50% coming from CH_4 (FAO, 2010). It has been reported that enteric CH_4 is the most important GHG emitted at farm scale (50-60%) in ruminant production systems (Ogino et al., 2007). The contribution of CO_2, CH_4 and N_2O to the different activities involved in livestock production has been estimated using the life cycle assessment (LCA) method (Martin et al., 2010).

The contribution of enteric fermentation to the global production of GHG and the projected increase in demand for ruminant products have led to the initiation of many programs to assess strategies to reduce GHG emissions from ruminants. Several reviews on enteric CH_4 and N_2O production and mitigation strategies have recently been published (Dalal et al., 2003; Beauchemin et al., 2008; de Klein and Eckard, 2008, McAllister and Newbold, 2008) Taking into account the increase in future demand for animal products, GHG emissions per unit of animal product will have to be reduced in

order to maintain the current impact of animal husbandry on global warming (Steinfeld et al., 2006).

1.2 THE RUMEN ENVIRONMENT

Ruminants can be distinguished from other mammalian species by their highly specialized, compartmentalized digestive system. The largest compartment is the reticulo-rumen, where the microbiota (a symbiotic population of bacteria, protozoa and fungi) secretes enzymes that allow breakdown of complex structural cell wall carbohydrates of plants (cellulose and hemicelluloses) and other ingested components by the host ruminant. Microbial digestion also occurs in the hindgut (large intestine), but its overall contribution is smaller (Waghorn et al., 2007).

The rumen functions as a large anaerobic fermenter which provides optimal environmental conditions in terms of pH (5,6-7,0) and temperature (39°C) for the microbiota responsible for the degradation of feeds (Morgavi et al., 2010). Rumen contractions move and mix the content ensuring contact between microorganisms and feed particles, while other mechanisms (chewing and particle size reduction through rumination) allow ingested feed to be retained for an extended period. Subsequently, the breakdown products, mainly sugars and amino acids, are fermented by microorganisms. This process yields energy for the microbes and the resulting fermentation end-products, volatile fatty acids (VFA), are absorbed by the host animal and used as its main energy source, as reported in figure 1.4. Moreover the microbial biomass forms a valuable source of protein to the ruminant as it escapes from the rumen to be digested in the small intestine. The main VFA are acetate, butyrate and propionate. The former two can be used by the animal metabolism during lipogenesis, whilst propionate is the principal precursor of glucose and glycogen (Waghorn et al., 2007). The proportion among different VFA is important because it affects the efficiency of energy use and the composition of the final products (meat and milk) deriving from the ruminant (Bannink and Tamminga, 2005). Rumen degradation of feeds also yields CO_2 and hydrogen (H_2), which are utilized by methanogenic *Archea* to form CH_4 during a process called methanogenesis (Section 1.3). These gases are released in the environment through animal eructation. The removal of these gases maintains a low H_2 partial pressure in the rumen and allows for optimal microbial fermentation and growth (Janssen, 2010). Most of enteric CH_4 (87-92%; Murray et al., 1976) is produced under anaerobic conditions in the rumen and, to a lesser extent, in the hindgut. Murray et al. (1976) reported that 98% of total CH_4 is released through eructation and the remaining 2% is excreted in the flatus.

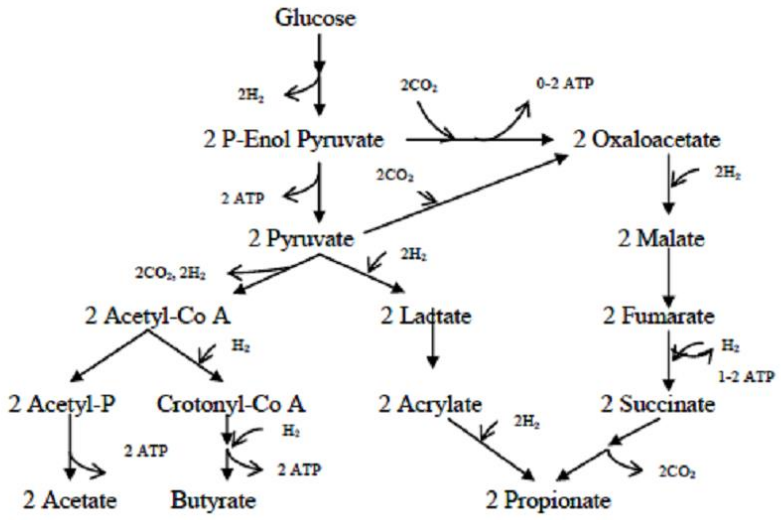

Figure 1.4: Fermentation pathways in the rumen (Ungerfeld and Kohn, 2006).

The ability to break down and utilize fibrous carbohydrates allows the ruminant to survive on vegetal substrates that are mostly inaccessible to mammalian digestive enzymes.

1.3 ENTERIC METHANE

In anaerobic environments, organic material is decomposed by bacteria through fermentation, where organic material is broken down to, among others, VFA and CO_2. Hydrogen, released during the production of VFA, accumulates in the fermentation system. In aerobic environments, oxygen (O_2) reduced to H_2O using the excess H_2, would be the terminal electron acceptor. The lack of O_2 in anaerobic systems necessitates the use of other terminal electron acceptors to remove H_2 (Russel and Wallace, 1997). Many oxidized compounds can be used for this purpose (e.g. ferric iron, sulfate, nitrate, manganese and CO_2). However, these compounds, except for CO_2, are present in low concentrations in many fermentation systems as the rumen. As a consequence, they are rapidly reduced and exhausted and CO_2 functions as the main terminal electron acceptor for the remaining excess H_2.

CO_2 is reduced to CH_4 in the fermentation system (equation 1), and the CH_4 in gaseous form subsequently dissipates from the system.

$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O$ [equation 1]

In the gastrointestinal tract of animals, fermentation and VFA production by the microorganisms is accompanied by methanogenesis. Many mammals, including livestock species for meat and milk production, partly depend on microbial fermentation to digest their diet. As a consequence they produce CH_4. However, the extent and dependency on microbial feed degradation varies widely among species. Consequently,

the amount of CH_4 produced per animal is variable. Ruminant species rely on microbial fermentation to a larger extent than monogastric species. For this reason, methanogenesis from ruminant species (expressed per kilogram of bodyweight) is relatively higher (table 1.1).

Table 1.1: Estimated annual enteric CH_4 emissions from the main domesticated livestock species (Sauvant, 1993)

	CH_4 emission (kg animal^{-1} year^{-1})	Assumed average Bodyweight (kg)	CH_4 emission (g kg BW^{-1} year^{-1})
Ruminants			
Dairy cows	90	600	150
Beef cattle	65	400	163
Sheep	8	50	160
Goats	8	50	160
Non Ruminants			
Swine	1	80	13
Poultry	<0,1	2	-
Horses	18	600	30

Within the rumen, CH_4 is produced by methanogenic *Archaea* (Chaban et al., 2006), a microbial group distinct from *Eubacteria* which represents the main (10^8-10^{10} cfu/L rumen fluid; Kumar et al., 2009) ruminal hydrogenotrophic microbial community (Wolin et al., 1997) which uses H_2 to reduce CO_2 into CH_4, as the only means of acquiring energy (Mathinson et al., 1998). As a consequence, methanogens consume the metabolic H_2 produced during microbial fermentation (McAllister and Newbold, 2008). Although H_2 and CO_2 are preferred substrates, formate, acetate, methanol and mono-, di- and tri-methylamine can also be utilized as substrates for CH_4 production (Wolin et al., 1997). Members of the *Archea* domain are found in a wide range of environments, however, those isolated from the rumen are strictly anaerobic (Janssen and Kris, 2008). Between 9 to 25% of methanogens in the rumen are reported to be associated with protozoa (Newbold et al., 1996; Takahashi, 2001) and this relationship has been shown to be responsible for up to 37% of total CH_4 produced in the rumen (Hegarty, 1999; Kumar et

al., 2009). McAllister and Newbold (2008) reported that protozoa are responsible for up to 50% of the fibrolytic activity within the rumen, and are an important H_2 source (Takahashi, 2001).

Methane production per unit of fermented feed is proportional to the pattern of VFA produced. Rumen fermentation of carbohydrates produces different VFA that are not equivalent in terms of H_2 output and CH_4 production: acetate (equation 2) and butyrate (equation 3) are H_2 sources and favor CH_4 synthesis, while the propionate formation (equation 4) is a H_2 sink and a competitive pathway for H_2 utilization other than methanogenesis.

$C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_3O_2^- + 2CO_2 + 4H_2 + 2H^+$ [equation 2]

$C_6H_{12}O_6 \rightarrow C_4H_7O_2^- + 2CO_2 + 2H_2 + H^+$ [equation 3]

$C_6H_{12}O_6 + 2H_2 \rightarrow 2C_3H_5O_2^- + 2H^+ + 2H_2O$ [equation 4]

A higher proportion of propionate therefore results in a lower CH_4 production (Ellis et al., 2008) and this property can be used as a mitigation strategy. Moreover propionate production determines an increase in the efficiency of energy capture from hexoses relative to acetate (Beever, 1993). However, acetate and butyrate production always exceed propionogenesis, resulting in a net surplus of H_2 within the rumen.

Fermentation is an oxidative process, during which reduced cofactors (NADH, NADPH, and FADH) are re-oxidized trough dehydrogenation reactions that release H_2 in the rumen. Methanogenesis is an essential metabolic pathway for efficient degradation of plant cell wall carbohydrates and subsequently for an optimal rumen function, because it avoids H_2 accumulation (Moss et al., 2000) and maintains a low redox potential. If H_2 accumulates, the re-oxidation of cofactors is inhibited, reducing microbial growth, rumen degradation of feeds, and the associated production of VFA (Joblin, 1999). Therefore, although methanogens do not directly contribute to fiber digestion, their presence enhances this process.

Typically, CH_4 represents an energy loss to the dairy cow ranging from 2% to 12% of gross energy (GE) intake, depending on level of feed intake and diet composition (Johnson and Johnson, 1995; Boadi et al., 2004). Daily CH_4 excretion is estimated to be 400-500 L in an adult cow (Chaucheyras-Durand et al., 2008). Therefore, reducing enteric CH_4 production may also lead to production benefits.

1.4 FACTORS AFFECTING ENTERIC METHANE PRODUCTION

In general, CH_4 production from ruminant enteric fermentation can be described in three different ways : 1) as absolute CH_4 production (total CH_4 produced per day;

g/day); 2) as CH_4 yield expressed per unit of dry matter intake (g/kg DMI) or as energy loss as percentage of gross energy intake (% GEI); 3) as CH_4 yield per unit of final product (g/kg milk or meat). Eructation of CH_4 by cattle begins approximately 4 weeks after birth, when solid feeds are retained in the reticulo-rumen (Anderson et al., 1987).
In recent years there have been a number of reviews and meta-analyses of published data from beef and dairy cattle and sheep (Boadi et al., 2004; Beauchemin et al., 2008; Ellis et al., 2007; Martin et al., 2010) that have been used to explore the major dietary factors that determine CH_4 emission from dairy cows and develop regression equations and other models to predict CH_4 emission (Reynolds et al., 2010). This section briefly summarizes the main dietary factors affecting methanogenesis.

1.4.1 EFFECT OF FEED INTAKE

For all the models previously mentioned the major determinant of total CH_4 excretion is the amount of DMI, or more precisely the fermentable organic matter consumed (Reynolds et al., 2010). In fact, in a recent meta-analysis of 1335 measurements of CH_4 emission from energy metabolism studies with cattle, Mills et al. (2009) reported that DMI was the major determinant of CH_4 emission (Figure 1.5). These results are consistent with those reported by Tamburini et al. (2010). The relationship between DMI and CH_4 emission reflects the relationship between DMI and fermentable organic matter, but in the analysis of Mills et al. (2009) the prediction of methanogenesis was only slightly improved when digestible energy intake was used instead of DMI. Tyrrell et al. (1990) found that CH_4 production (L/kg DMI) was lower in lactating than non-pregnant dry dairy cows fed the same diet (27.2 vs 35.1 L, respectively): this difference was associated with differences in dry matter digestibility of the diet (64.5 vs 72.0%, respectively). This is due to the fact that dry cows are generally fed at lower levels of DMI and they have lower rates of rumen turnover (passage rate of the *digesta*, k_p) and consequently a greater extent of ruminal digestion of diet components, differences in fermentation profile and rumen pH relatively to lactating cows. Current evidence indicate that the retention time of *digesta* in the rumen influences the rate of CH_4 production and yield. In fact, increased k_p values of feeds, particularly of forages, which have been ground or pelleted, likely contribute to the reduced CH_4 production (Johnson and Johnson, 1995).

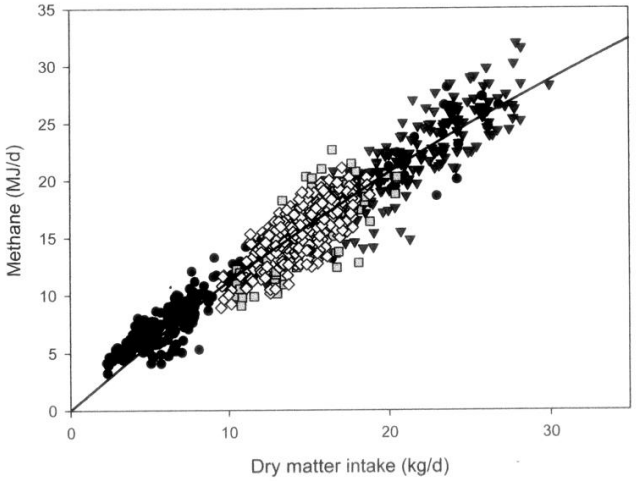

Figure 1.5: Relationship between dry matter intake and CH_4 energy emission in beef and dairy cattle (Mills et al., 2009).

In their meta-analysis, Mills et al. (2009) found that for beef and dairy cattle CH_4 emission as a percentage of GE intake generally decreased with increasing DMI (figure 1.6), which likely reflects the influence of level of intake on rumen dynamics and diet component digestion previously mentioned. Johnson and Johnson (1995) reported that as the daily feed eaten by any animal increases, the percentage of dietary GE lost as CH_4 decreases by an average of 1,6% per level of intake. Although much of the variation in CH_4 emission (≈90%; Reynolds et al., 2010) by cattle can be explained by differences in DMI, there is still considerable variation in CH_4 production at a given level of DMI (Figure 1.5 and 1.6), which reflects the influence of other factors as animal variation and diet composition (e.g. "forage:concentrate" ratio of the ration, Johnson et al., 1993).

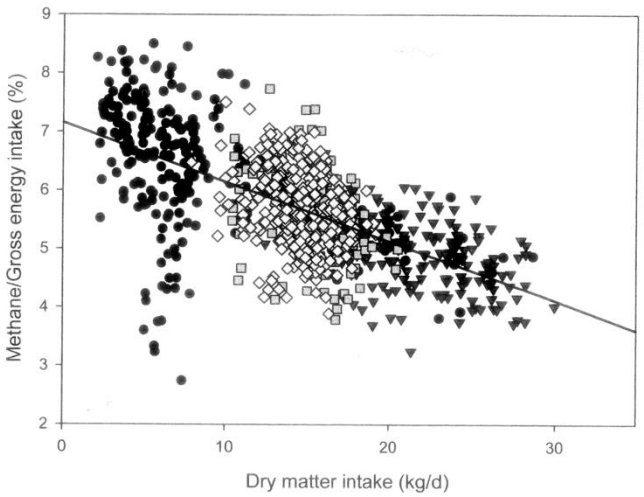

Figure 1.6: Relationship between daily matter intake (kg/d) and CH_4 excretion as percentage of gross energy intake in beef and dairy cattle (Mills et al., 2009).

1.4.2 EFFECT OF DIET COMPOSITION

In addition to total DMI, numerous other dietary aspects have significant effects on CH_4 production, including the amount and type of carbohydrates (fiber, starch and soluble sugars; Kebreab et al., 2006), dietary protein and fat. In a summarization of calorimetry studies that included measurements of intake of diet nutrients and CH_4 production by lactating dairy cows, Kirchgeßner et al. (1995) reported that, considering a wide range of basal diet types and levels of production, the primary determinant of CH_4 emission was the amount of DMI, but the consumption of individual diet components, based on proximate analysis, gave a better prediction of methanogenesis. In this regard the amounts of crude fiber and, to a lesser extent nitrogen free extract, had a positive effect on CH_4 emission, whilst the consumption of crude protein had little effect. These results are consistent with the analysis of data coming from lactating dairy cows by Moe and Tyrrell (1979), who found that in addition to DMI, the major dietary components determining methanogenesis were the intake of fiber (cellulose and hemicellulose) and non-fibrous carbohydrates (NFC). The prediction of CH_4 emission was improved as the amounts of these components digested was included in the regression.

In recent years, analyses of dietary factors determining CH_4 emission (Ellis et al., 2007; 2009) agreed with the results obtained by Moe and Tyrrell (1979) and Kirchgeßner et al. (1995) regarding the influence of relative amounts of fibrous and non-fibrous carbohydrates on methanogenesis. Mills et al. (2001) suggested that the replacement of soluble sugars with starch, and replacing grass silage with corn silage, as valuable approaches that would reduce CH_4 excretion from lactating dairy cows. These conclusions are based on the stoichiometry of the pattern of VFA production during rumen fermentation: as previously observed (section 1.3), the production of propionate and branched chain VFA acts as a H_2 sink, whereas the synthesis of acetate and butyrate is a H_2 source. On this regard, Bannink et al. (2010) reported that the least CH_4 is formed from starch and protein fermentation. Moreover Mills et al. (2003), in a meta-analysis of CH_4 emissions measurements from calorimetry studies, reported that methanogenesis was best predicted primarily on the basis of DMI and an adjustment was made for the ratio between starch and acid detergent fiber (ADF; cellulose and lignin) concentrations in diets.

The conclusions of Mills et al. (2001) have been supported by recent experimental observations: Hindrichsen et al. (2005) and Hindrichsen and Kreuzer (2009) have shown that soluble sugars have a higher methanogenic potential than starch, particularly at high rumen pH values. Furthermore, the rapid fermentation of readily fermentable carbohydrates, as starch, is associated with lower rumen pH values, which render the rumen environment less favorable for methanogens, which prefer a pH of 6.0-6.4

(Johnson and Johnson, 1995; Lee et al., 2000; Moss et al., 2000; Jarvis et al., 2000). Recent experimental data have also shown that CH_4 production per kg DMI is lower in lactating dairy cows fed diets based on corn silage compared to grass silage (McCourt et al., 2007; Reynolds et al, 2010a), and even lower when whole-crop wheat silage is fed (McCourt et al., 2007). In the study of Reynolds et al. (2010a), diets were formulated to be similar in starch and neutral detergent fiber (NDF) concentrations by manipulating the "forage:concentrate" ratio of the diets, thus differences in methanogenesis per kg DMI between the corn and grass based diets may be attributable to differences in the rate and extent of degradation of carbohydrates. In this regard, the extent of rumen carbohydrate degradation may vary with forage maturity and, consequently, CH_4 production may be reduced as the extent of NDF lignification increases (Hindrichsen et al., 2005). In this regard, in a growing beef cattle trial McGeough et al. (2010) reported a linear reduction in CH_4 production per kg DMI with increasing maturity of corn silage associated with a linear decrease in NDF digestibility.

As regards the effect of dietary protein on CH_4 excretion, Moe and Tyrrell (1979) and Kirchgeßner et al. (1995) reported that the amount of total or digestible protein consumed did not have a large effect on methanogenesis compared to the effects of digestible carbohydrates. These results are consistent with those reported by Ellis et al. (2009), Mills et al. (2009) and Reynolds et al. (2010a). These results apply to the protein concentration of the ration fed to animals. As regards the nitrogen fertilization rate and the resulting protein concentration of forages fed to lactating dairy cows, Bannink et al. (2010) reported a negative correlation between grass protein concentration and CH_4 emissions. This may relate to a lower CH_4 production from fermented protein compared to most carbohydrates, or associated changes in the carbohydrate composition and degradability of the forage fed.

Considering the effects of dietary fat on methanogenesis, Moe and Tyrrell (1979) found that diet ether extract intake did not have significant effects on the reduction of CH_4 production. In a recent meta-analysis of measurements deriving from calorimetry trials, Mills et al. (2009) also found that diet ether extract concentration (or ether extract intake) was not significantly related to methanogenesis. On the other hand, Kirchgeßner et al. (1995) reported that ether extract consumption had a large negative effect on CH_4 emission from dairy cattle. In the former two summarizations there were relatively few data concerning trials where supplemental fat was fed and, according to Mills et al. (2009), the influence of dietary fat concentration on CH_4 emission is of greater relevance when rations are supplemented with fat, as the variability in basal diet fat concentration is fairly low and does not exert a strong mitigation against CH_4 production. In this regard it has long been known that the use of supplemental fat sources reduces CH_4 production

(among others Andrew et al., 1991; Tyrrell et al., 1990), and more recently many studies have focused on the effects of different supplemental dietary fats on CH_4 emission (Beauchemin et al., 2008; Martin et al., 2010).

Several *in vitro* and *in vivo* experiments have demonstrated marked reductions in methanogenesis as levels of supplemental ether extract are increased. The effects vary according to basal diet, type of fat and level of inclusion in the diet. Effects of supplemental fat on CH_4 production are multifactorial (Reynolds et al., 2010) as there are several mechanisms involved in lowering CH_4 losses, including the reduction of rumen fermentation, biohydrogenation of unsaturated fatty acids, changes in VFA pattern, and microorganisms inhibition (Johnson and Johnson, 1995). Firstly, fat supplies digestible energy that is not fermented in the rumen, thus reduces the amount of CH_4 emitted relatively to DMI or energy intake. Secondly, the use of H_2 to saturate the dietary fatty acids acts as an alternative sink for H_2 that reduces the need for CH_4 production. Finally, the inhibitory effects of specific fatty acids on rumen bacteria and fiber fermentation, and associated changes in fermentation dynamics that together can have pronounced inhibitory effects on rumen methanogenesis (Reynolds et al., 2010). Recent research has focused specifically on the CH_4 suppressing effects of the medium chain fatty acids due to their ability to achieve a suppression in methanogenesis whilst exerting lesser negative influences on DMI and animal performance than longer chain polyunsaturated fatty acids (Reynolds et al., 2010). For example Beauchemin et al. (2008) have demonstrated large reductions in CH_4 emission for C12:0 and C14:0 rich oils supplemented to ruminant diets. Unfortunately, in a recent trial with lactating dairy cows (Crompton et al., 2010), decreases in CH_4 emission with supplemental coconut oil were obtained with concomitant decreases in DMI.

In practice supplemental fat and oils are often fed to dairy cows in order to increase the dietary energy density and also to manipulate milk fat composition. In particular, there has been much interest in the effects of supplemental fats on milk fatty acid profile (Reynolds et al., 2010). It can generally be assumed that when supplemental fat is used, there will be a reduction in CH_4 emission per kg DMI or per kg milk yield and fatty acid composition may be altered (Beauchemin et al., 2008; Martin et al., 2010). However, recent studies suggest that dietary fats containing C18:3 and longer chain polyunsaturated fatty acids, such as linseed and fish oil are particularly effective in suppressing CH_4 emissions (Fievez et al., 2003; Martin et al., 2010). This may relate to their degree of unsaturation, their reactivity in the rumen, and their effects on specific rumen microorganisms (e.g. cellulolytic bacteria and protozoa).

1.5 MITIGATION STRATEGIES TO REDUCE ENTERIC METHANOGENESIS

When expressed per kg of final product (milk or meat), CH_4 emissions vary widely between different parts of the world. This is largely a consequence of the level of productivity in different geographical regions (FAO, 2010). Over the past decades, in developed countries, progresses in animal husbandry (e.g. genetics, farm management and animal nutrition) have considerably increased animal production efficiency. Although total CH_4 production (kg/animal) have generally increased, CH_4 output per unit of consumable product has decreased significantly.

Capper et al. (2009) reported that in the US a dairy cow produced 13.5 kg CO_2-eq./d in 1944. In 2007, GHG production per cow had more than doubled (27.8 kg CO_2-eq./d). However, annual milk production increased from 2070 kg to 9152 kg and, as a consequence, the carbon footprint of milk was reduced from 3.66 kg CO_2-eq./kg milk in 1944 to 1.35 kg CO_2-eq./kg milk in 2007 (figure 1.7). Although these values were estimated using a fairly simplistic model, they demonstrate the potential of increasing productivity to reduce GHGs emissions. As a consequence, it is likely that the adoption of better management practices (e.g. enhance productivity by improving diet quality, eliminating nutrient deficiencies, utilization of effective additives and appropriate genotypes) in countries that currently have low levels of milk productivity may reduce the carbon footprint per unit of milk produced (figure 1.8) and likely decrease livestock CH_4 emissions over the long term (Johnson and Johnson, 1995). Enhancing the level of productivity decreases the maintenance subsidy and, thus, decreases the obligatory CH_4 emissions from fermentation of the feed associated with animal maintenance. Moreover, the increase in GHG production associated with ruminants will probably increase at a slower rate than the demand for ruminant products. However, additional measures will still be necessary to avoid large increases in GHG emissions from animal husbandry systems and large research programs have been initiated globally to explore dietary strategies to directly lower enteric methanogenesis.

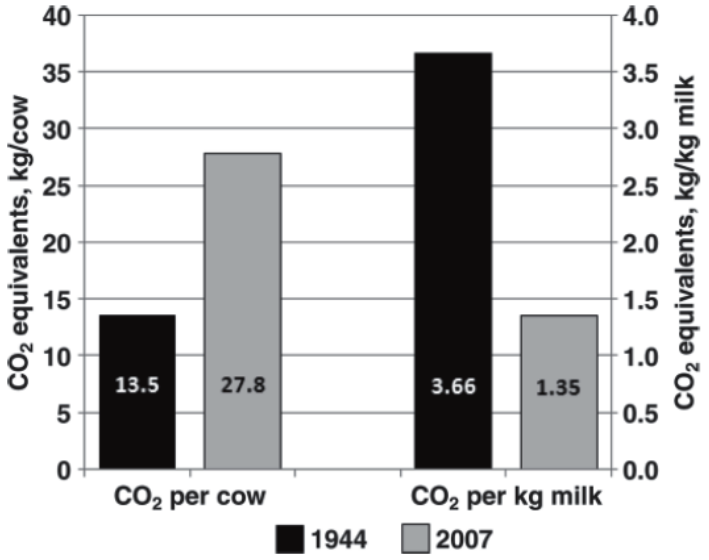

Figure 1.7: Carbon footprint per cow and per kilogram of milk for 1944 and 2007 US dairy production systems. The carbon footprint per kilogram of milk includes all sources of GHG emissions from milk production including animals, cropping, fertilizer and manure (Capper et al., 2009).

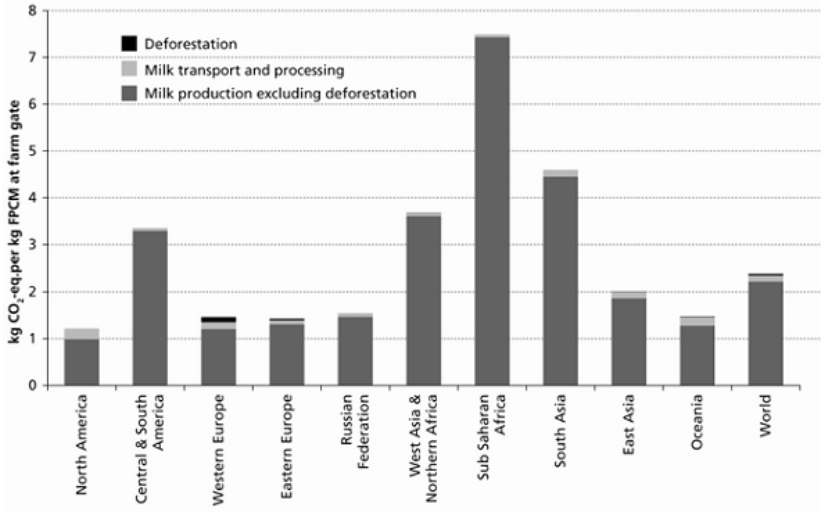

Figure 1.8: Estimated GHG emissions per kg of fat and protein corrected milk produced in different regions of the world (FAO, 2010).

Nutritional strategies to reduce enteric CH_4 emissions should be mainly focused on one or more of the following principles (Martin et al., 2010): 1) a direct inhibition of methanogenesis; 2) lowering of the production of H_2 during enteric fermentation that should be achieved without impairing feed digestibility; 3) providing alternative pathways for H_2 use within the rumen that can result in alternative end products beneficial for the animal. Therefore, any mitigation strategy that reduces methanogen populations must also include an alternative pathway for H_2 removal from the rumen. In this section the most promising nutritional strategies to reduce CH_4 production from ruminants will be discussed.

1.5.1 MITIGATION THROUGH ADDITIVES

Probiotics (acetogens and yeasts)

Probiotic utilization or the stimulation of rumen microbial populations capable to decrease rumen CH_4 production is potentially an interesting approach (Martin et al., 2010). Reductive acetogenesis is a mechanism of H_2 utilization that coexists with methanogenesis in the gastrointestinal tract of many animals (human, rabbit, hamster and rat), that could shift H_2 utilization from methanogenesis to acetate production. Indeed, homoacetogenic bacteria produce acetate from CO_2 and H_2 (Chaucheyras-Durand et al., 2008). The final product of the reaction, acetate, is an energetic source for the animal. However, in the rumen environment, reductive acetogens are less abundant and efficient than methanogen bacteria in the competition for H_2 uptake. This is likely because acetogens need a higher H_2 concentration to reduce CO_2 into acetate than that required by methanogens to reduce CO_2 into CH_4 (Martin et al., 2010). Moreover, the former reaction is thermodynamically less favorable than the latter one (McAllister and Newbold, 2008; Weimer, 1998). Attempts to increase the rumen population of acetogens and the use of acetogens as probiotic has been assayed but results have been unsatisfactory and inconclusive. In fact research into acetogenesis as a CH_4 abatement option is still largely conceptual, with extensive research still required to understand the physiology and ecology of acetogens (Eckard et al., 2010).

While there are many studies on rumen fermentation and animal performance, the use of live yeasts in ruminant production has not been extensively tested to date for their impact on rumen CH_4 production (Chaucheyras-Durand et al., 2008). Literature data are based on the utilization of strains selected for effects other than CH_4 reduction and results are quite contradictory. In a meta-analysis, Sauvant (2005) reported no effects of yeasts on reduction of methanogenesis, however yeasts show a great functional and metabolic diversity and some strains have been reported to reduce *in vitro* the CH_4 production (Newbold and Rode, 2006; Lynch and Martin, 2002). The mechanism by which yeasts decrease methanogenesis may be related to a stimulation of microbial synthesis and to the stimulation of reductive acetogenesis (Chaucheyras et al., 1995). A recent evaluation of various yeast strains in rumen-simulating fermenters on CH_4 production reported a strong strain effect, with results ranging from no effect in one study to a 58% decrease in another (Newbold and Rode, 2006). This suggests that more research is required before it can be concluded that live yeasts decrease CH_4 production *in vivo*.

Elimination of protozoa (Rumen defaunation)

Hydrogen is the key element to consider for reducing CH_4 production (Joblin, 1999). Rumen protozoa are large producers of this metabolic end product. Moreover, in the

rumen environment, there is a physical association between protozoal cells and methanogens that favours H_2 transfer. Methanogens, both attached and inside the ciliate protozoal cells, have been reported to contribute between 9 and 37% of the total rumen methanogenesis (Newbold et al., 1995). Some lipids, saponins and tannins have the ability to reduce protozoal numbers. Indeed, the removal of protozoa from the rumen (defaunation) has been shown to reduce methanogenesis by up to 50% depending on the diet (Hegarty, 1999). However these results are not consistent with other studies that found no reduction in methanogenesis (Wang et al., 1998; Hess et al., 2003), which may be related to different basal diets, saponin concentration and number of day of adaptation used within each trial (Hart et al., 2008). Rumen protozoa are proteolytic and actively ingest rumen bacteria. As a consequence, they are considered to be the most important cause of bacterial protein turnover in the rumen (Greathead, 2003). For this reason, N retention can be improved by defaunation (Wallace, 2004), particularly with low-protein diets, due to slower protein turnover in the rumen and less bacteria lyses (Hart et al., 2008). The rumen defaunation to mitigate CH_4 production appears interesting, but this option should be carefully evaluated in terms of animal performance (Martin et al., 2010): as protozoa are cellulolytic and maintain a stable rumen pH, there are implications in fiber fermentation, with negative consequences to overall rumen digestion, particularly in case of fiber rich diets.

Use of organic acids

The use of organic acids has been proposed as a means to provide an alternative H_2 sink to methanogenesis. Malate and fumarate can be converted into propionate in the rumen, consuming H_2 in the process. Fumarate and acrylate have been shown to be the most effective *in vitro* (Newbold et al., 2005). A number of *in vivo* studies with these compounds have been performed, but the effects on CH_4 production have been conflicting (Bayaru et al., 2001, Kolver and Aspin, 2006, Wallace et al., 2006, Wood et al., 2009). For example, Wallace et al. (2006) reported an exceptional decrease in CH_4 production (up to 75%) with 10% encapsulated fumarate in diets for lambs, without any negative effect on animal growth. In contrast, encapsulated fumarate had no significant effects in another trial with dairy cattle (McCourt et al., 2008).

Plant extracts: condensed tannins, saponins and essential oils

Plants produce an extensive variety of organic compounds deriving from their secondary metabolism, which seem to have no direct function in their growth and development (Balandrin and Klocke, 1985). These substances can be classified into 3 groups: saponins, tannins and essential oils. These plant extracts have been screened *in vitro* for their potential to directly inhibit methanogenesis (e.g. Calsamiglia et al., 2007, García-

González et al., 2008). The results of these screenings are promising, but *in vivo* verification of their efficacy remains necessary.

Tannins

The anti-methanogenic activity of tannins has been attributed primarily to the group of condensed tannins. Hydrolysable tannins can also affect methanogens (Field et al., 1989), however they are considered more toxic to the animal and have not been extremely tested. Condensed tannins have been shown to reduce CH_4 production by 13-16% (on DMI basis) (Waghorn et al., 2002; Woodward et al., 2004; Carulla et al., 2005; Grainger et al., 2009). After *in vitro* trials, Tavendale et al. (2005) proposed two modes of action of tannins on methanogenesis: a direct effect on ruminal methanogens and an indirect effect on H_2 production, due to a lower feed degradability. However, high concentrations of condensed tannins (>55 g/kg DM) can reduce voluntary DMI and diet digestibility (Min et al., 2003; Beauchemin et al., 2008).

Saponins

Chemically, saponins are high molecular weight glycosides in which sugars are linked to a triterpene or steroidal aglycone portion (Wallace, 2004). Saponins are secondary metabolites found in different parts (roots, bark, leaves, seeds and fruits) (Hart et al., 2008) of many plants that have effects on rumen microorganisms, decreasing feed protein degradation and increasing microbial protein synthesis (Makkar and Beker, 1996), resulting in a reduced availability of H_2 for CH_4 production (Dijkstra et al., 2007). However their mode of action seems to be primarily related to their anti-protozoal effects occurring mainly in the digestive tract, since they are poorly absorbed (Newbold and Rode, 2006; Cheeke, 1996; Beauchemin et al., 2008). Numerous studies have demonstrated that saponins and saponin-containing plants have toxic effects on rumen protozoa: the sensitivity of ciliate protozoa towards saponins may be related to the presence of sterols in protozoal, but not in bacterial membranes. The sterol-binding capacity of saponins probably causes the destruction of protozoal cell membranes. Saponin from *Yucca schidigera* has been observed to reduce protozoal numbers but not bacterial numbers in a 22d semi-continuous system (Valdez et al., 1986). The same results were obtained by Wallace et al. (1994) during *in vitro* experiments, however these effects were not always confirmed *in vivo*. In an *in vitro* trial, Guo et al. (2008) found that a tea saponin decreased methanogenesis (-8%) as well as protozoa abundance (-50%). Also saponins from *Quillaja saponaria* and *Acacia auriculoformis* have been observed to be antiprotozoal *in vitro* (Newbold et al., 1997). However, their antiprotozoal effects may be transient (Koenig et al., 2007) due to a possible adaptation of the mixed microbial population to saponins or to their degradation in the rumen (Teferedegne et al., 1999) and it is not always accompanied by a decrease in CH_4

production (Pen et al., 2006), suggesting that many aspects such as the source of saponin, diet characteristics, the preparation, the dose and the experimental settings are important aspects to be considered (Hess et al., 2004).

Essential oils

Essential oils are secondary metabolites with an oily appearance obtained from plant volatile fraction by steam distillation. They are found throughout the plant, including roots, flowers, leaves, fruit bodies and stems. The term "essential" relates to their properties of providing specific flavours and odours to many plants, as they are not essential for nutrition or metabolism nor are they oils in the sense of being glycerol based lipids (Hart et al., 2008). Some of the more common essential oils compounds include: thymol, eugenol, carvacrol, guaiacol, cinnamaldehyde, anethol and capsaicin. The most important active compounds are included in 2 chemical groups: terpenoids and phenylpropanoids (Greathead, 2003).

Essential oils have been reported to have antiseptic, antimicrobial and fungicidal activities (Calsamiglia et al., 2007; Hart et al., 2008). These compounds develop their action against bacteria through interactions with the cell membrane due to their hydrophobic nature (affinity for lipids), which allows them to interact with phospholipidic membranes. This interaction causes conformational changes in the membrane structure (Griffin et al., 1999) resulting in a loss of stability (Greathead, 2003; Benchaar and Greathead, 2011) and a decrease in the transmembrane ionic gradient which causes a slower bacterial growth. Although their main action is primarily centred on the activity on membranes, this is not the only mechanism of action. Gustafson and Bowen (1997) and Burt (2004) reported the potential of essential oils to coagulate cytoplasmatic constituents, probably through denaturation of proteins. A change in growth rates results in altered ruminal microbial populations, resulting in changes in the fermentation profile. Helander et al. (1998) reported the ability of thymol and carvacrol to disrupt the external membrane of gram-negative bacteria and observed the release of membrane lipopolysaccharides and an increased permeability of cytoplasmatic membrane. The small molecular weight of these compounds may allow them to be active against gram positive and negative bacteria, reducing their selectivity against specific microbial populations, making the modulation of rumen microbial fermentations more difficult. Also the effects of essential oils on rumen protozoa have been studied. Benchaar et al. (2003) and Newbold et al. (2004) in *in vivo* trials and McIntosh et al. (2003) in an *in vitro* trial found no effects of feeding moderate doses of blends of essential oils on protozoa activity. On the other hand, Ando et al. (2003) found that feeding high doses of essential oils (to levels that seem to be impractical in terms of feeding) significantly decreased protozoa numbers. The effect of these additives on

rumen protozoa varies with the oil tested, although relatively high concentrations seem to be required to have an effect (Hart et al., 2008). Even rumen anaerobic fungi are sensitive to essential oils at concentrations similar to those for sensitive bacteria (Wallace, 2004). Thus, fiber degradation may be decreased by essential oils under conditions in which the fungi are prevalent, such as high-fiber low quality diets.

Borchers (1965) was the first to report the potential benefits of essential oils on ruminal microbial fermentations. The Author observed that the addition of thymol to the rumen fluid, during *in vitro* incubations, determined the accumulation of amino acidic N and the reduction of ammonia N, suggesting a inhibition of deamination. Oh et al. (1967) using a 24h *in vitro* procedure evaluated the activity of essential oil of *Pseudostuga menziesii* and demonstrated that the inclusion in the *inoculum* of 12 mL/L of active compound determined a reduction in gas production during fermentation. Some of the principal effects of the use of essential oils are related to the stimulation of ruminal fermentation and inhibition of methanogenesis (Broudiscou et al., 2000, 2002; Mohammed et al., 2004), to the modification of the production and profile of ruminal VFA (lower acetate and higher propionate and butyrate concentrations), to N metabolism (inhibition of deamination), or both (Cardozo et al., 2004; 2005; Busquet et al., 2005; 2006). Only recently the effects of pure essential oils on rumen microbial fermentation have been studied in order to identify additives that can increase propionate production and decrease acetate and CH_4 production without lowering total VFA production, and compounds to reduce ruminal proteolysis and deamination, primarily through *in vitro* procedures. In addition to pure and naturally occurring essential oils, blends of oils are available as commercial rumen manipulators (Hart et al., 2008): specific combinations of essential oils may result in additive and/or synergetic effects, which may enhance their efficiency in microbial fermentation manipulation (Benchaar and Greathead, 2011).

In vitro studies are useful in screening the effects of a wide variety of molecules, however the limitations of these methods have to be taken into account. In general, doses used *in vitro* (mg/L) are much higher than those reported to work *in vivo*. This is likely because the activity of these molecules depends on the probability of the active component to interact with the target (bacteria), which depends on the concentration of both the component and bacteria. Because the concentration of bacteria in *in vitro* methods is lower than what observed *in vivo*, *in vitro* procedures require a higher concentration of additive (Chow et al., 1994). However, the complexity of rumen ecosystem requires that recommended doses for animals have to be tested *in vivo*. On the other hand, from published *in vitro* studies, it appears that high concentrations of essential oils (> 300 mg/L of *inoculum*) are required to reduce rumen methanogenesis.

Many essential oils tested at high doses *in vitro* have inhibited rumen microbial fermentation, confirming their antimicrobial activity and their potential to reduce rumen methanogenesis. In most cases, when the concentrations of active components in *in vitro* procedures were higher than 500 mg/L, the effects were detrimental. Because of these high dosages required to lower CH_4 production, beneficial effects in terms of reduced methanogenesis are often counterbalanced by an overall inhibition of total VFA production and rumen degradability, which is not desirable due to negative consequences on animal performance (Benchaar and Greathead, 2011). At moderate doses (50-500 mg/L), some essential oils modified rumen fermentation by changing the VFA production and N metabolism, or both. In general, the reduction of the "acetate:propionate" ratio and the increased level of butyrate as a result of supplementation suggest that the mechanism of action may reduce methanogenesis. However, the effects are diet and pH dependent: Calsamiglia et al. (2007) reported a more pronounced antimicrobial activity of essential oils at low pH levels, which may shift these molecules to a more hydrophobic status.

The aforementioned levels are likely too high to be used *in vivo* and are impractical in terms of feeding due to potentially deleterious effects on efficiency of rumen fermentation and palatability, possible toxicity issues, and high cost. One of the major inconsistencies in explaining the action of essential oils *in vivo* is the knowledge of the effective concentration in the rumen. Moreover *in vivo* application of essential oils as feed additives may be limited by the adaptation of rumen microbiota to degrade and metabolise these compounds that may occur with time. The strong and wide-spectrum activity against bacteria populations, the narrow range between the optimal and the toxic dose which allows to move from a selective effect to a general inhibition if the additive is added in excess, and the variable effects reported in literature suggest that their antimicrobial activity might be too strong and nonspecific to modulate the fermentation in a complex microbial environment as the rumen (Calsamiglia et al., 2007). As for any other additive, the presence of residues in the final product should be evaluated: little information is available on transfer of these plant metabolites into animal products (Benchaar and Greathead, 2011). The use of any additive can be only justified if there is a beneficial effect larger than the cost of the product. This aspect depends on the cost of the additive, the dose required and the resulting improvement in animal performance.

1.5.2 MITIGATION THROUGH FEEDING PRACTICES

Effect of forage quality, concentrate characteristics and feeding level

Improving forage quality, either through feeding forage with a lower fiber content and higher level of non-fibrous carbohydrates (NFC) (e.g. starch and soluble carbohydrates) can decrease CH_4 production (Beauchemin et al., 2008). Fiber (cellulose and hemicellulose) ferments at slower rates than do NFC but yields more CH_4 per unit of substrate digested (McAllister et al., 1996). Consequently, the addition of grain to forage based diets increases starch and reduces fiber intake, reducing the rumen pH and favoring the production of propionate rather than acetate in the rumen (McAllister and Newbold, 2008). Improving forage quality (e.g. NDF digestibility) also tends to increase the voluntary DMI and reduce the retention time in the rumen, reducing the proportion of dietary energy content converted to CH_4 in the rumen. CH_4 emissions are also commonly lower with higher proportion of forage legumes in the diet, partly because of the lower fiber and higher protein contents, the faster k_p rate, and in some cases, the presence of condensed tannins (Waghorn, 2007; Beauchemin et al., 2008).

It is well known that CH_4 production (expressed as g CH_4/kg DMI, as a percentage of energy intake or expressed per unit of final product) can be reduced feeding diets containing higher levels of grain concentrate compared with forage based diets (Johnson and Johnson, 1995). In a meta-analysis, Sauvant and Ginger-Reverdin (2007) showed that the relationship between concentrate level in the diet and CH_4 production is curvilinear: CH_4 losses appear relatively constant for rations containing up to 30-40% concentrate (6-7% of GE intake) and then decrease rapidly to lower values (2-3% of GE intake) as the percentage of concentrates raises to 80-90% of DMI (Lovett et al., 2003; Beauchemin and McGinn, 2005; Martin et al., 2007a). As reported in section 1.4.2, NFC (e.g. starch) fermentation promotes propionate production, creating an alternative H_2 sink to methanogenesis, lowers ruminal pH and, consequently, inhibits the growth of rumen methanogens (Van Kessel and Russel, 1996) and protozoa.

An alternative to feeding grains, the use of cereal forages (e.g. corn silage and whole crop winter silages) that contain high quantities of starch has been proposed as a means to increase the starch content of rations and lower CH_4 emissions (Beauchemin et al., 2008). Furthermore, intake of whole crop silages is often higher than that of grass forages, and thus shorter residence time in the rumen could reduce CH_4 per kg of DMI (Grainger and Beauchemin, 2011). In a study with growing beef cattle, McGeough et al. (2010) compared four diets (24% concentrate and 76% silage on DM basis) containing 4 different corn silages to a higher concentrate diet (16.6% grass silage and 83.4% concentrate). The corn silages were harvested at increasing stages of maturity: the starch content ranged from 31.5 to 38.6% DM and NDF decreased from 48.5 to 43.4%. Animals fed the high concentrate diet (starch content of 36.9% on DM basis) produced 19% less CH_4 (g CH_4/kg DMI) than animals fed the corn silage diets. For corn silages, CH_4

output relative to DMI tended to linearly decrease to a 10.9% reduction in response to increasing the "starch:NDF" ratio. Thus the higher starch content of forages can help decrease methanogenesis, but CH_4 production of forage fed animals is still significantly higher than for concentrate fed cattle (Grainger and Beauchemin, 2011).

In conclusion, improving forage and diet quality can improve the animal production efficiency, enhancing performances and reducing CH_4 production per unit of animal product.

Supplementation with lipids: characteristics and feeding levels

The supplementation of lipids to the ration may offer a means of increasing the energy density of the diet that is not fermented in the rumen, improving the animal performance whilst lowering CH_4 (Beauchemin et al., 2008; Eugene et al., 2008; Martin et al., 2010; Grainger and Beauchemin, 2011): in fact less H_2 is produced per unit of feed ingested when higher fat levels are included in the diets for ruminants, as reported in section 1.4.2. Moreover, individual fatty acids have been considered to have specific anti-methanogenic properties: some refined oils (e.g. coconut oil) contain high concentrations of medium-chain fatty acids, particularly lauric and myristic fatty acids (Machmuller et al., 2001). Reductions in CH_4 yield can be attributed to these fatty acids (Dohme et al., 2001), as they have anti-microbial properties and are toxic to methanogens. Machmuller (2006) reported reductions in both protozoa and methanogens communities in response to medium chain fatty acids. Lipid sources containing high concentrations of unsaturated long chain fatty acids as linoleic acid (C18:2 from soybean and sunflower) and linolenic acid (C18:3 from linseed) are reported to have greater effects on CH_4 emissions compared with medium chain fatty acids (Machmuller, 2006). This can be partially attributed to a reduction in fiber degradation and lower DMI levels as rations are supplemented with these products (Beauchemin et al., 2008). Other fatty acids present in fish oil or in some algae also have a negative effect on methanogenesis (Martin et al., 2010). Hexadecatrienoic acid (C16:3), eicosapentaenoic acid (C20:5) and docosahexaenoic acid (C22:6) (Fievez et al., 2003, 2007) had a strong CH_4-suppressing effect when tested *in vitro*. Present data are scarce and there is a need of further research on the effects of these lipids sources on animals. Differently from the anti-methanogenic mechanism proposed for medium chain fatty acids, polyunsaturated fatty acids can even lower CH_4 production by consuming H_2 as they undergo hydrogenation in the rumen. The success and degree of CH_4 mitigation is likely to be dependent on the level of lipid supplementation and on the interaction between the diet characteristics and the physical form of the supplement. Beauchemin et al. (2008) reported that, over a range of 17 experiments with sheep and cattle including different lipid sources and diet characteristics, CH_4 yield was reduced by 5.6%

as the lipid addition was increased by 1%. Also Martin et al. (2010) found a similar relationship between the level of added fat (% of DMI) and the CH_4 decrease (g/kg DMI): the authors reported a mean decrease in CH_4 of 3.8% with each 1% addition of supplemental fat (figure 1.9).

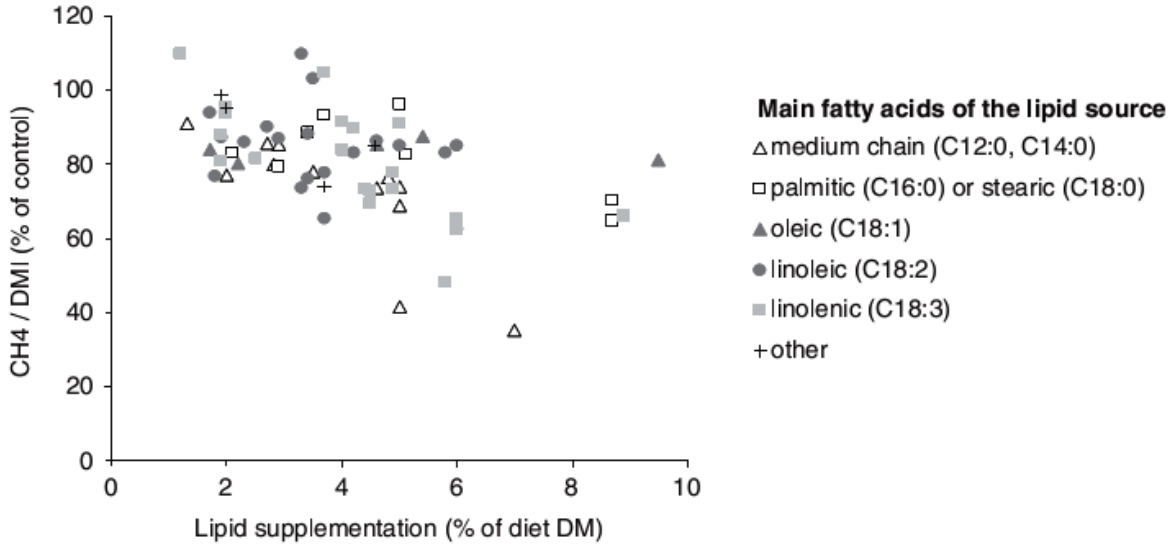

Figure 1.9: Review of 28 publications on the effect of lipid supply on CH_4 production in ruminants (Martin et al., 2010).

Moreover, Martin et al. (2008) reported a reduction in CH_4 yield (22.0 to 10.5 g CH_4/kg of organic matter intake) as cows were fed a mixed ration supplemented with linseed oil. The physical form of the lipid source affected CH_4 yield, with refined oil having a higher impact on methanogenesis than crude or extruded linseed (10.5, 19.8 and 16.3 g CH_4 per kg of organic matter intake, respectively) (Martin et al., 2008). Almost all experiments carried out with lipid supplements were short-term experiments: a research priority is to evaluate the long-term effect of different lipid sources.

1.6 METHODS TO MEASURE METHANE EMISSIONS FROM RUMINANTS

Over the last 100 years several different methods have been developed with the purpose of measuring or estimating CH_4 emissions from ruminants. These include CH_4 estimations from the VFA production (Blümmel et al., 1999; Hegarty and Nolan, 2007), *in vitro* techniques, isotopic or non-isotopic tracer techniques, ventilated hood techniques, and measurement in confined respiration chambers (Frankenfield, 2010). All these methods have various aims of application, advantages and disadvantages (Storm et al., 2012). This section briefly presents the most common methods for measuring methanogenesis from ruminants, using both *in vitro* and *in vivo* assays.

1.6.1 *IN VITRO* GAS PRODUCTION TECHNIQUE FOR CH_4 MEASUREMENTS

The *in vitro* gas production technique (GP) has been used to simulate ruminal fermentation of feeds and rations (Rymer et al., 2005) for decades. In recent years, with the increasing interest in GHGs emissions from agriculture, the traditional GP has been modified to include measurement of CH_4 production (Pellikaan et al., 2011; Navarro-Villa et al., 2011).

Figure 1.10: *In vitro* gas production technique

The basic principle of GP techniques is to ferment feed under controlled laboratory conditions employing rumen microorganisms. Feedstuffs are incubated at 39°C with a mixture of rumen fluid, buffer and minerals solutions for a certain time period, typically 24, 48, 72, 96 or 144 h (figure 1.10). The amount of total gas produced during incubation is measured and then sampled and its composition analyzed through gas-chromatography which is highly accurate and precise, to obtain data on the *in vitro* CH_4 production. At the same time, it is possible to determine *in vitro* digestibility of feedstuffs, making it possible to determine whether a reduction in methanogenesis is at the expense of feed digestibility. The output of GP experiments is usually reported as the amount of CH_4 per mL of total GP (the percentage of CH_4 in total GP), per gram of dry matter (DM), per gram of degraded DM (dDM), or per gram of degraded NDF (dNDF).

Various GP systems have been employed for CH_4 determination, as for example glass syringes (Blümmel and Ørskov, 1993; Bhatta et al., 2006), Rusitec (Bhatta et al., 2006), closed vessel batch fermentations (Navarro-Villa et al., 2011) and lately fully automated systems (Pellikaan et al., 2011). Depending on the system and other laboratory constraints, it is possible to conduct up to several tens parallel incubations

simultaneously, which allow for sufficient amounts of repetitions in experiments to support statistically significant differences between treatments.

The typical time frame for conducting an *in vitro* experiment is 1–4 weeks, which makes it possible to screen many different feedstuffs and potential additives relatively fast and cheaply. The system can also be used to explore dose-response curves for potential additives. Compared to *in vivo* experiments, it is also easier to control fermentation parameters: the chemical, physical and microbial environment can be kept more constant between *in vitro* replications (within runs) than between individual animals in *in vivo* experiments. The variability among different animals observed *in vivo* can be avoided using the same ruminal i*noculum* for all the treatments to be compared *in vitro*. Preferably the *inoculum* is obtained mixing rumen fluid collected from several (2-3) donor animals to include as many different rumen microorganisms as possible. The method requires access to fresh rumen fluid, which is typically obtained from ruminally fistulated animals. Alternative methods of collecting rumen fluid are by esophageal probe on intact animals or from slaughtered animals.

Two studies comparing GP measurements of CH_4 production to the SF_6-technique and the respiration chamber technique, respectively, show good agreement between *in vivo* techniques and GP assays (Bhatta et al., 2006; Bhatta et al., 2008). Other studies, reporting both *in vitro* and *in vivo* results, give examples of both good agreement between methods as well as the opposite (Klevenhusen et al., 2011; Patra and Saxena, 2010).

A clear disadvantage of GP is that it can only simulate the ruminal fermentation of feeds, not emissions and digestibility by the entire animal, and cannot take into account physical factors as the passage rate of *digesta* or the physical form of the diet fed (Storm et al., 2012). Furthermore, under normal conditions it does not include long-term adaptation of the ruminal microorganisms to the tested feedstuffs. It is common to use rumen fluid from animals on a standard feed ration. During *in vivo* experiments it is a common practice to have adaptation periods to new dietary treatments of at least 14 days. The animals response is not considered stable before that time period. For rumen methanogens there are indications that the adaptation period to a new dietary treatment is more than 30 days (Williams et al., 2009). Even if GP results should always be interpreted with care, this method is a very useful technique relatively labor saving and affordable (Getachew et al., 2005) as first approach to test potential feedstuffs and additives. The GP results can then be used to optimize larger and more expensive *in vivo* experiments.

1.6.2 *IN VIVO* TECHNIQUES FOR CH_4 MEASUREMENTS

The two most commonly employed *in vivo* techniques are the respiration chamber technique and the SF_6 –tracer technique.

During the last decades, different indirect calorimetry techniques or respiration chambers have been used with the main purpose of studying the energy metabolism of animals (Johnson et al., 2003; McLean and Tobin, 1987) through the measurement of O_2 consumption and CO_2 expiration. Methane loss is an inherent part of the energy metabolism in ruminants, and various types of chambers are valuable tools in the investigation of mitigation strategies for CH_4 emissions.

The respiration chamber technique entails confining one or more animals in airtight chambers and collect all exhaled breath from the animal to determine its composition. Calorimetric systems, where air composition is measured, are divided into two types: the closed-circuit and the open-circuit, with the latter being the dominating one (McLean and Tobin, 1987; Pinares-Patiño and Clark, 2008). Figure 1.11 shows an outline of the open-circuit system. This kind of facility involves the continuous flow of air through the chamber in which an animal is housed: the pump pumps air from the chamber through a flow-meter and O_2, CO_2 and CH_4 gas analyzers. Gases are analyzed both in inlet and outlet air. Fresh air for the animal is taken from outside. In some systems fresh air is drawn through an air conditioning system to control humidity and temperature within the chamber, whist others simply take air from outside the chamber. CH_4 emission is calculated from flow and gas concentration in inlet and outlet air from the chamber. The difference between the outgoing and incoming amount of CH_4 corresponds to the CH_4 emission from the animal (Johnson and Johnson, 1995).

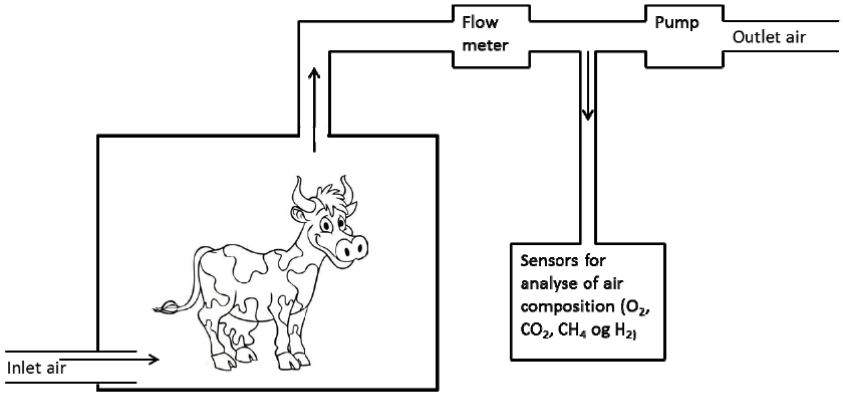

Figure 1.11: Open circuit respiration chamber scheme (Storm et al., 2012)

Chambers are regarded as the "gold-standard" method for measurement of CH_4 emission from ruminants, because the environment can be controlled and the reliability and stability of instruments can be measured (McLean and Tobin, 1987; Johnson and

Johnson, 1995). However, there is a risk of creating an artificial environment, which could affect animal behavior (e.g. DMI). As DMI is one of the main drivers of CH_4 production, a reduction in DMI would not only effect total emission but also the derived estimates like loss of gross energy (Ellis et al., 2007). Therefore, it has been queried that results obtained in chambers cannot be applied to free ranging animals (e.g. animals on pasture) (Johnson et al., 1994; Okelly and Spiers, 1992). On the other hand, investigations have shown that chambers give more precise estimates of CH_4 emissions than the SF_6 tracer technique (Pinares-Patiño et al., 2011).

Classical chambers for energy metabolism with air conditioning, internal mixing of air and careful tightening to reduce the risk of air losses to the surroundings (Derno et al., 2009) are expensive to build. With this kind of facility, data on CH_4 emission can be combined with data on rumen metabolism and digestibility (Johannes et al., 2011). In fact feces and urine can be separately collected, allowing the determination of feed digestibility and of full energy and nitrogen balances. As a consequence, nearly all aspects of feeding and nutrition and their role in CH_4 production can be investigated in a chamber system, including the level of feeding (restricted vs ad libitum), effects of different feedstuffs, effects of chemical composition and physical form of the ration, different feeding schedules and possibly the effect of different additives. Also changes in emission during the day can be described with the system, but resolution depends on the number of measurements during the day.

The variability in measurements is affected by instrument variation as well as within and between animal variations. Within animal variation (day-to-day variation) will affect the number of days for measurements. The day-to-day coefficient of variation (CV) has been reported to be 4,3% for dairy cows (Grainger et al., 2007) and 4,7% in sheep (Pinares-Patiño et al., 2011). For CH_4 and energy metabolism studies periods of three to five days have been used (Pinares-Patiño et al., 2011; Grainger et al., 2007). A high between animal variation will increase the number of animals needed to document differences among treatments.

The chamber method can be used to examine nearly all aspects of nutrition with good accuracy and precision in assessing the daily CH_4 production (deriving from ruminal and hindgut fermentations) from housed animals, but has limited capacity considering the number of animals to be included in the experiment. It is therefore best suited for comparisons among distinct mitigation strategies in crossover or latin square experimental designs. However, due to the precision and accuracy of measurements from respiration chambers, fewer animals per treatment group and fewer experimental days are required to ensure adequate statistical power relative to other CH_4 measurement techniques. Considerations about design and placement of the chambers

can eliminate the risk of reduced DMI. Moreover, it is not applicable to free ranging animals. There is no doubt that this system gives quantitative measurements of CH_4 emission but establishment and maintenance costs, high labor input required, limited capacity of the system and the need for trained animals restricts the number of treatments which can be examined experimentally.

The SF_6 tracer-technique was first developed by Johnson et al. (1994) and it is specifically designed to measure CH_4 from free ranging ruminants (Johnson et al., 2007), because it had been queried that results obtained in respiration chambers could not be applied to animals on pasture (Johnson et al., 1994; Okelly and Spiers, 1992). The basic idea behind the method is that CH_4 emission can be measured if the emission rate of a tracer gas from the rumen is known. A permeation tube containing the tracer gas sulfur hexafluoride (SF_6) is inserted into the rumen of the animal. The release rate of SF_6 from the permeation tube is carefully determined by gravimetric weighing for at least two months before insertion of the tube into the animal whilst permeation tube is kept at 39°C. Subsequently, air is sampled from the nostrils of the ruminant into a vacuum collection canister over a 24 h-period. In the collection canister, a representative sample of the expired and eructed breath of the animal is collected (figure 1.12).

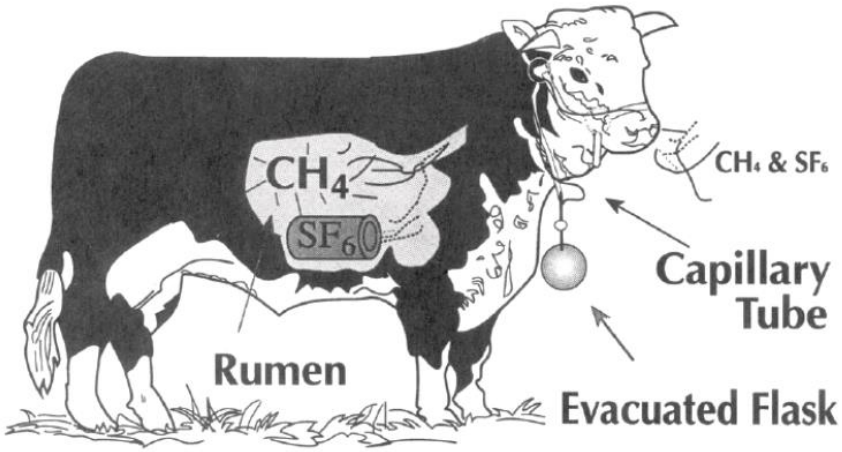

Figure 1.12: Illustration of the SF_6 tracer technique (Johnson et al., 1994).

The air in the canister is analyzed for SF_6 and CH_4 concentrations via gas-chromatography (Storm et al., 2012). Because the absolute amount of SF_6 released from the animal is known, the volume of CH_4 produced can be calculated from the CH_4 and SF_6 concentration in the canister and the known quantity of SF_6 released from the permeation tube. Relative to indirect calorimetry, this technique is less expensive and allows for measurements in large numbers of grazing animals. CH_4 production is unlikely affected by changes in animals behavior.

The SF_6 technique has been compared with the respiration chamber technique and measurements with both techniques showed good correlations in most (Johnson et al., 1994, McGinn et al, 2006), but not all studies (Lassey et al., 1997, Wright et al., 2004). The accuracy of the SF_6 tracer method in estimating CH_4 production depends on several assumptions, which are: 1) the SF_6 gas simulates CH_4 emissions and is uniformly mixed; 2) dilution rates of SF_6 and CH_4 gases are identical; 3) SF_6 is inert and its release from the permeation tube follows a constant linear pattern; 4) there are no interactions between rumen content and SF_6 gas (Johnson et al., 1994; Pinares-Patiño and Clark, 2008). In general, the SF_6 technique is known to underestimate CH_4 emissions relative to the chamber technique (because it does not measure all of the hindgut CH_4; Johnson and Johnson, 1995) and to have a larger standard deviation (Johnson et al., 1994, McGinn et al, 2006). Variation in CH_4 production within and between animals is more pronounced with the SF_6 than the chamber technique (Pinares-Patiño and Clark, 2008). For this reason, more animals will have to be included in a study using this technique in comparison with the respiration chamber technique. Moreover, the animals need to carry equipment around and must therefore be selected and trained before the experiments start.

In this thesis, CH_4 production was determined using both the *in vitro* and respiration chamber techniques. The former was chosen at first to study the effects of chemical composition of lactating cows diets and the use of additives on rumen CH_4 production; the latter was chosen because of its higher accuracy and the possibility to quantify the effect of lowering CH_4 production on the overall energy and nitrogen metabolism of animals.

1.7 REFERENCES

- Anderson, K.L., Nagaraja, T.G., Morrill, J.L., Avery, T.B., Galitzer, S.J., Boyer, J. E., 1987. Ruminal microbial development in conventionally or early-weaned calves. J. Anim. Sci. 64:1215.
- Ando, S., Nishida, T., Ishida, M., Hosoda, K., Bayaru, E., 2003. Effect of peppermint feeding on the digestibility, ruminal fermentation and protozoa. Livest. Prod. Sci. 82:245–248.
- Andrew, S.M., Tyrrell, H.F., Reynolds, C.K., Erdman, R.A., 1991. Net energy value for lactation of a dietary fat, calcium salts of long chain fatty acids, for cows fed silage based diets. J. Dairy Sci. 74:2588-2600.
- Balandrin, M.F., Klocke, J.A., 1985. Natural plant chemicals: Sources of industrial and medicinal materials. Science 228:1154–1160.

- Bannink, A., Tamminga, S., 2005. Rumen function. In J Dijkstra, J Forbes, J France (eds.) Quantitative Aspects of ruminant digestion and metabolism, CABI Publishing, Cambridge, UK, pp. 263-288.
- Bannink, A., Smits, M.C.J., Kebreab, E., Mills, J.A.N., Ellsi, J.L., Klop, A., France, J., Dijkstra, J., 2010. Simulating the effects of grassland management and grass ensiling on methane emission from lactating cows. J. Agric. Sci. 148: 55-72.
- Bayaru, E., Kanda, S., Kamada, T., Itabashi, H., Andoh, N., Nishida, T., Ishida, M., Itoii, T., Nagara, K., Isobe, Y., 2001. Effect of fumaric acid on methane production, rumen fermentation and digestibility of cattle fed roughage alone. J. Anim. Sci. 72:139-146.
- Beauchemin, K.A., McGinn, S.M., 2005. Methane emissions from feedlot cattle fed barley or corn diets. J. Anim. Sci. 83:653–661
- Beauchemin, K.A., Kreuzer, M., O'Mara, F., McAllister, T.A., 2008. Nutritional management for enteric methane abatement: a review. Aust. J. Ep. Agric. 48:21–27.
- Beever, D.E., 1993. Rumen function. In J Forbes, J France (eds.) Quantitative Aspect of rumen digestion and Metabolism, CABI International, Wallingford, UK, pp. 87-215.
- Benchaar, C., Petit, H.V., Berthiaume, R., Ouellet, D.R.,J. Chiquette, D.R., 2003. Effects of essential oil supplement on ruminal fermentation, rumen microbial populations and in sacco degradation of dry matter and nitrogen in the rumen of lactating dairy cows. Can. J. Anim. Sci. 83: 637-638.
- Benchaar, C., Greathead, H., 2011. Essential oils and opportunities to mitigate enteric methane emissions from ruminats. Anim. Feed Sci. Tech. 166-167:338-355.
- Bhatta, R., Tajima, K., Takusari, N., Higuchi, K., Enishi, O., Kurihara, M., 2006. Comparison of sulfur hexafluoride tracer technique, rumen simulation technique and in vitro gas production techniques for methane production from ruminant feeds. Int. Congr. Ser. 1293:58–61.
- Bhatta, R., Enishi, O., Takusari, N., Higuchi, K., Nonaka, I., Kurihara, M., 2008. Diet effects on methane production by goats and a comparison between measurement methodologies. J. Agric. Sci. 146:705–715.
- Blümmel, M., Ørskov, E.R., 1993. Comparison of in vitro gas production and nylon bag degradability of roughages in predicting feed intake in cattle. Anim. Feed Sci. Technol. 40:109–119.

- Blümmel, M., Aiple, K.P., Steingass, H., Becker, K., 1999. A note on the stoichiometrical relationship of short chain fatty acid production and gas formation in vitro in feedstuffs of widely differing quality. J. Anim. Physiol. An. N. 81:157–167.
- Boadi, D., Benchaar, C., Chiquette, J., Masse, D., 2004. Mitigation strategies to reduce enteric methane emissions from dairy cows: update review. Can. J. Anim. Sci. 84:319-335.
- Borchers, R., 1965. Proteolytic activity of rumen fluid in vitro. J. Anim. Sci. 24:1033–1038.
- Broudiscou, L.P., Papon, Y., Broudiscou, A.F., 2000. Effects of dry plant extracts on fermentation and methanogenesis in continuous culture of rumen microbes. Anim. Feed Sci. Tech. 87:263–277.
- Broudiscou, L.P., Papon, Y., Broudiscou, A.F., 2002. Effects of dry plant extracts on feed degradation and the production of rumen microbial biomass in a dual outflow fermenter. Anim. Feed Sci. Tech. 101:183–189.
- Burt, S., 2004. Essential oils: Their antibacterial properties and potential applications in foods—A review. Int. J. Food Microbiol. 94:223–253.
- Busquet, M., Calsamiglia, S., Ferret, A., Kamel, C., 2005. Screening for the effects of natural plant extracts and secondary plant metabolites on rumen microbial fermentation in continuous culture. Anim. Feed Sci. Tech. 123-124:597–613.
- Busquet, M., Calsamiglia, S., Ferret, A., Kamel, C., 2006. Plant extracts affect in vitro rumen microbial fermentation. J. Dairy Sci. 89:761–771.
- Calsamiglia, S., Busquet, M., Cardazo, P.W., Castillejos, L., Ferret, A., 2007. Invited review: essential oils as modifiers of rumen microbial fermentation. J. Dairy Sci. 90:2580–2595.
- Capper, J.L., Cady, R.A., Bauman D.E., 2009. The environmental impact of dairy production: 1944 compared with 2007. J. Anim Sci. 87:2160-2167.
- Cardozo, P.W., Calsamiglia, S., Ferret, A., Kamel, C., 2004. Effects of natural plant extracts on protein degradation and fermentation profiles in continuous culture. J. Anim. Sci. 82:3230–3236.
- Cardozo, P.W., Calsamiglia, S., Ferret, A., Kamel, C. 2005. Screening for the effects of natural plant extracts at different pH on in vitro rumen microbial fermentation of a high-concentrate diet for beef cattle. J. Anim. Sci. 83:2572–2579.
- Carulla, J.E., Kreuzer, M., Machmüller, A., Hess, H.D., 2005. Supplementation of *Acacia mearnsii* tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. Aust. J. Agric. Res. 56: 961–970.

- Chaban, B., Ng, S.Y., Jarrell, K.F., 2006. Archaeal habitats-from the extreme to the ordinary. Can. J. Microbiol. 52:73-116.
- Chaucheyras, F., Fonty, G., Bertin, G., Gouet, P., 1995. In vitro H_2 utilization by a ruminal acetogenic bacterium cultivated alone or in association with an archaea methanogen is stimulated by a probiotic strain of *Saccharomyces cerevisiae*. Appl. Environ. Microb. 61:3466–3467.
- Chaucheyras-Durand, F., Walker, N.D., Bach, A., 2008. Effects of dry yeasts on the rumen microbial ecosystem: past, present and future. Anim. Feed Sci. Tech. 145:5-26.
- Cheeke, P.R., 1996. Biological effects of feed and forage saponins and their impacts on animal production. In: G.R. Waller, K. Yamasaki (eds.) Saponins Used in Food and Agriculture, Plenum Press, New York. pp 377–385.
- Chow, J.M., Van Kessel, J.A.S., Russell, J.B., 1994. Binding of radiolabeled monensin and lasolacid to ruminal microorganisms and feed. J. Anim. Sci. 72:1630–1635.
- Crompton, L.A., Mills, J.A.N., Reynolds, C.K., 2010. Effect of feeding frequency and replacing calcium salts of palm oil with crushed rapeseed or coconut oil on methane emissions in lactating dairy cows. Proc. Nutr. Soc. 69:E329.
- Dalal, R., Wang, W., Robertson, G., Parton, W., 2003. Nitrous oxide emission from Australian agricultural lands and mitigation options: a review. Aust. J. Soil Res. 41:165–195.
- Derno, M., Elsner, H.G., Paetow, E.A., Scholze, H., Schweigel, M., 2009. Technical note: A new facility for continuous respiration measurements in lactating cows. J. Dairy Sci. 92:2804–2808.
- de Klein, C.A.M., Eckard, R.J., 2008. Targeted technologies for nitrous oxide abatement from animal agriculture. Aust. J. Exp. Agric. 48:14–20.
- Dijkstra, J., Bannink, A., France, J., Kebreab, E., 2007. Nutritional control to reduce environmental impacts of intensive dairy cattle systems. In: Q.X. Meng, L.P. Ren, Z.J. Cao (eds.) Proc. VII International Symposium on the Nutrition of Herbivores. China Agricultural University Press, Beijing, China. pp. 411–435.
- Dohme, F., Machmuller, A., Wasserfallen, A., Kreuzer, M., 2001. Ruminal methanogenesis as influenced by individual fatty acids supplemented to complete ruminants diets. Lett. Appl. Microbiol. 32:47-51.
- Eckard, R.J., Grainger, C., de Klein, C.A.M., 2010. Options for the abatement of methane and nitrous oxide from ruminant production: a review. Livest. Sci. 130:47-56.

- Ellis, J.L., Kebreab, E., Odongo, N.E., McBride, B.W., Okine, E.K., France, J., 2007. Prediction of methane production from dairy and beef cattle. J. Dairy Sci. 90:3456-3467.
- Ellis, J.L., Dijkstra, J., Kebreab, E., Bannink, A., Odongo, N.E., McBride, B.W., France, J., 2008. Aspects of rumen microbiology central to mechanistic modeling of methane production in cattle. J. Agric. Sci. 146:213-233.
- Ellis, J.L., Kebreab, E., Odongo, N.E., Beauchemin, K., McGinn, S., Nkrumah, J.D., Moore, S.S., Christopherson, R., Murdock, G.K., McBride, B.W., Okine, E.K., France, J., 2009. Modeling methane production from beef cattle using linear and nonlinear approaches. J. Anim. Sci. 87:1334-1345.
- Eugene, M., Masse, D., Chiquette, J., Benchaar, C., 2008. Short communication: Meta-analysis on the effects of lipid supplementation on methane production in lactating dairy cows. Can. J. Anim. Sci. 88:331-334.
- FAO, 2010. Greenhouse gas emissions from the dairy sector. A life cycle assessment. Food and Agriculture Organization of the United Nations, Rome, Italy.
- Field, J.A., Kortekaas, S., Lettinga, G., 1989. The tannin theory of methanogenic toxicity. Biol. Waste 29:241–262.
- Fievez, V., Dohme, F., Danneels, M., Raes, K., Demeyer, D., 2003. Fish oils as potent rumen methane inhibitors and associated effects on rumen fermentation in vitro and in vivo. Anim. Feed Sci. Tech. 104:41-58.
- Fievez, V., Boeckaert, C., Vlaeminck, B., Mestdagh, J., Demeyer, D., 2007.In vitro examination of DHA-edible micro-algae: 2. Effect on rumen methane production and apparent degradability of hay. Anim. Feed Sci. Tech.136:80–95.
- Forster, P., Ramaswamy, V., Artaxo, P., Berntsen, T., Betts, R., Fahey, D.W., Haywood, J., Lean, J., Lowe, D.C., Myhre, G., Nganga, J., Prinn, R., Raga, G., Schulz, M., Van Dorland, R., 2007. Changes in atmospheric constituents and in radiative forcing. Cambridge University Press, Cambridge, UK.
- Frankenfield, D.C., 2010. On heat, respiration, and calorimetry. Nutrition 26:939-950.
- García-González, R., López, S., Fernández, M., Bodas, R., González, J.S., 2008. Screening the activity of plants and spices for decreasing ruminal methane production in vitro. Anim. Feed Sci. Tech. 147:36-52.
- Getachew, G., DePeters, E.J., Robinson, P.H., Fadel, J.G., 2005. Use of an in vitro rumen gas production technique to evaluate microbial fermentation of ruminant

feeds and its impact on fermentation products. Anim. Feed Sci. Tech. 123:547-559.

- Grainger, C., Clarke, T., Mcginn, S.M., Auldist, M.J., Beauchemin, K.A., Hannah, M.C., Waghorn, G.C., Clark, H., Eckard, R.J., 2007. Methane emissions from dairy cows measured using sulfur hexafluoride (SF6) and chamber techniques. J. Dairy Sci. 90:2755–2766.
- Grainger, C., Clarke, T., Auldist, M.J., Beauchemin, K.A., McGinn, S.M., Waghorn, G.C., Eckard, R.J., 2009. Mitigation of greenhouse gas emissions from dairy cows fed pasture and grain through supplementation with Acacia mearnsii tannins. Can. J. Anim. Sci. 89:241–251.
- Grainger, C., Beauchemin, K.A., 2011. Can enteric methane emissions from ruminants be lowered without lowering their production? Anim. Feed Sci. Tech. 166-167:308-320.
- Greathead, H., 2003. Plants and plant extracts for improving animal productivity. Proc. Nutrition Society. 62:279-290.
- Griffin, S.G., Wyllie, S.G., Markham, J.L., Leach, D.N., 1999. The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. Flavour Fragr. J. 14:322–332.
- Guo, Y.Q., Liu, J.X., Lu, Y., Zhu, W.Y., Denman, S.E., McSweeney, C.S., 2008. Effect of tea saponin on methanogenesis, microbial community structure and expression of mcrA gene, in cultures of rumen micro-organisms. Lett. Appl. Microbiol. 47:421–426.
- Gustafson, R.H., Bowen, R.E., 1997. Antibiotic use in animal agriculture. J. Appl. Microbiol. 83:531–541.
- Hart, K.J., Yáñes-Ruiz, D.R., Duval, S.M., McEwan, N.R., Newbold, C.J., 2008. Plant extract to manipulate rumen fermentation. Anim. Feed Sci. Tech. 147:8-35.
- Hegarty, R.S., 1999. Mechanisms of competitively reducing ruminal methanogenesis. Aust. J. Agr. Res. 50:1299-1305.
- Hegarty, J., Nolan, J., 2007. Estimation of ruminal methane production from measurement of volatile fatty acid production. In: H.P.S. Makkar, P.E. Vercoe (eds.) Measuring methane production from ruminants. Springer, Dordrecht, the Netherlands.
- Helander, I.M., Alakomi, H., Latva-Kala, K. , Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M., Wright, A., 1998. Characterization of the action of selected essential oil components on gram-negative bacteria. J. Agric. Food Chem. 46:3590–3595.

- Hess, H.D., Monsalve, L.M., Lascano, C.E., Carulla, J.E., Diaz, T.E., Kreuzer, M., 2003. Supplementation of a tropical grass diet with forage legumes and Sapindus saponaria fruits: effects on in vitro ruminal nitrogen turnover and methanogenesis. Aust. J. Agric. Res. 54:703–713.
- Hess, H.D., Beuret, R.A., Lotscher, M., Hindrichsen, I.K., Machmuller, A., Carulla, J.E., Lascano, C.E., Kreuzer, M., 2004. Ruminal fermentation, methanogenesis and nitrogen utilization of sheep receiving tropical grass hay-concentrate diets offered with *Sapindus saponaria* fruits and *Cratylia argentea* foliage. Anim. Sci. 79:177–189.
- Hindrichsen, I.K., Wettstein, H.R., Machmuller, A., Jorg, B., Kreuzer, M., 2005. Effect of the carbohydrate composition of feed concentrates on methane emission from dairy cows and their slurry. Environ. Monitoring Assessment. 107:329-350.
- Hindrichsen, I.K., Kreuzer, M., 2009. High methanogenic potential of sucrose compared with starch at high ruminal pH. J. Anim. Physiol. 93:61-65.
- Intergovernmental Panel on Climate Change, 2007. Climate Change 2007: Mitigation of climate change. Contribution of working group III to the fourth assessment report of the Intergovernmental Panel on Climate Change (IPCC). Cambridge Univ. Press, Cambridge, UK.
- Janssen, P.H., Kris, M., 2008. Structure of the archeal community of the rumen. Appl. Environ. Microb. 74:3619-3625.
- Janssen, P.H., 2010. Influence of hydrogen on rumen methane formation and fermentation balances through microbial growth kinetics and fermentation thermodynamics. Anim. Feed Sci. Tech. 160:1-22.
- Jarvis, G.N., Strompi, C., Burgess, D.M., Skillman, L.C., Moore, E.R.B., Joblin, K.N., 2000. Isolation and identification of ruminal methanogens from grazing cattle. Curr. Microbiol. 40:327-332.
- Joblin, K.N., 1999. Ruminal acetogens and their potential to lower ruminant methane emissions. Aust. J. Agric. Res. 50:1307–1313.
- Johannes, M., Lund, P., Weisbjerg, M.R., Hellwing, A.L.F., Hvelplund, T., 2011. The effect of different physical forms of rapeseed as fat supplement on rumen NDF digestion and methane emission in dairy cows. Adv. Anim. Biosci. 2:516.
- Johnson, D.E., Hill, T.M., Ward, G.M., Johnson, K.A., Branine, M.E., Carmean, B.R., Lodman, D.W., 1993. Ruminants and other animals. In: M.A.K. Khaill (ed.) Atmospheric methane: sources, sinks and role in global change. Springer-Verlag Berlin, Heidelberg, Germany, pp 199-226.

- Johnson, K.A., Huyler, M., Westberg, H., Lamb, B., Zimmerman, P., 1994. Measurement of methane emissions from ruminant livestock using a SF_6 tracer technique. Environ. Sci. Technol. 28:359–362.
- Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. J. Anim. Sci. 73:2483–2492.
- Johnson, D.E., Ferrell, C.L., Jenkins, T.G., 2003. The history of energetic efficiency research: Where have we been and where are we going? J. Anim. Sci. 81:E27–E38.
- Johnson, K.A., Westberg, H.H., Michal, J.J., Cossalman, M.W., 2007. The SF_6 tracer technique: Methane measurement from ruminants. In: H.P.S. Makkar, P.E. Vercoe (eds.) Measuring methane production from ruminants. Springer, Dordrecht, the Netherlands , pp 33-67.
- Kebreab, E., Clark, K., Wagner-Riddle, C., France, J., 2006. Methane and nitrous oxide emissions from Canadian animal agriculture: a review. Can. J. Anim. Sci. 86:135-158.
- Kirchgeßner, M., Windisch, W., Müller, H.L., 1995. Nutritional factors for the quantification of methane production. In: W. Englehardt, S. Leonhard-Marek, G. Brevers, D. Giesecke (eds.) Ruminant physiology: digestion, metabolism, growth and reproduction. Proceedings of the 8th International Symposium on Ruminant Physiology. Ferdinand Enke Verlag, Stuttgart, Germany, pp 333-348.
- Klevenhusen, F., Zeitz, J.O., Duval, S., Kreuzer, M., Soliva, C.R., 2011. Garlic oil and its principal component diallyl disulfide fail to mitigate methane, but improve digestibility in sheep. Anim. Feed Sci. Tech. 166–167:356–363.
- Koenig, K.M., Ivan, M., Teferedegne, B.T., Morgavi, D.P., Rode, L.M., Ibrahim, I.M., Newbold, C.J., 2007. Effect of dietary Enterolobium cyclocarpum on microbial protein flow and nutrient digestibility in sheep maintained fauna-free, with total mixed fauna or with Entodinium caudatum monofauna. Brit. J. Nutr. 98:504–516.
- Kolver, A.S., Aspin, P.W., 2006. Supplemental fumarate did not influence milk solids or methane production from dairy cows fed high quality pasture. Proc. N. Z. Soc. Anim. Prod. 66:409-415.
- Kumar, S., Puniya, A.K., Puniya, M., Dagar, S.S., Sirohi, S.K., Singh, K., Griffith, G.W., 2009. Factors affecting rumen methanogens and methane mitigation strategies. World J. Microb. Biot. 205:1557-1566.

- Lassey, K.R., Ulyatt, M.J., Martin, R.J., Walker, C.F., Shelton, I.D., 1997. Methane emissions measured directly from grazing livestock in New Zealand. Atmos. Environ. 31:2905-2914.
- Lee, S.C., Lee, H.J., Oh, Y.K., 2000. Methane production from enteric fermentation in ruminants. J. Anim. Sci. 13 (Special issue):171-181.
- Lovett, D.K., Lovell, S., Stack, L., Callan, J., Finlay, M., Conolly, J., O'Mara, F.P., 2003. Effect of forage/concentrate ratio and dietary coconut oil level on methane output and performance of finishing beef heifers. Livest. Prod. Sci. 84:135–146.
- Lynch, H.A., Martin, S.A., 2002. Effects of *Saccharomyces cerevisiae* culture and *Saccharomyces cerevisiae* live cells on in vitro mixed ruminal microorganism fermentation. J. Dairy Sci. 85:2603-2608.
- Machmuller, A., Dohme, F., Soliva, C.R., Wanner, M., Kreuzer, M., 2001. Diet composition affects the level of ruminal methane suppression by medium-chain fatty acids. Austr. J. Agric. Res. 52:713-722.
- Machmuller, A., 2006. Medium chain fatty acids and their potential to reduce methanogenesis in domestic ruminants. Agr.Ecosyst. Environ. 112(S.I.):107-114.
- Makkar, H.P.S., Becker, K., 1996. Effect of pH, temperature, and time on inactivation of tannins and possible implications in detannification studies. J. Agric. Food Chem. 44:1291–1295.
- Martin, C., Dubroeucq, H., Micol, D., Agabriel, J. Doreau, M., 2007a. Methane output from beef cattle fed different high-concentrate diets. Page 46 in Proc. Brit. Soc. Anim. Sci., Southport, UK.
- Martin, C., Rouel, J., Jouany, J.P., Doreau, M., Chilliard Y., 2008. Methane output and diet digestibility in response to feeding dairy cows crude linseed, extruded linseed, or linessed oil. J. Anim. Sci. 86:2642-2650.
- Martin, C., Morgavi, D.P., Doreau, M., 2010. Methane mitigation in ruminants: from microbe to the farm scale. Anim. 4-3:351-365.
- Mathinson, G.W., Okine, E.K., McAllister, T.A., Dong, Y., Galbraith, J., Dmytruk, O.I.N., 1998. Reducing methane emissions from ruminant animals. J. Appl. Anim. Res. 14:1-28.
- McAllister, T.A., Okine, E.K., Mathinson, G.W., Cheng, K.J., 1996. Dietary environmental and microbiological aspects of methane production in ruminants. Can. J. Anim. Sci. 76:231-243.
- McAllister, T.A., Newbold, C.J., 2008. Redirecting rumen fermentation to reduce methanogenesis. Aust. J. Exp. Agric. 48:7–13.

- McCourt, A.R., Yan, T., Mayne, C.S., 2007. Effect of forage type on methane production from dairy cows. Proc. Brit. Soc. Anim. Sci. 48.
- McCourt, A.R., Yan, T., Mayne, C.S., Wallace, J., 2008. Effect of dietary inclusion of encapsulated fumaric acid on methane production from grazing dairy cows. Proc. Brit. Soc. Anim. Sci. 64.
- McGeough, E.J., O'Kiely, P., Foley, P.A., Hart, K.J., Boland, T.M., Kenny, D.A., 2010. Methane emissions, feed intake, and performance of finishing beef cattle offered maize silage harvested at 4 different stages of maturity. J. Anim. Sci. 88:1479-1491.
- McGinn, S.M., Beauchemin, K.A., Iwaasa, A.D., McAllister, T.A., 2006. Assessment of the sulfur hexafluoride (SF_6) tracer technique for measuring enteric methane emissions from cattle. J. Environ. Qual. 35:1686-1691.
- McIntosh, F.M., Williams, P., Losa, R., Wallace, R.J., Beever, D.E., Newbold, C.J., 2003. Effects of essential oils on ruminal microorganisms and their protein metabolism. Appl. Environ. Microb. 69:5011-5014.
- McLean, J.A., Tobin, G., 1987. Animal and Human Calorimetry. Cambridge University Press, Cambridge, UK.
- Mills, J.A.N., Dijkstra, J., Bannink, A., Cammell, S.B., Kebreab, E., France, J., 2001. A mechanistic model of whole-tract digestion and methanogenesis in the lactating dairy cow: model development, evaluation and application. J. Anim. Sci. 79:1584-1597.
- Mills, J.A.N., Kebreab, E., Yates, C.M., Crompton, L.A., Cammell, S.B.,Dhanoa, M.S., Agnew, R.E., France, J., 2003. Alternative approaches to predicting methane emissions from dairy cows. J. Anim. Sci. 81:3141-3150.
- Mills, J.A.N., Crompton, L.A., Bannink, A., Tamminga, S., Moorby, J.M., Reynolds, C.K., 2009. Predicting methane emissions and nitrogen excretion from cattle. In: L.A. Crompton, T.R. Wheeler (eds.) Proceedings of the 41st meeting of the agricultural research modellers' group. J. Agric. Sci. 147,741.
- Min, B.R., Barry, T.N., Attwood, G.T., McNabb, W.C., 2003. The effect of condensed tannins on the nutrition and health of ruminants fed fresh temperate forages: a review. Anim. Feed Sci. Tech. 106:3–19.
- Moe, P.W., Tyrrell, H.F., 1979. Methane production in dairy cows. J. Dairy Sci. 62:1583-1586.
- Mohammed, N., Ajisaka, N., Lila, Z.A., Mikuni, K., Hara, K., Kanda, S., Itabashi, H., 2004. Effect of Japanese horseradish oil on methane production and ruminal fermentation in vitro and in steers. J. Anim. Sci. 82:1839–1846.

- Morgavi, D.P., Forano, E., Martin, C., Newbold, C.J., 2010. Microbial ecosystem and methanogenesis in ruminants. Anim. 4:1024-1036.
- Moss, A.R., Jouany, J.P., Newbold, J., 2000. Methane production by ruminants: its contribution to global warming. Ann. Zootech. 49, 231-253.
- Murray, R.M., Bryant, A.M., Leng, R.A., 1976. Rates of production of methane in the rumen and large intestines of sheep. Brit. J. Nutr. 36:1–14.
- Navarro-Villa, A., O'Brien, M., Lopez, S., Boland, T.M., O'Kiely, P., 2011. Modifications of a gas production technique for assessing in vitro rumen methane production from feedstuffs. Anim. Feed Sci. Tech. 166–167:163–174.
- Newbold, C.J., Lassalas, B., Jouany, J.P., 1995. The importance of methanogens associated with ciliate protozoa in ruminal methane production in vitro. Lett. Appl. Microbiol. 21:230–234.
- Newbold, C.J., Ushida, K., Morvan, B., Fonty, G., Jouany, J.P., 1996. The role of ciliate protozoa in the lysis of methanogenic archea in rumen fluid. Lett. Appl. Microbiol. 23:421-425.
- Newbold, C.J., El Hassan, S.M., Wang, J., Ortega, M.E., Wallace, R.J., 1997. Influence of foliage from African multipurpose trees on activity of rumen protozoa and bacteria. Brit. J. Nutr. 78:237–249.
- Newbold, C.J., McIntosh, F.M., Williams, P., Losa, R., Wallace, R.J., 2004. Effects of a specific blend of essential oils on rumen fermentation. Anim. Feed Sci. Tech. 114:105-112.
- Newbold, C.J, Lopez, S., Nelson, N., Ouda, J.O., Wallace, R.J., Moss, A.R., 2005. Propionate precursors and other metabolic intermediates as possible alternative electron acceptors to methanogenesis in ruminal fermentation in vitro. Brit. J. Nutr. 94:27–35.
- Newbold, C.J., Rode, L.M., 2006. Dietary additives to control methanogenesis in the rumen. In: C.R. Soliva, J. Takahashi, M. Kreuzer (eds.) Greenhouse gases and animal agriculture: an update. Elsevier International Congress Series 1293, Elsevier, Amsterdam, The Netherlands, pp 138–147.
- Ogino, A., Orito, H., Shimadad, K., Hirooka, H., 2007. Evaluating environmental impacts of the Japanese beef cow–calf system by the life cycle assessment method. J. Anim. Sci. 78:424–432.
- Oh, H.K., Sakai, T., Jones, M.B., Longhurst, W.M., 1967. Effect of various essential oils isolated from Douglas fir needles upon sheep and deer rumen microbial activity. Appl. Microbiol. 15:777–784.

- Okelly, J.C., Spiers, W.G., 1992. Effect of monensin on methane and heat productions of steers fed lucerne hay either ad-libitum or at the rate of 250 g/hour. Aust. J. Agric. Res. 43:1789–1793.
- Patra, A.K., Saxena, J.A., 2010. New perspective on the use of plant secondary metabolites to inhibit methanogenesis in the rumen. Phytochemistry. 71:1198–1222.
- Pellikaan, W.F., Hendriks, W.H., Uwimana, G., Bongers, L.J.G.M., Becker, P.M., Cone, J.W., 2011. A novel method to determine simultaneously methane production during in vitro gas production using fully automated equipment. Anim. Feed Sci. Tech. 168:196–205.
- Pen, B., Sar, C., Mwenya, B., Kuwaki, K., Morikawa, R., Takahashi, J., 2006. Effects of *Yucca schidigera* and *Quillaja saponaria* extracts on in vitro ruminal fermentation and methane emission. Anim. Feed Sci. Tech. 129:175–186.
- Pinares-Patiño, C.S., Clark, H., 2008. Reliability of the sulfur hexafluoride tracer technique for methane emission measurement from individual animals: an overview. Aust. J. Exp. Agr. 48:223-229.
- Pinares-Patiño, C.S., Lassey, K.R., Martin, R.J., Molano, G., Fernandez, M., MacLean, S., Sandoval, E., Luo, D., Clark, H., 2011. Assessment of the sulphur hexafluoride (SF_6) tracer technique using respiration chambers for estimation of methane emissions from sheep. Anim. Feed Sci. Tech., 166–167:201–209.
- Pulina, G., Dias Francesconi, A.H., Mele, M., Ronchi, B., Stefanon, B., Sturaro, E., Trevisi, E., 2011. Sfamare un mondo di nove miliardi di persone: le sfide per una zootecnia sostenibile. Ital. J. Agron. 6(Suppl.2):e7.
- Reynolds, C.K., Mills, J.A.N., Crompton, L.A., Givens, D.I., Bannink, A., 2010. Ruminant nutrition regimes to reduce greenhouse gas emissions in dairy cows. In: G.M. Crovetto (ed.) Energy and protein metabolism and nutrition. EAAP publication No. 127, Wageningen Academic Publishers, Wageningen, the Netherlands, pp 427-437.
- Reynolds, C.K., Crompton, L.A., Mills, J.A.N., Humphries, D.J., Kirton, P., Relling, A.E., Misselbrook, T.H., Chadwick, D.R., Givens, D.I., 2010a. Effects of diet protein level and forage source on energy and nitrogen balance and methane and nitrogen excretion in lactating dairy cows. In: G.M. Crovetto (ed.) Energy and protein metabolism and nutrition. EAAP publication No. 127, Wageningen Academic Publishers, Wageningen, the Netherlands, pp 463-464.
- Russell, J.B., Wallace, R.J., 1997. Energy-yielding and energy consuming reactions. In: P.N. Hobsen and C.S. Stewart (eds.). The rumen microbial ecosystem. Second edition. Blackie academic and professional, pp. 246-282.

- Rymer, C., Huntington, J.A., Williams, B.A., Givens, D.I., 2005. In vitro cumulative gas production techniques: History, methodological considerations and challenges. Anim. Feed Sci. Tech., 123–124:9–30.
- Sauvant, D., 1993. La production de méthane dans la biosphère: Le rôle des animaux d'élevage. Courrier de la Cellule Environnement 18:67-70.
- Sauvant, D., 2005. Rumen acidosis: modeling ruminant response to yeast culture. In T.P. Lyons, K.A. Jacques (eds.) Nutritional biotechnology in the feed and food industries. Nottingham University Press, Nottingham, UK, pp. 221–228.
- Sauvant, D., Giger-Reverdin, S., 2007. Empirical modelling meta-analysis of digestive interactions and CH_4 production in ruminants. In: I. Ortigues-Marty, N. Miraux, W. Brand-Williams (eds.) Energy and protein metabolism and nutrition, EAAP publication no. 124, Wageningen Academic Publishers, Wageningen, The Netherlands, pp 561.
- Smith, P., Martino, D., Cai, Z., Gwary, D., Janzen, H., Kumar, P., McCarl, B., Ogle, S., O'Mara, F., Rice, C., Scholes, B., Sirotenko, O., 2007. Agriculture. In: B. Metz, O.R. Davidson, P.R. Bosch, R. Dave, L.A. Meyer, (eds.) Climate Change 2007: Mitigation. Contribution of Working Group III to the Fourth Assessment Report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, UK, pp. 498–540.
- Steinfeld, H., Gerber, P., Wassenaar, T., Castel, V., Rosales, M., de Haan, C., 2006. Livestock's role in climate change and air pollution. In H Steinfeld, P Gerber, T Wassenaar, V Castel, M Rosales, C de Haan (eds.) Livestock's long shadow: environmental issues and options. Food and Agriculture Organization of the United Nations, Rome, Italy, pp. 79–123.
- Storm, I.M.L.D., Hellwing, A.L.F., Nielsen, N.I., Madsen, J., 2012. Methods for measuring and estimating methane emission from ruminants. Animals. 2:160-183.
- Takahashi, J., 2001. Nutritional manipulation of methanogenesis in ruminants. Asian-Aust. J. Anim. Sci. 14:131-135.
- Tamburini, A., Colombini, S., Penati, C., Zucali, M., Roveda, P., Rapetti, L., Crovetto, G.M., 2010. Methane emission in livestock and diet characteristics. In: G.M. Crovetto (ed.) Energy and protein metabolism and nutrition. EAAP publication No. 127, Wageningen Academic Publishers, Wageningen, the Netherlands, pp 465-466.
- Tavendale, M.H., Meagher, L.P., Pacheco, D., Walker, N., Attwood, G.T., Sivakumaran, S., 2005. Methane production from in vitro rumen incubations with

Lotus pedunculatus and *Medicago sativa*, and effects of extractable condensed tannin fractions on methanogenesis. Anim. Feed Sci. Tech. 123–124:403–419.

- Teferedegne, B., Osuji, P.O., Odenyo, A.A., Wallace, R.J., Newbold, C.J., 1999. Influence of foliage of different accessions of the sub-tropical leguminous tree, Sesbania sesban, on ruminal protozoa in Ethiopian and Scottish sheep. Anim. Feed Sci. Tech. 78:11–20.
- Tyrrell, H.F., Reynolds, C.K., Baxter, H.D., 1990. Energy metabolism of Jersey and Holstein cows fed total mixed diets with or without whole cottonseed. J. Dairy Sci. 73:192.
- Ungerfeld, E.M., Kohn, R.A., 2006. The role of thermodynamics in the control of ruminal fermentation. In K Sejrsen, T Hvelplund, M.O. Nielsen (eds.) Ruminant physiology digestion, metabolism and impact of nutrition on gene expression, immunology and stress, Wageningen Academic Publisher, the Netherlands, pp. 55-86.
- Valdez, F.R., Bush, L.J., Goetsch, A.L., Owens, F.N., 1986. Effect of steroidal sapogenins on ruminal fermentation and on production of lactating dairy cows. J. Dairy Sci. 69:1568–1575.
- Van Kessel, J.A.S., Russell, J.B., 1996. The effect of pH on ruminal methanogenesis. FEMS Microbiol. Ecol. 20:205-210.
- Waghorn, G.C., Tavendale, M.H., Woodfield, D.R., 2002. Methanogenesis from forages fed to sheep. Proc. N.Z. Grassl. Assoc. 64:167–171.
- Waghorn, G.C., Burke, J.L., Kolver, E.S., 2007. Principles of feeding value. In P.V. Rattray, I.M. Brookers, A.M. Nicol (eds.) New Zealand Society of Animal Production, Hamilton, New Zealand.
- Waghorn, G.C., 2007. Beneficial and detrimental effects of dietary condensed tannins for sustainable sheep and goat production: progress and challenges. Anim. Feed Sci. Tech. 147:116–139.
- Wallace, R.J., Arthaud, L., Newbold, C.J., 1994. Influence of Yucca shidigera extract on ruminal ammonia concentrations and ruminal microorganisms. Appl. Environ. Microbiol. 60:1762-1767.
- Wallace, R.J., 2004. Antimicrobial properties of plant secondary metabolites. Proc. Nutrition Society 63:621-629.
- Wallace, R.J., Wood, T.A., Rowe, A., Price, J., Yanez, D.R., Williams, S.P., Newbold, C.J., 2006. Encapsulated fumaric acid as a means of decreasing ruminal methane emissions. Int. Congr. Ser. 1293:148-151.

- Wang, Y., McAllister, T.A., Newbold, C.J., Rode, L.M., Cheeke, P.R., Cheng, K.J., 1998. Effects of *Yucca schidigera* extract on fermentation and degradation of steroidal saponins in the rumen simulation technique (RUSITEC). Anim. Feed Sci. Tech. 74:143-153.
- Weimer, P.J., 1998. Manipulating ruminal fermentation: a microbial ecological perspective. J. Anim. Sci. 76:3114–3122.
- Williams, Y.J., Popovski, S., Rea, S.M., Skillman, L.C., Toovey, A.F., Northwood, K.S., Wright, A.D., 2009. A vaccine against rumen methanogens can alter the composition of archaeal populations. Appl. Environ. Microbiol. 75:1860–1866.
- Wolin, M.J., Miller, T.L., Stewart, C.S., 1997. Microbe-microbe interactions. In P.N. Hobson, C.S. Stewart (eds.) The rumen microbial ecosystem, 2nd ed. Chapman & Hall, London, UK, pp. 467-491.
- Wood, T.A., Wallace, R.J., Rowe, A., Price, J., Yáñez-Ruiz, D.R., Murray, P., Newbold, C.J., 2009. Encapsulated fumaric acid as a feed ingredient to decrease ruminal methane emissions. Anim. Feed Sci. Tech. 152:62-71.
- Woodward, S.L., Waghorn, G.C., Laboyrie, P., 2004. Condensed tannins in birdsfoot trefoil (*Lotus corniculatus*) reduced methane emissions from dairy cows. Proc. N.Z. Soc. Anim. Prod. 64:160–164.
- Wright, A.D.G., Kennedy, P., O'Neill, C.J., Toovey, A.F., Popovski, S., Rea, S.M., Pimm, C.L., Klein, L., 2004. Reducing methane emissions in sheep by immunization against rumen methanogens. Vaccine 22:3976-3985.

2. METHANE YIELD FROM DRY AND LACTATING COWS DIETS IN THE PO PLAIN (ITALY) USING AN *IN VITRO* GAS PRODUCTION TECHNIQUE.

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2.1 ABSTRACT

The aim of the study was to measure total gas and methane (CH_4) production from 30 total mixed rations (TMRs) fed to dry and lactating cows in 20 commercial dairies in the Po Plain (Italy). Samples were analysed for chemical composition, *in situ* 48 h fibre digestibility (NDFD) and *in vitro* gas production (GP) and CH_4 concentration at 24 h of incubation. NDFD of TMRs from dry and lactating cows was identical (52.1%; P=0.995). The TMRs fed to dry and lactating cows differed for GP (43.0 and 54.4 mL/200 mg DM, respectively; P<0.001) and CH_4 (7.24 and 8.85 mL/200 mg DM, respectively; P=0.001), but not for CH_4 as percentage of GP (24.3 and 23.7%, respectively; P=0.286). The data were analysed dividing the TMRs into quartiles depending on starch:ADF ratio; the average ratios of the groups 1, 2, 3 and 4 were 37, 77, 116 and 138, respectively. Increasing starch:ADF ratio determined a higher GP: 42.2, 51.4, 55.1 and 56.2 mL/200 mg DM for groups 1, 2, 3 and 4, respectively (P<0.001), whilst CH_4 (mL/200 mg DM) was lower (P<0.001) for group 1 (7.12) in comparison with the others (8.82 on average). Acetate (% on total VFA) decreased for increasing starch:ADF ratio (P=0.009), whereas butyrate tended to increase (from 8.11 to 9.23% on total VFA; P=0.069) and the acetate:propionate ratio to decrease (from 3.35 to 3.09; P=0.082). The lack of a higher CH_4 concentration in GP from diets richer in fibre might be attributed mainly to the relatively short time of incubation.

Keywords**:** methane, cow, TMR, *in vitro.*

2.2 INTRODUCTION

Methane (CH_4) is one of the most important greenhouse gases (GHG) emitted from anthropogenic sources with a contribute to climatic change and global warming 21 times more effective than carbon dioxide (CO_2) (IPCC, 2007). Agriculture accounts for approximately 10 to 12% of the estimated anthropogenic greenhouse effect, producing about 45% of overall anthropogenic CH_4 emissions, with a wide range of uncertainty in the estimates of both the agricultural contribution and the anthropogenic total (IPCC, 2007). Domestic ruminants are the main responsible of these emissions, which derive primarily from enteric fermentations (Ellis et al., 2007) but also from the fermentation of organic matter in manure. Methane emissions from gastrointestinal tract are an indirect result of ruminal fermentation processes performed by microorganisms that digest and ferment carbohydrates into energy sources such as volatile fatty acids (VFA) (Getachew et al., 2005b; Hariadi and Santoso, 2010). Ruminal CH_4 production is energetically a wasteful process (Getachew et al., 2005b), since the proportion of feed converted to CH_4 represents a loss of approximately 2% to 12% of the gross energy intake (Johnson and Johnson, 1995). In the European Union approximately two-thirds of

annual regional methane emissions, amounting to about 6.8 million tonnes, have been attributed to enteric fermentation in ruminants (Moss et al., 2000). Among European countries, Italy has a legally binding commitment under the Kyoto Protocol to reduce GHG emissions by 6.5% below base-year levels, on average, over the first commitment period, 2008-2012. There is still uncertainty about the real impact of animal husbandry on GHG: from the first very high estimates (18%) of Steinfeld et al.(2006) lower impacts (12 to 13%) were reported by IPCC (2007), even lower (3 to 8%) by Capper et al. (2009) and eventually to an impact of 3% reported by Pulina et al.(2011) for Italy.

The amount of enteric CH_4 production is determined by many factors including: dry matter intake (Reynolds et al., 2010; Tamburini et al., 2010), diet characteristics such as supplementation with lipids (Grainger and Beauchemin, 2011) and type and level of carbohydrates fermented (Ellis et al., 2007), technological processes applied to feeds, and management practices that manipulate ruminal population (number of protozoa and methanogens) (Odongo et al., 2007). The adoption of these factors can reduce CH_4 production improving nutrient and energy utilization efficiency and decreasing environmental pollution. Hence, it is essential to be able to quantify the CH_4 produced from ruminal fermentation of different diets fed to dairy cows. Methane emissions are determined using both *in vivo* and *in vitro* assays. However, direct techniques are expensive, require complex equipment and are labour expensive and time consuming. Consequently, *in vitro* gas production (GP) method is an alternative relatively labour saving and affordable technique (Getachew et al., 2005a) which allows different diets to be tested simultaneously, alone or in presence of additives and inhibitors, for their effect on methanogenesis.

Due to lack of measured data on methane enteric emission by dairy cows in the Po Plain (Lombardy, Italy), aim of this study was to measure CH_4 production from total mixed rations (TMRs) fed to dairy cows in commercial dairies in this area using an *in vitro* GP technique.

2.3 MATERIALS AND METHODS

Sample collection and preparation

Thirty TMRs (22 and 8 samples for lactating and dry cows, respectively) were collected from 20 Italian Friesian commercial dairy farms in the Po plain (Italy) in the period November-December 2010. Average milk yield of cows in the selected farms was 30±4.6 kg/d. Samples were dried at 55°C in a forced air oven for 48 h and then ground to pass a 1 mm sieve using a Wiley mill (Pulverisette, Fritsch, Idar-Oberstein, Germany).

Chemical analysis, dry matter and fibre digestibility

Dry matter (DM) was determined following the AOAC procedure (AOAC, method 945.15, 1995), and organic matter was calculated as weight lost upon ignition at 600°C (AOAC, method 942.05, 1995). The crude protein (CP) content was determined by the macro-Kjeldahl technique (AOAC, method 984.13, 1995) using a 2300 Kjeltec Analyzer Unit (FOSS, Hillerød, Denmark). Ether extract was determined following the method 920.29 of the AOAC (1995). Neutral detergent fibre (NDF) was determined according to Mertens (2002), with addition of sodium sulphite and α-amylase to the neutral detergent solution. Acid detergent fibre (ADF), determined not sequentially to NDF, and acid detergent lignin (ADL) were calculated according to the method of Van Soest et al. (1991) using the Ankom 200 fibre apparatus (ANKOM Technology Corporation, Fairport, NY). Fibre fractions are reported on an ash-free basis. Neutral detergent insoluble crude protein and acid detergent insoluble crude protein were determined according to Licitra et al. (1996). Starch content was determined using Megazyme kit K-TSTA (Megazyme International Ireland Ltd., Wicklow, Ireland) for total starch assay procedure (AOAC, method 996.11, 1998). Ruminal NDF digestibility (NDFD) was determined using an *in situ* assay as reported by Spanghero et al. (2010) with an incubation time of 48 h. Each sample was weighed (250 mg as fed) in duplicate into Ankom F57 filter bags (ANKOM Technology Corporation, Fairport, NY). Samples were incubated in 2 incubation runs repeated on different days and using for each sample 2 rumen fistulated dry Italian Friesian cows fed a ration composed of meadow hay, flaked maize and a commercial protein concentrate (forage:concentrate ratio 65:35 on DM) twice daily. After 48 h of incubation, bags were collected from the rumen, washed thoroughly using cold tap water and then analysed using the Ankom 200 fibre apparatus (ANKOM Technology Corporation, Fairport, NY) according to Mertens (2002), with addition of sodium sulphite and α-amylase to the neutral detergent solution.

Animals on trial were handled as outlined by the guidelines of the 116/92 Italian law about animal welfare on experimental animals (Italian Regulation, 1992).

***In vitro* gas production and calculations of energetic value of rations**

Gas production was determined using a semi-automatic system (Theodorou et al., 1994), based on the measurement of the headspace gas pressure in the incubation bottles. Samples (250 mg as fed) were weighed in duplicate into 120 mL serum bottles. Buffered mineral solution and reducing solution were prepared according to Menke and Steingass (1988), stored in a water-bath at 39°C and purged with CO_2. Rumen fluid was collected before the morning feeding from two fistulated dry Italian Friesian cows fed as previously described. At the barn rumen fluid was squeezed through a cheesecloth layer and stored into a pre-warmed thermos flask, transferred to the laboratory, strained

through four layers of cheesecloth and flushed with CO_2. The rumen fluid was added to the buffered mineral solution (rumen fluid:buffer solution ratio=1:2) with constant stirring, while maintained at 39°C. Thirty mL of *inoculum* was dispensed into the bottles containing the TMR samples, for a corresponding headspace volume of 90 mL. The procedures were conducted under anaerobic conditions, flushing the bottle headspace with CO_2. The serum bottles were closed hermetically with rubber tops and placed in a shaking water-bath (75 RPM) at 39°C for 24 h. Each sample was analysed in 2 incubation runs. Headspace pressure was recorded after 2, 4, 6, 8, 10 and 24 h of incubation using a digital manometer (model 840082, Sper Scientific, Scottsdale, AZ, USA), avoiding that headspace pressure exceeded 48 kPa to preserve the normal microbial activity, as reported by Theodorou et al. (1994). The gas pressure data recorded at each time-point were converted to moles of gas using the ideal gas law (n=p*(V/R*T), were: n: gas produced (mol), p: pressure (kPa), V: headspace volume in bottles (L), R: gas constant (8.314 $L*kPa*K^{-1}*mol^{-1}$), T: temperature (K)), converted to millilitres of gas and then cumulated to obtain GP at 24 h of incubation. The GP values at 24 h were corrected for blanks and standard feeds included in each run in order to calculate the rumen organic matter digestibility (OMD, %) and the energetic value of TMRs (NE_L, Mcal/kg DM) according to Menke and Steingass (1988). At each reading time, after gas pressure data recording, a fixed-volume sample of gas (5 mL/bottle) was also collected for subsequent methane analysis using gas-tight syringes fitted with needles through the bottle top. After collection, remaining gas was released by needles and the pressure was brought back to atmospheric value.

Methane measurement

The gas composition of the headspace was determined by injecting 5 mL of gas into an Agilent 3000A micro GC gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) using N as carrier. An external standard mixture of CO_2 and CH_4 prepared by SIAD (SIAD S.p.A, Bergamo, Italy) was used for instrument calibration. Peak areas were calculated by automatic integration. The CH_4 volume (mL) produced between two time points and final cumulated volume were calculated as reported by Tavendale et al. (2005).

Ruminal volatile fatty acids determination

At the end of the incubation period, 10 mL of *inoculum* was sampled and clarified by centrifugation at 3,500 × *g* for 10 minutes. After centrifugation 5 mL of supernatant was sampled and added with 25% meta-phosphoric acid at a ratio of 5 parts of *inoculum* to 1 part of acid. The mixture was covered, held at room temperature for 30 minutes and centrifuged again at 3,500 × *g* for 10 minutes. A 1 mL aliquot of supernatant was pipetted into a GC auto-sampler vial containing 100 µL of internal standard solution (2

mL of 2-ethylbutyric acid in 15 mL of 90% ethanol brought to a final volume of 500 mL with deionized water), sealed and placed in an auto-sampler tray. Volatile fatty acid concentrations were determined using a Varian 3800 gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA) using a Nukol fused silica capillary column (30 m length; 0.25 mm diameter; 0.25 µm film thickness; Supelco, Bellefonte, PA, USA). Initial column temperature was 80°C for 2 minutes, increased at a rate of 5°C/min to 100°C maintained for 1 minute, increased at a rate of 7°C/min to 140°C and finally increased at a rate of 15°C/min to a final temperature of 200°C, with a total running time of 21 minutes. Injector and FID temperatures were 220°C and 250°C respectively, with a carrier gas (He) flow of 4 mL/min and a split ratio of 50:1. VFA were identified and quantified from chromatograph peaks areas using calibration with internal standard, according to a modified procedure introduced by Moore et al. (2002).

Statistical analysis

Statistical analysis was carried out using one-way variance analysis procedure of IBM SPSS Statistics 18 (IBM Corporation, Armonk, NY, USA). Data deriving from biological analyses were analysed considering the type of TMR (dry vs lactating) as factor. The Pearson correlation between the starch:ADF ratio of the all 30 diets and the GP and the CH_4 production was first studied. Then the entire population (30 TMRs) was divided into quartiles considering starch:ADF ratio as parameter to take into account the effects of both rapidly and slowly fermentable carbohydrates. Data were analysed considering the quartile as main factor (fixed effect), the period (incubation run) as random effect, and their interaction. Differences among means with $P<0.05$ were declared significant. Subsequent post-hoc multiple comparisons were performed using Bonferroni test (equal variances assumed) or Dunnett (T3) test in case of heteroskedasticity of variances.

2.4 RESULTS AND DISCUSSION

Diet composition and chemical analysis

The feeds used to formulate dry cows TMRs were primarily meadow hay, corn silage, wheat straw and protein concentrate (39±14.8, 32±2.32, 26±12.5 and 14±9.68% of total DM, respectively). For lactating TMRs there was a wide range in terms of feeds used and levels of inclusion. Corn silage (29±7.60% of total DM), corn (both ground and high moisture, 15±5.25 and 14±4.74% of total DM, respectively), protein concentrate (20±8.63% of total DM) and soybean meal (12±5.99% of total DM) were the main ingredients included in the majority of the TMRs fed to lactating cows. The average chemical analysis of the TMRs fed to dry and lactating cows is reported in table 2.1. The

CP content was 11.5±3.07 and 15.6±1.73% DM in dry and lactating TMRs, respectively and it seems to be fairly low as required by the need to meet the European Union Nitrate Directive constraints (European Commission, 1991). The average NDF content was 49.2±5.27 in dry TMRs, whereas in lactating TMRs ranged from 25.5 and 41.3, with a mean value of 32.1±4.17% DM basis. Starch content of lactating TMRs (23.5±4.41% on DM) is slightly lower than the level normally registered (28% on DM) in the Po plain area for high producing cows (Crovetto and Colombini, 2010), but non-fibrous carbohydrate (NFC) content (42.4±2.93% on DM) is consistent with that found in previous experiments (Getachew et al., 2005b; Masoero et al., 2006).

Table 2.1: Chemical composition of total mixed rations fed to dry and lactating cows.

		DRY				LACTATING			
		Mean	SD	Min	Max	Mean	SD	Min	Max
Dry Matter	%	62.4	10.9	47.8	77.5	49.9	5.01	35.3	56.4
Ash	% DM	9.19	1.12	7.70	11.5	7.00	0.69	6.13	8.32
Crude protein	% DM	11.5	3.07	8.12	15.7	15.6	1.73	11.8	18.6
Ether extract	% DM	2.39	0.50	1.89	3.32	3.76	0.83	2.75	5.49
NDF	% DM	49.2	5.27	45.9	61.8	32.1	4.17	25.5	41.3
NDFIP[1]	% DM	2.63	0.68	1.75	3.73	2.01	0.73	1.21	4.60
	% CP	23.6	6.46	14.7	33.4	13.0	4.91	7.21	30.4
ADF	% DM	31.7	3.82	27.6	40.2	21.1	2.83	15.0	26.5
ADL	% DM	4.83	0.75	3.68	5.88	3.57	0.76	2.40	5.89
Starch	% DM	12.5	4.29	5.89	19.4	23.5	4.41	16.1	33.4
NFC	% DM	28.4	4.76	18.4	35.3	42.4	2.93	35.6	47.4
Starch:NDF	%	26.0	9.88	9.53	42.3	75.4	21.6	38.9	128
Starch:ADF	%	40.4	15.2	14.7	66.0	115	35.7	60.5	223

[1]: Protein bound to NDF fraction

The diets for both groups appear well balanced to meet the nutritional requirements of the animals in the first phase of the dry period and adequate for lactating cows producing 30 kg milk daily, on average (Fox et al., 2004).

Nutritive value and methane production of the total mixed rations for dry and lactating cows

The different composition and analysis of the two groups of TMRs (dry and lactating) significantly influence GP, OMD and consequently the energetic value (Table 2.2). There were differences between dry and lactating TMRs in total GP at 24h of incubation ($P<0.001$) and in CH_4 production, expressed as mL/200 mg DM ($P\leq0.001$).

Table 2.2: Total gas and methane production, organic matter and neutral detergent fibre digestibility, and volatile fatty acids production of total mixed rations fed to dry and lactating cows.

		DRY	LACTATING	SE	P
GP[1]	mL/200 mg DM	43.0	54.4	1.11	<0.001
Methane[2]	mL/200 mg DM	7.24	8.85	0.19	0.001
	% total GP	24.3	23.7	0.22	0.286
OMD[3]	%	64.3	74.8	1.03	<0.001
NE_L[4]	Mcal/kg DM	1.25	1.62	0.04	<0.001
NDFD	%	52.1	52.1	1.04	0.995
dNDF	% DM	25.6	16.8	0.95	<0.001
VFA	mmol/L	49.2	47.7	0.99	0.516
Acetate	mmol/L	34.9	33.2	0.77	0.340
	% VFA	70.6	69.3	0.26	0.025
Propionate	mmol/L	10.4	10.3	0.21	0.750
	% VFA	21.3	21.6	0.20	0.448
Butyrate	mmol/L	3.89	4.25	0.09	0.066
	% VFA	8.14	9.08	0.17	0.011
Acetate:Propionate		3.34	3.24	0.04	0.314

[1]: Gas Production at 24 h of incubation corrected for blank and standard feeds gas production.
[2]: Cumulated methane production at 24 h of incubation. The percentage on total GP is referred to the raw GP, not corrected for standard feeds gas production.
[3]: Organic matter digestibility calculated according to Menke and Steingass GP technique (1988).
[4]: Energetic value of TMRs estimated using equation 17f for compounds and roughage feeds, Menke and Steingass (1988).

The higher CH_4 emission of TMRs for lactation is explained by the higher starch and NFC contents in comparison with the TMRs for dry animals. Higher CH_4 production is normally associated with fibre fermentation, however, for highly digestible feeds, such as TMRs for lactation, a higher quantity of CH_4 is produced in early hours of fermentation. In the present experiment GP and CH_4 productions were registered at 24 h, a time sufficient for a complete fermentation of the readily fermentable carbohydrates, but not for the fibrous fractions. These results are consistent with those reported by Getachew et al. (2005a) who found a positive correlation between CH_4 production and organic matter, NFC, dNDF, and DM digestibility in the first 24 h of *in vitro* incubation. Similarly Lee et al. (2003) registered a comparative higher CH_4 production for grains among other feed ingredients rich in fibre or protein or oil, that might be attributed to the high content of easily fermentable sugars, starch and pectins of grains. Similar results were obtained by Navarro-Villa et al. (2011) who found an increase in CH_4 output with feeds rich in rapidly fermentable carbohydrates.

Unexpectedly, no difference was observed in the percentage of CH_4 of total GP (24.3±1.32 and 23.7±1.18 for dry and lactation TMRs, respectively; P=0.286). Consistently with the present results, a study of Getachew et al. (2005b) did not show

any difference in CH_4 percentage between 7 diets for lactation in the first 24 hours of *in vitro* incubation, although significant differences among diets and an overall increase in CH_4 proportion were registered at 48 and 72 h. No differences were detected among groups for NDFD (P=0.995) (Table 2.2), however, due to the higher NDF content in dry cows TMRs, digestible NDF (dNDF, % on DM) in this group resulted significantly higher (P<0.001). This resulted in a significant higher acetic acid concentration, expressed as percentage of total VFA produced, for dry cows TMRs (P=0.025) although the difference between the two groups was numerically quite small. On the contrary, butyric acid concentration, as percentage of total VFA, resulted significantly higher for lactating cows TMRs. No significant difference between groups was observed for the acetate:propionate ratio (P=0.314) (Table 2.2).

Methane production and dietary starch:ADF ratio

The period (random effect) and the interaction period x starch:ADF ratio were never significant and hence removed from the statistical model. Considering the entire population of TMR tested, divided into quartiles using the starch:ADF ratio as parameter, significant differences among groups in total GP (P<0.001) and in CH_4 production, expressed as mL/200 mg DM (P<0.001) were observed (Table 2.3). The volume of CH_4 produced increased significantly for increasing starch:ADF ratio, confirming the positive effect of starch on methanogenesis within the first 24 h of *in vitro* incubation. This is confirmed by the highly significant correlation between the starch:ADF ratio and the GP (r=0.87; P<0.001) and the volume of CH_4 produced (r=0.66; P<0.001). Similarly, Singh et al.(2011), in an *in vitro* study, showed that dry roughages with a higher content of non-structural and soluble carbohydrates produced more CH_4 than roughages with higher levels of fibre. Overall, the average volume of CH_4 produced after 24 h of incubation was consistent with the values reported by Lee et al.(2003) for cereal grains (6.9 to 11.6 mL/200 mg DM).

Table 2.3: Total gas and methane production, organic matter and neutral detergent fibre digestibility, and volatile fatty acids production of total mixed rations divided into quartiles using starch:ADF as parameter.

		group 1	group 2	group 3	group 4	SE	P
	Starch:ADF, %	37	77	116	138		
GP[1]	mL/200 mg DM	42.2 [c]	51.4 [b]	55.1 [ab]	56.2 [a]	1.51	<0.001
Methane[2]	mL/200 mg DM	7.12 [b]	8.54 [a]	9.04 [a]	8.87 [a]	0.19	<0.001
	% total GP	24.2	24.2	23.8	23.2	0.22	0.382
OMD[3]	%	63.9 [c]	71.5 [b]	75.6 [a]	76.5 [a]	1.51	<0.001
NE_L[4]	Mcal/kg DM	1.23 [c]	1.51 [b]	1.63 [a]	1.69 [a]	0.06	<0.001
NDFD	%	52.1	51.5	52.7	52.0	3.21	0.985
dNDF	% DM	25.9 [a]	19.1 [b]	16.5 [b]	15.3 [b]	1.82	<0.001
VFA	mmol/L	49.4	48.6	47.6	46.7	3.00	0.820
Acetate	mmol/L	35.1	34.2	33.1	32.2	2.29	0.596
	% VFA	70.7 [a]	70.2 [ab]	69.3 [ab]	68.5 [b]	0.64	0.009
Propionate	mmol/L	10.4	10.2	10.2	10.3	0.64	0.976
	% VFA	21.2	21.1	21.5	22.3	0.55	0.136
Butyrate	mmol/L	3.89	4.17	4.31	4.21	0.25	0.382
	% VFA	8.11	8.75	9.19	9.23	0.45	0.069
Acetate:Propionate		3.35	3.36	3.26	3.09	0.12	0.082

[a,b,c]: means in a row sharing different superscript letters differ significantly (P<0.05).

[1]: Gas Production at 24 h of incubation corrected for blank and standard feeds gas production.

[2]: Cumulated methane production at 24 h of incubation.

[3]: Organic matter digestibility calculated according to Menke and Steingass Gas Production Technique (1988).

[4]: Energetic value of TMR estimated using equation 17f for compounds and roughage feeds, Menke and Steingass (1988).

As previously observed, no differences were detected for CH_4 as percentage of total GP. This is confirmed by the low correlation coefficient (r=-0.28; P=0.138) between the starch:ADF ratio and the percentage of CH_4. The lack of difference can be partially due to the short incubation time. Furthermore *in vitro* fermentation is essentially a type of enrichment culture, in which the particular set of environmental conditions used (pH, substrate type and concentration, presence of growth stimulants or inhibitors, etc.) are likely to set the stage for preferential rate or extent of growth of some microbial species over other species present in the original *inoculum* (Madigan et al., 2000). The strongly buffered *in vitro* system also did not determine the low pH that is associated with inhibition of fibrolytic bacteria (Argyle and Baldwin, 1988) and methanogens (Van Kessel and Russell, 1996). Russel (1998) showed that CH_4 production *in vivo* decreased dramatically at pH below 6.3. In our experiment, as in most of *in vitro* studies, the use of a buffer solution avoided such a drop in pH which was maintained in the range of 6.5-7.0, optimal for the fermentation of the cellulolytic microbes (McGeough et al., 2011). In the present experiment all diets in every incubation run were treated with the same

inoculum, therefore the comparison between treatments is valid. However, a possible interaction between *inoculum* characteristics and the diet fed to the donor animals can be expected. Demeyer and Fievez (2000) reported that a greater proportion of cellulolytic bacteria and methanogenic *Archea* is expected in rumen fluid obtained from ruminants fed high forage diets. Consequently, the *in vitro* fermentation pattern can be indirectly influenced by the diet of the donor animals. Martinez et al.(2010), in a study on 24 h *in vitro* methanogenesis, found that the use of an *inoculum* obtained from animals fed diets with a 30:70 forage:concentrate ratio on DM determined a higher CH_4 production in comparison with an *inoculum* obtained from animals fed diets with a 70:30 forage:concentrate ratio. The NDFD did not differ among groups but the proportion of dNDF significantly decreased ($P<0.001$) as the starch:ADF ratio increased, due to a subsequent lower NDF content. As a consequence acetic acid as percentage of total VFA slightly decreased from 70.7±1.67 (group 1, lowest starch:ADF ratio) to 68.5±0.82% (group 4, highest starch:ADF ratio) ($P=0.009$), whilst butyric acid percentage numerically increased with increasing starch:ADF ratio. The proportion of propionic acid did not differ among groups; consequently only a tendency ($P=0.082$) in acetate:propionate ratio decrease was registered as starch:ADF ratio increased (Table 2.3).

2.5 CONCLUSIONS

In the present experiment methane production of different TMRs resulted positively related to the starch and the NE_L concentrations. Unexpectedly, CH_4 production as a percentage of total GP was not influenced by diet characteristics. This might be attributed to the short incubation time, to the characteristics of the *inoculum* and to the methodology applied, which hampered an effective simulation of rumen fermentation. The long-term *in vivo* technique (e.g. with respiration chambers) is the most accurate in determining the CH_4 production, but it is very cumbersome, expensive and time consuming. On the contrary, the *in vitro* technique, despite some limitations, permits a precise prediction of the fermentation pattern.

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2.7 REFERENCES

- AOAC, 1995. Official Methods of Analysis of the Association of Official Analytical Chemists. 15th ed., Washington, DC, USA.

- AOAC, 1998. Official Methods of Analysis, 16th ed., Association of Official Analytical Chemists, Washington, DC, USA.
- Argyle, J.L., Baldwin, R.L., 1988. Modeling of rumen water kinetics and effects of rumen pH changes. J. Dairy Sci. 71:1178-1188.
- Capper, J.L., Cady, R.A., Bauman D.E., 2009. Demystifying the environmental sustain-ability of food production. pp 174-190 in Proc. Int. Cornell Nutrition Conf., East Syracuse, NY, USA.
- Crovetto, G.M., Colombini, S., 2010. Feeding and nitrogen excretion in dairy cattle. In: G.M. Crovetto and A. Sandrucci (eds.) Allevamento animale e riflessi ambientali. Fondazione Iniziative Zooprofilattiche e Zootecniche, Brescia, Italy, pp 27-54.
- Demeyer, D., Fievez, V., 2000. Ruminants et environment: la méthanogénèse (Ruminants and environment: methanogenesis). Ann. Zootech. 49:95-112.
- Ellis, J.L., Kebreab, E., Odongo, N.E., McBride, B.W., Okine, E.K., France, J., 2007. Prediction of methane production from dairy and beef cattle. J. Dairy Sci. 90:3456-3467.
- European Commission, 1991. Directive of the Council of December 12 concerning the protection of waters against pollution caused by nitrates from agricultural sources, 91/676/EEC. In: Official Journal L 375, 31/12/1991, pp 1-8.
- Fox, D.G., Tedeschi, L.O., Tylutki, T.P., Russell, J.B., Van Amburgh, M.E., Chase, L.E., Pell, A.N., Overton, T.R., 2004. The Cornell net carbohydrate and protein system model for evaluating herd nutrition and nutrient excretion. Anim. Feed Sci. Tech. 112:29-78.
- Getachew, G., DePeters, E.J., Robinson, P.H., Fadel, J.G., 2005a. Use of an in vitro rumen gas production technique to evaluate microbial fermentation of ruminant feeds and its impact on fermentation products. Anim. Feed Sci. Tech. 123:547-559.
- Getachew, G., Robinson, P.H., DePeters, E.J., Taylor, S.J., Gisi, D.D., Higginbotham, G.E., Riordan, T.J., 2005b. Methane production from commercial dairy rations estimated using an in vitro gas technique. Anim. Feed Sci. Tech. 123:391-402.
- Grainger, G., Beauchemin, K.A., 2011. Can enteric methane emissions from ruminants be lowered without lowering their production? Anim. Feed Sci. Tech. 166-167:308-320.
- Hariadi, B.T., Santoso, B., 2010. Evaluation of tropical plants containing tannin on in vitro methanogenesis and fermentation parameters using rumen fluid. J. Sci. Food Agric. 90:456-461.

- Intergovernmental Panel on Climate Change, 2007. Climate Change 2007: Mitigation of climate change. Contribution of working group III to the fourth assessment report of the Intergovernmental Panel on Climate Change (IPCC). Cambridge Univ. Press, Cambridge, UK.
- Italian Regulation, 1992. Application of the Council Directive (EEC) No. 86/609 regarding the protection of animals used for experimental and other scientific purposes. LD 116/1992. In: Official Journal No. 294, 18/2/1992, pp 5-24.
- Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. J. Anim. Sci. 73:2483-2492.
- Lee, H.J., Lee, S.C., Kim, J.D., Oh, Y.G., Kim, K., Kim, C.W., Kim, K.J., 2003. Methane production potential of feed ingredients as measured by in vitro gas test. Asian-Aust. J. Anim. Sci. 16:1143-1150.
- Licitra, G., Hernandez, T.M., Van Soest, P.J., 1996. Standardization of procedures for nitrogen fractionation of ruminant feeds. Anim. Feed Sci. Tech. 57:347-358.
- Madigan, M.T., Martinko, J.M., Parker, J., 2000. Brock biology of microorganisms. Prentice Hall Publ., Upper Saddle River, NJ, USA.
- Martinez, M.E., Ranilla, M.J., Tejido, M.L., Saro, C., Carro, M.D., 2010. The effect of the diet fed to donor sheep on in vitro methane production and ruminal fermentation of diets of variable composition. Anim. Feed Sci. Tech. 158:126-135.
- Masoero, F., Moschini, M., Fusconi, G., Piva, G., 2006. Raw, extruded and expanded pea (Pisum sativum) in dairy cows diets. Ital. J. Anim. Sci. 5:237-247.
- McGeough, E.J., O'Kiely, P., O'Brien, M., Kenny, D.A., 2011. An evaluation of the methane output associated with high-moisture grains and silages using the in vitro total gas production technique. Anim. Prod. Sci. 51:627-634.
- Menke, K.H., Steingass, H., 1988. Estimation of the energetic feed value obtained from chemical analysis and in vitro gas production using rumen fluid. Anim. Res. Dev. 28:7-55.
- Mertens, D.R., 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds using refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85:1217-1240.
- Moore, J.A., Poore, M.H., Luginbuhl, J.M., 2002. By-product feeds for meat goats: Effects on digestibility, ruminal environment, and carcass characteristics. J. Anim. Sci. 80:1752-1758.
- Moss, A.R., Jouany, J.P., Newbold, J., 2000. Methane production by ruminants: its contribution to global warming. Ann. Zootech. 49:231-253.

- Navarro-Villa, A., O'Brien, M., Lopez, S., Boland, T.M., O'Kiely, P., 2011. Modifications of a gas production technique for assessing in vitro rumen methane production from feedstuffs. Anim. Feed Sci. Tech. 166-167:163-174.
- Odongo, N.E., Bagg, R., Vessie, G., Dick, P., Or-Rashid, M.M., Hook, S.E., Gray, J.T., Kebreab, E., France, J., McBride, B.W., 2007. Long-term effects of feeding monensin on methane production in lactating dairy cows. J. Dairy Sci. 90:1781-1788.
- Pulina, G., Dias Francesconi, A.H., Mele, M., Ronchi, B., Stefanon, B., Sturaro, E., Trevisi, E., 2011. Sfamare un mondo di nove miliardi di persone: le sfide per una zootecnia sostenibile. Ital J. Agron. 6(Suppl.2):e7.
- Reynolds, C.K., Mills, J.A.N., Crompton, L.A., Givens, D.I., Bannink, A., 2010. Ruminant nutrition regimes reduce greenhouse gas emissions in dairy cows. In: G.M. Crovetto (ed.) Energy and protein metabolism and nutrition. Wageningen Academic Publ., Wageningen, The Netherlands, pp 427-437.
- Russell, J.B., 1998. The importance of pH in the regulation of ruminal acetate to propionate ratio and methane production in vitro. J. Dairy Sci. 81:3222-3230.
- Singh, S., Kushwaha, B.P., Nag, S.K., Mishra, A.K., Bhattacharya, S., Gupta, P.K., Singh, A., 2011. In vitro methane emission from Indian dry roughages in relation to chemical composition. Curr. Sci. India 101:57-65.
- Spanghero, M., Berzaghi, P., Fortina, R., Masoero, F., Rapetti, L., Zanfi, C., Tassone, S., Gallo, A., Colombini, S., Ferlito, J.C., 2010. Technical note: Precision and accuracy of in vitro digestion of neutral deter-gent fiber and predicted net energy of lactation content of fibrous feeds. J. Dairy Sci. 93:4855-4859.
- Steinfeld, H., Gerber, P., Wassenaar, T., Castle, V., Rosales, M., de Haan, C. 2006. Livestock's long shadow: environmental issues and options. FAO Publ., Roma, Italy.
- Tamburini, A., Colombini, S., Penati, C., Zucali, M., Roveda, P., Rapetti, L., Crovetto, G.M., 2010. Methane emission in livestock and diet characteristics. In: G.M. Crovetto (ed.) Energy and protein metabolism and nutrition. Wageningen Academic Publ., Wageningen, The Netherlands, pp 465-466.
- Tavendale, M.H., Meagher, L.P., Pacheco, D., Walker, N., Attwood, G.T., Sivakumaran, S., 2005. Methane production from in vitro rumen incubations with Lotus pedunculatus and Medicago sativa, and effects of extractable condensed tannin fractions on methanogenesis. Anim. Feed Sci. Tech. 123-124:403-419.

- Theodorou, M.K., Williams, B.A., Dhanoa, M.S., McAllan, A.B., France, J., 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Tech. 48:185-197.
- Van Kessel, J.A.S., Russell, J.B., 1996. The effect of pH on ruminal methanogenesis. FEMS Microbiol. Lett. 20:205-210.
- Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods of dietary fiber, neutral detergent fiber and non-polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597.

3 EFFECTS OF A SELECTION OF ADDITIVES ON *IN VITRO* RUMINAL METHANOGENESIS AND *IN SITU* AND *IN VIVO* NDF DIGESTIBILITY

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3.1 ABSTRACT

The effects of 18 essential oils, yeast, *Quebracho* tannin and *Quillaja* saponin on ruminal methane and gas production (GP) were studied *in vitro*.

A lactating cow diet was incubated with rumen *inoculum*. Doses of the additives (mg/L) were: essential oils=500; tannin=67; yeast=8.35 and 16.7; saponin=300.

Lemongrass, estragole, eugenol, geraniol, limonen, thyme oil and thymol produced less gas (overall mean 33.8 mL/200 mg DM) than control (43.6 mL/200 mg DM; P<0.001). Methane produced (mL/200 mg DM) by guaiacol (10.7), lemongrass (9.6), limonene (11.4), thyme oil (10.9) and thymol (2.1) was lower than control (12.5) (P<0.001). Methane percentage on total GP was lower (P<0.001) for cinnamic alcohol (25.4), guaiacol (24.5), thymol (19.7) and vanillin (26.3) than control (28.8).

In a second experiment, thymol, guaiacol and yeast were added to the diet of dry fistulated cows to determine *in situ* NDF digestibility (NDFD) of six forages and *in vivo* dietary NDFD. Thymol and yeast decreased *in situ* NDFD after 24 h (33.9% and 33.5% vs 38.1%; P=0.008). Thymol decreased *in vivo* total tract NDFD (40.8% vs. 51.4%; P=0.02).

Differences in GP and methane levels were registered within classes of additives. A careful selection of additives may allow for the manipulation of ruminal fermentation.

Key words**:** methane; rumen; additives; essential oils; NDF digestibility

3.2 INTRODUCTION

In recent years, concern has risen over the accumulation of greenhouse gases (GHG) in the atmosphere and the resultant increase in average global temperatures. Enteric methane (CH_4) is the most important GHG emitted at the level of the farm (50-60%) in ruminant production systems (Ogino et al., 2007), and enteric CH_4 contributes to approximately 30-40% of the total CH_4 produced by agricultural sources (Moss *et* al., 2000). Methane emitted from the rumen represents 2-12% of the gross energy lost from the host animal (Johnson and Johnson, 1995); the amount of methane emitted depends on dietary factors such as feed intake (Reynolds et al., 2010; Shibata and Terada, 2010; Tamburini et al., 2010), supplementation with lipids (Grainger and Beauchemin, 2011) and the type and level of carbohydrates fermented in the rumen (Ellis et al., 2007; Shibata and Terada, 2010). Given the ecological significance of CH_4 and the negative correlation between methane emission and energy utilization in ruminants, considerable efforts have been undertaken to develop strategies to reduce CH_4 emissions from domestic ruminants and to re-channel hydrogen to increase the production of ruminal volatile fatty acids (VFA) and microbial biomass (Lila et al., 2003), thus increasing the efficiency of dietary energy utilization in the animal. Several reviews on enteric CH_4 production and strategies to mitigate the emission of CH_4 have recently been published (e.g., Beauchemin et al., 2008; McAllister and Newbold, 2008; Eckard et

al., 2010; Martin et al., 2010; Grainger and Beauchemin, 2011). Nutritional strategies aimed at reducing CH_4 production include the use of additives such as essential oils (Evans and Martin, 2000), saponins (Santoso et al., 2004), tannins (Carulla et al., 2005; Grainger et al., 2009), organic acids (McAllister and Newbold, 2008) and probiotics such as live yeast (Lynch and Martin, 2002; Newbold and Rode, 2006). The inclusion of essential oils, tannins and saponins in ruminant diets has received wide interest, as these additives decrease rumen protozoal counts (Wallace et al., 1994; Hart et al., 2008) and methanogenesis (Hess et al., 2003, 2004; Santoso et al., 2004). These beneficial effects, however, could be offset by a decrease in total VFA production and feed digestibility (Busquet et al., 2006; Martinez et al., 2006). Several studies (most of which were conducted *in vitro*) have been published on the effects of essential oils on rumen microbial fermentation, with a focus on nitrogen (N) metabolism and the production of VFAs (e.g., Benchaar et al., 2007; Busquet et al., 2006; Castillejos et al., 2006). However, the ability of essential oils to selectively inhibit ruminal methanogenesis has only recently been evaluated (Benchaar and Greathead, 2011). For example, a number of studies have investigated the effects of thymol on ruminal fermentation (e.g., Castillejos et al., 2006; Martinez et al., 2006); however, only a few studies have specifically evaluated the effects of thymol on ruminal methanogenesis (Benchaar and Greathead, 2011). Castillejos et al. (2006) found that 500 mg guaiacol/L of rumen *inoculum* reduced the proportion of produced acetate without reducing total VFA concentrations. To the best of our knowledge, no information is available on the effect of guaiacol on CH_4 production. It is often unclear which chemical moieties confer antimicrobial activity on a secondary molecule; however, the presence of oxygen, a hydroxyl group and a phenolic ring appear to be important (Ultee et al., 2002; Burt, 2004; Benchaar and Greathead, 2011). For this reason, there is great interest in studying products with the described chemical structure (such as guaiacol, thymol, etc.) to verify their potential effect on the production of CH_4.

The aims of the present study were to 1) evaluate the effects of certain additives (essential oils, saponins, live yeast and tannin) on total gas and CH_4 production *in vitro* and 2) evaluate the effects of selected additives (thymol, guaiacol and live yeast) fed to rumen-fistulated dry cows on fiber digestibility *in vivo* and *in situ*.

3.3 MATERIALS AND METHODS

Experiment 1. *In vitro* trial

Experimental design

Sixteen essential oils (cinnamic alcohol, anethole, anisole, carvone, lemongrass, dihydrocarvone, estragole, eugenol, geraniol, guaiacol, humulene, lavender, limonen, thyme oil, thymol and vanillin), two commercial blends of essential oils (Crina® Ruminants, DSM Nutritional Products Ltd., Basel, Switzerland, and Agolin® Ruminant Agolin SA, Bière, Switzerland), a live yeast strain (Levucell® SC, Lallemand Animal Nutrition SA, Blagnac, France), a commercial hydrolyzable *Quebracho* tannin (Silvafeed By PROQ®, Silvateam S.p.a., San Michele Mondovì, Italy) and *Quillaja saponaria* saponin were tested *in vitro* for their effects on total gas and CH_4 production. The pure essential oils and the commercial blends of essential oils were dissolved in ethanol before being added to the *inoculum* solution, whereas the other products were suspended directly in the *inoculum* solution. The additives were added at the following concentrations: essential oils - 500 mg/L *inoculum* (for guaiacol, a lower dose (250 mg/L) was also tested); commercial blends of essential oils - 16.7 mg/L of *inoculum* (corresponding to an approximate *in vivo* dose of 1 g/head/d according to the manufacturer's instructions); live yeast - 8.35 and 16.7 mg/L of *inoculum* (corresponding to an approximate *in vivo* dose of 0.5 g or 1 g/head/d); tannin - 67 mg/L of *inoculum* and saponin - 300 mg/L of *inoculum*. The additives were tested in duplicate in three incubation runs, and a total mixed ration (TMR) for lactating dairy cows was used as a substrate. Each additive was tested against a negative control (TMR + *inoculum* + ethanol for essential oils; TMR + *inoculum* for the other additives). For each additive, a corresponding blank (*inoculum* + ethanol + additive for essential oils; *inoculum* + additive for the other additives) was incubated. The diet (93.4% OM, 14.4% CP, 4.1% EE, 35.6% NDF, 19.5% ADF, 26.2% starch and 40.3% NFC on a DM basis) was constituted (% of DM) of corn silage (33.7), a commercial protein supplement (29.0), corn meal (17.7), alfalfa hay (8.6), Italian ryegrass silage (5.3), alfalfa silage (5.1) and corn gluten meal (0.6). The sample was dried in a forced air oven (55°C for 48 h) and ground to pass through a 1 mm sieve using a Wiley mill (Pulverisette 19, Fritsch, Idar-Oberstein, Germany).

<u>*In vitro* gas production technique and methane analysis</u>

Ruminal fluid was collected from two fistulated dry Italian Friesian dairy cows fed a diet composed of meadow hay, flaked corn and a commercial protein supplement (forage:concentrate ratio of 65:35 on a DM basis). Throughout the experimental period, the animals were handled as outlined by the guidelines of the 116/92 Italian law on animal welfare for experimental animals (Italian Regulation, 1992) and the guidelines of the University of Milan Ethics Committee for animal use and care; the experimental

procedure conformed to the provisions for animal welfare of the Declaration of Helsinki revised in Seoul (2008).

Gas production (GP) was measured using a semi-automatic system (Theodorou *et al.,* 1994) based on the measurement of the headspace gas pressure in the incubation bottles as reported by Pirondini et al. (2012). The headspace pressure was recorded using a digital manometer (model 840082, Sper Scientific, Scottsdale, AZ, USA) after 2, 4, 6, 8, 10 and 24 h of incubation. A fixed-volume sample of gas (5 mL/bottle) was also collected using gas-tight syringes for the subsequent CH_4 analysis. The gas composition of the headspace was determined by injecting 5 mL of gas into an Agilent 3000A micro gas chromatograph (Agilent Technologies, Santa Clara, CA, USA) using N as a carrier. The volume (mL) of CH_4 produced between two time points and the final cumulative volume were calculated following the methods of Tavendale *et al.* (2005).

Analysis of diet and rumen samples

The dry matter and ash contents were determined following the procedures (methods 945.15 and 942.05, respectively) of the Association of Official Analytical Chemists (AOAC, 1995). The CP content was determined with the macro-Kjeldahl technique (method 984.13; AOAC, 1995). The ether extract content was determined following method 920.29 of the AOAC (1995). The neutral detergent fiber content was determined according to the method described by Mertens (2002), with the addition of sodium sulfite and α-amylase to the neutral detergent solution. The acid detergent fiber (ADF) and acid detergent lignin (ADL) contents were calculated using the Ankom 200 fiber apparatus (ANKOM Technology Corporation, Macedon, NY, USA) according to the methods described by Van Soest *et al.* (1991). The fiber fractions were reported on an ash-free basis. The starch content was determined using the Megazyme Kit K-TSTA (Megazyme International Ireland Ltd., Wicklow, Ireland) for the total starch assay procedure according to method 996.11 of the AOAC (1998).

At the end of the incubation period (24 h), 10 mL of the *inoculum* was collected from each serum bottle and clarified by centrifugation at 3,500 × *g* for 10 minutes. The supernatants were analyzed for VFA levels with a Varian 3800 gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA) equipped with a flame ionization detector (FID) according to the method described by Moore *et al.* (2002) and a Nukol fused silica capillary column (30 m length; 0.25 mm diameter; 0.25 µm film thickness; Supelco, Bellefonte, PA, USA) as reported by Pirondini *et al*. (2012).

Experiment 2. *In vivo* and *in situ* trials

The three additives used in this experiment were chosen based on the results of the *in vitro* analysis to have one additive with a strong antimicrobial effect (thymol), one

additive with a selective anti-methanogenic effect (guaiacol) and one additive with a supposed positive effect on rumen microbial growth, which, in turn, could improve the performance of the animal (live yeast).

Animals, experimental design and diets

Four dry Italian Friesian cows fitted with ruminal *cannulae* (average body weight of 750±21 kg) were used in a 4 x 4 Latin square experimental design over four 25 d periods. The animals were housed in individual tie stalls with free access to water throughout the experiment; feces were collected daily. The cows were fed a balanced TMR twice daily (7:30 AM and 6:30 PM) to achieve a dry matter intake (DMI) of 10.6 kg/d. The diet (% DM) was composed of grass hay (39.2), corn silage (32.4), wheat bran (9.1), a commercial protein supplement (8.8), ground and flaked corn (6.8), a commercial mineral/vitamin premix (2.0) (Ca 4.6, P 3.7, Mg 4.5, and Na 3.3% DM; Cu 680, Zn 2550, I 8.7 and Se 21.7 ppm; Vit. A 1630 and Vit. D 174 KIU/kg DM; and Vit. E 8150 mg/kg DM) and cane molasses (1.7) without (Control) or with supplemental guaiacol (5 g/d; Sigma Aldrich, St. Louis, MO, USA), thymol (5 g/d; Sigma Aldrich, St. Louis, MO, USA) or *Saccharomyces cerevisiae* strain CNCM 1077 (5 g/d, which is equivalent to $10*10^{10}$ CFU/d; Levucell SC®, Lallemand Animal Nutrition SA, Blagnac, France). The additives were dissolved in 5 mL of ethanol, incorporated into 100 g of ground corn and administered to the cows once daily directly through the ruminal fistula. The control treatment was supplemented with ethanol (5 mL/d). The chemical composition of the experimental diet was as follows: 91.8% OM, 13.4% CP, 2.9% EE, 45.3% NDF, 29.2% ADF, and 17.7% starch on a DM basis. Orts were collected daily at 7:00 AM and weighed. For each 25 d period, the cows were allowed to adapt to the experimental treatments from days 1 to 14; from days 15 to 25, the *in situ* digestibility was determined and the data and samples were collected.

Apparent *in vivo* (total tract) digestibility and *in situ* ruminal NDF degradation

The dietary DMI was recorded daily. The TMR and ort samples were collected daily and frozen. The TMR and ort samples were composited by period, oven-dried (55°C) and ground through a 1 mm mill sieve (Pulverisette 19, Fritsch, Idar-Oberstein, Germany). For 10 consecutive days, the individual feces were weighed, sampled (5% on a weight basis), stored at -20°C, subsequently thawed, dried at 55°C for 48 h and ground through a 1 mm screen for chemical analysis. The *in situ* ruminal digestibility of the neutral detergent fiber (NDFD) of six feeds was determined using the same four cows following the procedures recommended by the NRC (2001) for a standardized *in situ* procedure. The six feeds tested were alfalfa hay, alfalfa silage, Italian ryegrass hay, Italian ryegrass silage and two corn silages. The samples were dried at 55°C in a forced air oven for 48 h

and ground using a Wiley mill (Pulverisette, Fritsch, Idar-Oberstein, Germany) to pass through a 2 mm sieve for the *in situ* analysis. The samples were weighed (3.50 g as fed) in nylon bags (53 µm pore size; 10 cm x 20 cm net surface area) that were placed in the rumen for three different periods of time (24, 48 and 240 h). No corrections were made for physical losses at time 0.

Chemical analysis and rumen samples

The chemical analyses of the experimental forages, TMR, orts and feces were performed as previously reported.

During each experimental period, the ruminal fluid was collected once from each cow 0, 3 and 7 h after the morning feeding. The pH of the ruminal fluid was measured immediately using a pH-meter (Eutech Instruments, Nijkerk, The Netherlands). The concentrations of NH_3-N in the ruminal fluid samples were analyzed with the macro-Kjeldahl technique using a 2300 Kjeltec Analyzer Unit (FOSS, Hillerød, Denmark).

Protozoal counts were performed on the ruminal fluid samples collected 3 h after the morning feeding. The ruminal fluid samples and solid *digesta* were collected individually from each cow and blended under CO_2 flux for three minutes. The samples were strained through four layers of cheesecloth, and a 5 mL portion of the ruminal fluid was preserved in 5 mL of 10% formaldehyde solution. The protozoa were enumerated microscopically in a Sedgewick Rafter counting cell (Cole-Parmer, Vernon Hills, IL, USA) as described by Dehority (1984). The enumerations were performed by counting 25 fields in duplicate.

Statistical analysis

Statistical analyses were carried out using the mixed procedure of the Statistical Analysis Systems (SAS Institute, 2000). The model used to analyze the *in vitro* data included the additive as the main effect and the incubation run as a random effect. The model used to analyze the *in situ* data included the additive, the forage, the time of incubation and the interactions (additive*forage; additive*forage*time) as main effects and the cow as a random effect. Because the interactions were never significant, they were eliminated from the model. The model used to analyze the *in vivo* data included the additive as the main effect and the cow as a random effect.

The data are reported as least square means and the separation of least squares means was conducted at $\alpha = 0.05$.

3.4 RESULTS

Experiment 1

***In vitro* trial**

The amounts of total gas and CH_4 produced by the diets incubated with the experimental additives are provided in table 3.1 (for the additives dissolved in ethanol) and table 3.2 (for the additives not dissolved in ethanol).

The GP at 24 h was significantly affected by the additives dissolved in ethanol ($P<0.001$); the GP in the *inocula* incubated with lemongrass, estragole, eugenol, geraniol, limonen, thyme oil and thymol was lower than in the control *inoculum*. Methane production was also affected by the additives; CH_4 production in the *inocula* incubated with lemongrass, guaiacol, limonen, thyme oil, thymol and vanillin was significantly lower ($P<0.001$) than in the control *inocula*. The contribution of methane to the total GP was lower ($P<0.001$) in the *inocula* incubated with cinnamic alcohol, guaiacol, thymol and vanillin. In contrast, incubation with geraniol resulted in a significantly increased percentage of CH_4 production.

Incubation with live yeast, tannin and saponin did not affect GP or methanogenesis.

Table 3.1: Effects of the incubation with additives dissolved in ethanol on total gas production (GP) and CH_4 from the TMR after 24 h of incubation.

Additive	GP (mL/200 mg DM)	CH_4 (mL/200 mg DM)	CH_4 (% GP)
Control (ethanol)	43.6 ^{AB}	12.5 ^{AB}	28.8 ^{B}
Cinnamic alcohol	43.4 ^{ABC}	11.5 ^{BCDEF}	25.4 ^{DE}
Anethole	44.0 ^{AB}	12.8 ^{AB}	29.2 ^{BC}
Anisole	43.7 ^{AB}	12.2 ^{BCDE}	27.4 ^{BCD}
Carvone	41.6 ^{BCDE}	11.6 ^{BCDEF}	28.1 ^{BCD}
Lemongrass	34.5 ^{F}	9.57 ^{G}	26.9 ^{BCDE}
Dihydrocarvone	42.6 ^{ABCD}	12.9 ^{ABC}	29.9 ^{B}
Estragole	40.5 ^{DE}	12.2 ^{ABCDE}	29.6 ^{B}
Eugenol	39.2 ^{E}	11.5 ^{BCDEF}	29.0 ^{BC}
Geraniol	35.5 ^{F}	12.4 ^{ABCD}	34.2 ^{A}
Guaiacol	43.2 ^{AB}	10.7 ^{FG}	24.5 ^{E}
Guaiacol (half dose)	43.3 ^{ABC}	12.4 ^{ABCDE}	27.8 ^{BCD}
Humulene	42.1 ^{BCD}	11.8 ^{BCDE}	28.1 ^{BCD}
Lavender	41.4 ^{BCDE}	11.6 ^{BCDEF}	28.3 ^{BCD}
Limonen	40.9 ^{CDE}	11.4 ^{DEF}	28.2 ^{BCD}
Thyme oil	36.1 ^{F}	10.9 ^{EFG}	29.3 ^{BC}
Thymol	9.85 ^{G}	2.06 ^{H}	19.7 ^{F}
Vanillin	42.2 ^{BCD}	11.4 ^{CDEF}	26.3 ^{CDE}
Crina® Ruminants	43.0 ^{AB}	12.6 ^{ABCD}	29.1 ^{BC}
Agolin® Ruminant	45.4 ^{A}	13.4 ^{A}	28.3 ^{BC}
P-value	<0.001	<0.001	<0.001
SE	1.153	0.575	1.276

^{A,B,C,D,E,F,G,H}: Values in the same column with different superscripts differ significantly (P<0.05)

Table 3.2: Effects of the incubation with additives not dissolved in ethanol on total gas production (GP) and CH_4 from the TMR after 24 h of incubation.

Additive	GP (mL/200 mg DM)	CH_4(mL/200 mg DM)	CH_4 (% GP)
Control	40.9	10.2	24.9
Live yeast 8.35 mg/L	41.5	11.5	27.4
Live yeast 16.7 mg/L	41.8	9.86	23.0
Quebracho tannin	39.2	10.6	26.4
Quillaja saponaria saponin	41.7	10.1	24.0
P-value	0.273	0.560	0.332
SE	1.011	1.036	2.228

The effects of the additives on the fermentative profile of the ruminal *inoculum* added to the TMR after 24 h of incubation are reported in table 3.3 (for the additives dissolved in ethanol) and table 3.4 (for the additives not dissolved in ethanol). The total VFA production, the propionic and butyric acid contents (expressed as percentages of VFA) and the acetate:propioniate ratio were significantly affected by incubation with certain essential oils but not by incubation with live yeast, tannin or saponin. The percentage of produced acetic acid was never affected by incubation with any additive (P=0.315 and P=0.821 for incubation with essential oils and other additives not dissolved in ethanol, respectively). The VFA production was lower (P<0.001) in the *inocula* incubated with anethole, carvone, lemongrass, dihydrocarvone, estragole, eugenol, geraniol, guaiacol, humulene, lavender, limonen, thyme oil, thymol, and Crina Ruminants® than in the control *inoculum* (incubated with ethanol). Moreover, incubation with carvone and lavender resulted in a higher percentage of propionate (16.9 and 17.3, respectively, vs. 14.9; P<0.05) and therefore a lower acetate:propionate ratio than in the control (4.26 and 4.42, respectively, vs. 5.14; P<0.05). In contrast, incubation with geraniol and thyme oil resulted in a significantly lower percentage of propionate than was produced in the control (11.4 and 12.8, respectively, vs. 14.9; P<0.05) and a consequent increase in the acetate:propionate ratio (6.52 and 5.79, respectively, vs. 5.14; P<0.05). Incubation with lemongrass, geraniol and thyme oil resulted in a higher (P=0.025) percentage of butyrate than was observed in the control (13.3, 14.3 and 14.1, respectively, vs. 8.84). Incubation with tannin resulted in a decreased (P=0.072) production of VFAs relative to the control.

Table 3.3: Effects of the incubation with additives dissolved in ethanol on the fermentative profile of the rumen *inoculum* added to the TMR after 24 h of incubation.

Additive	VFA (mmol/L)	Acetate (% VFA)	Propionate (% VFA)	Butyrate (% VFA)	Acetate: Propionate
Control (ethanol)	91.2 ^{AB}	76.3	14.9 ^{CDE}	8.84 ^{C}	5.14 ^{CD}
Cinnamic alcohol	82.4 ^{BCDE}	75.8	15.2 ^{ABCDE}	9.04 ^{C}	4.97 ^{CDEF}
Anethole	70.7 ^{FG}	75.5	15.8 ^{ABCD}	8.58 ^{C}	4.79 ^{CDEF}
Anisole	82.5 ^{BCD}	75.4	15.1 ^{BCDE}	9.63 ^{C}	4.99 ^{CDE}
Carvone	78.1 ^{CDEF}	72.3	16.9 ^{AB}	10.9 ^{ABC}	4.26 ^{F}
Lemongrass	68.7 ^{FG}	73.2	13.6 ^{EF}	13.3 ^{AB}	5.42 ^{BC}
Dihydrocarvone	77.5 ^{CDEF}	73.0	16.1 ^{ABCD}	11.0 ^{ABC}	4.53 ^{DEF}
Estragole	60.7 ^{G}	74.9	15.3 ^{ABCDE}	9.64 ^{BC}	4.92 ^{CDEF}
Eugenol	71.1 ^{EFG}	73.3	15.4 ^{ABCDE}	11.4 ^{ABC}	4.76 ^{CDEF}
Geraniol	67.7 ^{FG}	74.4	11.4 ^{G}	14.3 ^{A}	6.52 ^{A}
Guaiacol	68.1 ^{FG}	75.6	15.7 ^{ABCD}	8.56 ^{C}	4.82 ^{CDEF}
Guaiacol (half dose)	92.6 ^{AB}	76.0	15.1 ^{BCDE}	8.95 ^{C}	5.04 ^{CDE}
Humulene	73.4 ^{DEF}	75.4	16.2 ^{ABC}	8.43 ^{C}	4.67 ^{DEF}
Lavender	66.9 ^{FG}	73.3	17.3 ^{A}	9.55 ^{C}	4.42 ^{EF}
Limonen	62.8 ^{G}	75.5	16.4 ^{ABC}	7.99 ^{C}	4.66 ^{DEF}
Thyme oil	67.7 ^{FG}	73.2	12.8 ^{FG}	14.1 ^{A}	5.79 ^{AB}
Thymol	36.1 ^{H}	74.4	14.2 ^{DEF}	11.6 ^{ABC}	5.24 ^{BCD}
Vanillin	83.6 ^{BC}	75.5	15.3 ^{BCDE}	9.21 ^{C}	4.93 ^{CDEF}
Crina® Ruminants	70.8 ^{FG}	75.5	16.1 ^{ABCD}	8.23 ^{C}	4.70 ^{CDEF}
Agolin® Ruminant	93.4 ^{A}	76.2	15.1 ^{BCDE}	8.74 ^{C}	5.07 ^{CDE}
P-value	<0.001	0.315	<0.001	0.025	<0.001
SE	3.99	1.27	0.72	1.431	0.259

^{A,B,C,D,E,F,G,H}: Values in the same column with different superscripts differ significantly (P<0.05)

Table 3.4: Effects of the incubation with additives not dissolved in ethanol on the fermentative profile of the rumen *inoculum* added to the TMR after 24 h of incubation.

Additive	VFA (mmol/L)	Acetate (% VFA)	Propionate (% VFA)	Butyrate (% VFA)	Acetate: Propionate
Control	72.7	72.6	17.5	9.83	4.16
Live yeast 8.35 mg/L	69.1	73.4	16.9	9.77	4.36
Live yeast 16.7 mg/L	72.3	73.9	16.5	9.56	4.46
Quebracho tannin	63.6	73.1	17.5	9.34	4.17
Quillaja saponaria saponin	71.9	73.1	17.2	9.72	4.25
P-value	0.072	0.821	0.583	0.958	0.607
SE	2.53	1.07	0.63	0.631	0.192

A positive relationship between the VFA concentration (mmol/L) and the GP (mL/200 mg DM) was observed (VFA=1.318*GP24h + 19.8; r^2=0.58; P <0.001). A positive relationship between CH_4 production (% GP) and the acetate:propionate ratio was also observed (CH_4=2.615*acetate:propionate + 15.0; r^2=0.36; P<0.01).

Experiment 2:

Determination of the *in situ* NDF digestibility of the six forages tested

The chemical composition and the NDF digestibilities (presented as the mean of the four dietary treatments) of the six forages used for the *in situ* evaluation are reported in table 3.5.

The effects of the different additives on the *in situ* NDF digestibility of the six forages determined after 24, 48 and 240 h of incubation are reported in table 3.6. No significant interactions were observed between the "forage" and "additive" factors at any time point. The fiber digestibility determined after 24, 48 and 240 h of incubation was significantly affected by the forage (P<0.001). Ryegrass silage was characterized by the highest NDFD values at each time point. After 24 h of incubation, the corn silage samples had the lowest fiber digestibility values of all forages. After 48 and 240 h of incubation, the alfalfa hay sample had the lowest values of fiber digestibility.

Different additives had significantly different effects on the NDFD (Table 3.6) (P=0.008) after 24 h of incubation. In particular, guaiacol did not affect the NDFD value whereas live yeast and thymol significantly decreased fiber digestibility as compared to control. No differences in NDFD among the treatments were observed after 48 and 240 h of incubation.

Table 3.5: Chemical composition (% DM) and *in situ* NDF digestibility (%) of the forages used during the *in situ* trial after 24, 48 and 240 h of incubation. Each NDF digestibility value represents the mean of the four treatments (control and guaiacol, live yeast and thymol incubations).

Forage	OM	CP	EE	NDF	ADF	ADL	Starch	NDF Digestibility		
								24 h	48 h	240 h
Alfalfa silage	89.6	18.2	1.85	51.6	41.5	10.3	-	38.5 ^{B}	48.2 ^{C}	53.9 ^{D}
Alfalfa hay	92.2	18.7	1.16	46.9	42.5	11.0	-	32.9 ^{C}	40.4 ^{E}	42.8 ^{E}
Corn silage 1	95.6	7.50	3.56	42.0	26.4	4.71	33.4	25.3 ^{D}	44.4 ^{D}	70.3 ^{C}
Corn silage 2	95.5	8.85	2.89	37.3	23.6	4.07	35.3	26.3 ^{D}	48.3 ^{C}	71.5 ^{C}
Rye grass hay	95.1	4.13	0.62	56.8	34.8	5.18	-	35.2 ^{BC}	56.2 ^{B}	75.0 ^{B}
Rye grass silage	91.4	6.94	2.05	43.3	25.5	2.80	-	54.7 ^{A}	75.1 ^{A}	88.1 ^{A}
SE	-	-	-	-	-	-	-	1.97	1.94	0.55
P-Value	-	-	-	-	-	-	-	<0.001	<0.001	<0.001

$^{A, B, C, D, E}$: Values in the same column with different superscripts differ significantly (P<0.05)

Table 3.6: Effects of the incubation with different additives on the *in situ* NDF digestibility (%) after 24, 48 and 240 h of incubation. Each value represents the mean of the six forages tested.

Treatment	24 h	48 h	240 h
Control	38.1 ^{A}	53.2	67.3
Guaiacol	36.4 ^{AB}	52.2	67.3
Live yeast	33.5 ^{C}	52.3	66.4
Thymol	33.9 ^{BC}	50.7	66.7
SE	1.82	1.82	0.46
P-value	0.008	0.291	0.324

$^{A, B, C}$: Values in the same column with different superscripts differ significantly (P<0.05)

Determination of the *in vivo* total tract diet digestibility

The results of the *in vivo* total tract digestibility trial are reported in Table 3.7. The dietary thymol supplementation resulted in decreased DM, OM, NDF and ADF digestibility relative to the other dietary treatments.

Table 3.7: *In vivo* total tract DM, OM, NDF and ADF digestibility of the TMR fed during the *in vivo* and *in situ* trials.

Treatment	DM (%)	OM (%)	NDF (%)	ADF (%)
Control	63.9 ^{A}	67.0 ^{A}	51.4 ^{A}	47.7 ^{A}
Guaiacol	64.5 ^{A}	67.3 ^{A}	51.0 ^{A}	46.1 ^{A}
Live yeast	62.1 ^{A}	65.6 ^{A}	48.4 ^{A}	44.2 ^{A}
Thymol	56.9 ^{B}	60.9 ^{B}	40.8 ^{B}	35.8 ^{B}
SE	3.67	3.29	5.08	5.93
P-value	0.024	0.028	0.018	0.049

$^{A, B,}$: Values in the same column with different superscripts differ significantly (P<0.05)

The protozoal counts were 5.77, 5.69, 5.69 and 5.62 log/mL for the control, guaiacol, live yeast and thymol, respectively (P=0.29). The ruminal NH_3-N contents were affected by the time of sampling (P=0.005) but not by the diet (P=0.30) (11.8, 12.0, 13.7 and 12.2 mmol/mL for the control, guaiacol, live yeast and thymol, respectively). The ruminal pH was affected by the time of sampling (P<0.001) and by the diet (P=0.007). The mean ruminal pH values were 6.58, 6.60, 6.56 and 6.49 for the control, guaiacol, live yeast and thymol supplementation, respectively, with a significantly lower pH value for the thymol than for the other treatments.

3.5 DISCUSSION

The additives that lowered CH_4 production can be divided into two groups: 1) the additives that lowered both GP and CH_4 production (lemongrass, estragole, eugenol, geraniol, limonen, thyme oil and thymol) and 2) the additives that decreased CH_4 production (% total GP) without reducing total GP (cinnamic alcohol, guaiacol and

vanillin). The additives in the latter group are the most promising, as a decrease in the GP is associated with a decrease in the OM digestibility and consequently with the amount of energy supplied to the animal. Guaiacol and vanillin are phenolic compounds, whereas cinnamic alcohol is a phenylpropanol. These three molecules are processed in highly connected metabolic pathways; for example, vanillin is chemically synthesized from guaiacol, lignin and ferulic acid (Clark, 1990; Rosazza et al., 1995), which is an extremely abundant cinnamic acid derivative. In contrast, incubation with eugenol, an allyl chain-substituted guaiacol, decreased GP without affecting CH_4 production. Patra and Yu (2013) tested the effects of clove oil (1 g/L), an essential oil rich in eugenol, and found that this oil drastically decreased the ruminal microbial population *in vitro*, most likely due to the direct inhibition of ruminal bacteria. These authors also found that vanillin (0.76 and 1.52 g/L) lowered GP and CH_4 production; however, this effect was most likely not associated with the direct inhibition of bacteria, as the size of the microbial population (including methanogens) was not reduced. Castillejos et al. (2006) found that vanillin (0.5 g/L) slightly decreased the proportion of acetate produced (−2.0%) without affecting total VFA production; a reduction in the proportion of acetate produced might be correlated with lower CH_4 emissions. Although the effects of the additives on the microbial population were not evaluated in our experiment, we can hypothesize that these additives would have an effect similar to that of eugenol described by Patra and Yu (2013) (i.e., direct inhibition of bacteria).

Thymol, a phenolic compound and one of the major components of the essential oils of thyme and oregano, decreased methane production (% total GP) while negatively affecting the GP and *in situ* and *in vivo* NDFD. Several studies have demonstrated that thymol has broad-spectrum antimicrobial activity and can inhibit gram-positive and gram-negative bacteria by acting as a membrane permeabilizer (Helander et al., 1998; Dorman and Deans, 2000; Walsh et al., 2003). Few studies have specifically examined the effects of thymol on GP and methanogenesis. Benchar et al. (2007) found that the addition of thymol to the diet (200 mg/L) resulted in a decrease in GP, whereas other *in vitro* studies reported that thymol negatively affected CH_4 production only when the dosage of the additive was greater than 300 mg/L (Macheboeuf et al., 2008) or 400 mg/L (Evans and Martin, 2000). These results suggest that the range of effective doses of thymol is small compared to that of other active compounds (Cardozo et al., 2005); in addition, the narrow range between optimal and toxic doses suggests that the antimicrobial activity of thymol may be too strong and nonspecific to modulate fermentation in a complex environment such as the rumen (Calsamiglia et al., 2007). Benchaar et al. (2007) reported that supplementation with thymol and thyme oil resulted in a decrease in the *in vitro* NDFD. Castillejos et al. (2006) similarly

demonstrated that incubation with 500 mg/L of thymol reduced fiber digestibility, suggesting that thymol might also act on cellulolytic species. This result is consistent with the decrease in the pH and NDFD *(in situ* and *in vivo*) observed in the thymol treatment in our experiment. Unfortunately, we do not have data on the effects of essential oils on *in vivo* CH_4 production; however, we can speculate that thymol incubation might decrease CH_4 production and fiber digestibility. Such a hypothesis would be consistent with the findings of Tekippe et al. (2011) who found that oregano, a product rich in thymol, reduced the production of CH_4 *in vivo*.

To our knowledge, few data are available in the literature on the effects of guaiacol on CH_4 production. Guaiacol is a component of the essential oils of peppermint, celery, birch and juniper (Magyar et al., 2004). Castillejos et al. (2006) found that a range of doses of guaiacol reduced the production of VFAs. However, at a dose of 500 mg/L, incubation with guaiacol reduced the production of acetate (−2.5%) without reducing total VFA concentrations. The lack of negative effects of guaiacol is consistent with the results of the present experiment; indeed, we found that when a dose of 500 mg/L was used VFA production decreased. The lower proportion of acetate reported by Castillejos et al. (2006) might explain the reduction in the CH_4 production observed in the present study. In the *in situ* and *in vivo* trials, guaiacol supplementation did not negatively influence the 24 h NDF digestibility of the selected feeds, and the OM and fiber total tract digestibility of the diet.

No specific information on the effects of cinnamic alcohol was found in the literature. However, Jayanegara (2009) showed that the addition of cinnamic acid to an *in vitro* rumen system decreased the production of CH_4. Similarly, Macheboeuf et al. (2008) showed that GP and CH_4 production decreased linearly with increasing doses of cinnamaldehyde. Cinnamaldehyde has been shown to have antimicrobial properties similar to those of the phenolic compounds thymol and carvacrol (Helander et al., 1998). The cinnamaldehyde molecule consists of a phenyl group attached to an unsaturated aldehyde; therefore, unlike thymol, carvacrol and cinnamic alcohol, cinnamaldehyde does not have a hydroxyl or acid group that acts as a proton carrier and disrupts the outer membrane of bacteria or depletes their intracellular pool of ATP (Helander et al., 1998). It is possible that cinnamaldehyde is transformed to cinnamic alcohol/acid in the rumen and has a toxic effect on the ruminal microbial population.

It is unclear which chemical moieties confer antimicrobial properties on a secondary compound; however, the results of this study appear to confirm that the presence of oxygen, a hydroxyl group and a phenolic ring are important (Ultee et al., 2002; Burt, 2004; Benchaar and Greathead, 2011). In contrast, geraniol, a monoterpenoid alcohol, decreased GP but not CH_4 production, which resulted in an increase in the contribution

of CH_4 to total GP; this is consistent with the lower production of VFAs, the lower proportion of propionate and the higher proportions of butyrate observed in our study. Based on these results, we hypothesize that geraniol has a negative effect on ruminal amylolytic bacteria. Misharina (2002) demonstrated that starch granules can absorb certain components of essential oils that can affect ruminal bacterial fermentation patterns (Duval and Newbold, 2004). For example, Wallace et al. (2002) suggested that *Ruminobacter amylophilus* might be the primary target of a commercial blend of essential oils.

It has been hypothesized that live yeast can have a positive effect on ruminal microbial growth, which, in turn, could improve feed utilization; however, this hypothesis was not confirmed by the results of the *in situ/in vivo* portion of our study. The live yeast did not improve animal performance in terms of GP and NDFD either *in situ* or *in vivo*. In fact, the NDFD was decreased *in situ* after 24 h. However, this negative effect disappeared after 48 h of *in situ* incubation and *in vivo*. These results are consistent with those of Enjalbert et al. (1999) who demonstrated a significant cubic effect of the interaction between yeast and incubation time on *in situ* fiber degradation; indeed, cows supplemented with yeast cultures exhibited a lower NDFD after 12 h of incubation than cows fed a control diet. However, other studies (e.g., Chung et al., 2011 and Lopez-Soto et al., 2013) demonstrated a positive or a null effect of yeast on fiber digestion. The results reported in the literature are not consistent and appear to depend heavily on factors such as the strain and the dose of yeast and the chemical composition of the diet fed to the animals (Chaucheyras-Durand et al., 2008).

In conclusion, incubation with certain candidate additives used in the present study produced a negative effect on digestive parameters determined *in vitro*, *in situ* and *in vivo*. The results of this study suggest that attention must be paid when using feed additives to reduce ruminal methanogenesis. Major differences in digestive parameters can be observed within the same class of additives. However, results obtained in *in vitro* studies should always be confirmed by *in vivo* studies that evaluate the medium/long-term response to different additives added at different doses.

3.6 ACKNOWLEDGMENTS

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3.7 REFERENCES

- AOAC, 1995. Official Methods of Analysis of the Association of Official Analytical Chemists 15th edn. Washington, DC, USA.
- AOAC, 1998. Official Methods of Analysis 16th edn. Association of Official Analytical Chemists, Washington, DC, USA.
- Beauchemin, K.A., Kreuzer, M., O'Mara, F., McAllister, T.A., 2008. Nutritional management for enteric methane abatement: a review. Aust. J. Exp. Agr. 48:21–27.
- Benchaar, C., Chaves, A.V., Fraser, G.R., Wang, Y., Beauchemin, K.A., McAllister, T.A., 2007. Effects of essential oils and their components on in vitro rumen microbial fermentation. Can. J. Anim. Sci. 87:413–419.
- Benchaar, C., Greathead, H., 2011. Essential oils and opportunities to mitigate enteric methane emissions from ruminants. Anim. Feed Sci. Tech. 166-167:388-355.
- Burt, S., 2004. Essential oils: their antibacterial properties and potential applications in foods—a review. Int. J. Food Microbiol. 94:223–253.
- Busquet, M., Calsamiglia, S., Ferret, A., Kamel, C., 2006. Plant extracts affect in vitro rumen microbial fermentation. J. Dairy Sci. 89:761–771.
- Calsamiglia, S., Busquet, M., Cardozo, P.W., Castillejos, L., Ferret, A., 2007. Essential oils as modifiers of rumen microbial fermentation. J. Dairy Sci. 90:2580–2595.
- Cardozo, P.W., Calsamiglia, S., Ferret, A., Kamel, C., 2005. Screening for the effects at two pH levels on in vitro rumen microbial fermentation of a high-concentrate beef cattle diet. J. Anim. Sci. 83:2572–2579.
- Carulla, J.E., Kreuzer, M., Machmüller, A., Hess, H.D., 2005. Supplementation of Acacia mearnsii tannins decreases methanogenesis and urinary nitrogen in forage-fed sheep. Aust. J. Agr. Res. 56:961–970.
- Castillejos, L., Calsamiglia, S., Ferret, A., 2006. Effect of essential oil active compounds on rumen microbial fermentation and nutrient flow in in vitro systems. J. Dairy Sci. 89:2649–2658.
- Chaucheyras-Durand, F., Walker, N.D., Bach, A., 2008. Effects of active dry yeasts on the rumen microbial ecosystem: Past, present and future. Anim. Feed Sci. Tech. 145:5–26.
- Chung, Y.H., Walker, N.D., McGinn, S.M., Beauchemin, K.A., 2011. Differing effects of 2 active dried yeast (Saccharomyces cerevisiae) strains on ruminal acidosis and methane production in nonlactating dairy cows. J. Dairy Sci. 94:2431-2439.
- Clark, G.S., 1990. Vanillin. Perfume Flavor 15:45-54.
- Dehority, B.A., 1984. Evaluation of subsampling and fixation procedures used for counting rumen protozoa. Appl. Environ. Microb. 48:182-185.
- Dorman, H.J., Deans, S.G., 2000. Antimicrobial agents from plants: Antibacterial activity of plant volatile oils. J. Appl. Microbiol. 88:308–316.

- Duval, S.M., Newbold, C.J., 2009. Effect of a blend of essential oils on the fermentation of starch-rich substrate as estimated by its gas production profile. In: Chilliard Y, Glasser F, Faulconnier Y, Bocquier F, Veissier I, Doreau M (eds), Ruminant physiology: digestion, metabolism, and effects of nutrition on reproduction and welfare, pp 166-167. Proceedings of the 11th International Symposium on Ruminant Physiology, Wageningen Academic Publishers, The Netherlands.
- Eckard, R.J., Grainger, C., de Klein, C.A.M., 2010. Options for the abatement of methane and nitrous oxide from ruminant production: a review. Livest. Sci. 130:47-56.
- Ellis, J.L., Kebreab, E., Odongo, N.E., McBride, B.W., Okine, E.K., France, J., 2007. Prediction of methane production from dairy and beef cattle. J. Dairy Sci. 90:3456-3467.
- Enjalbert, F., Garrett, J.E., Moncoulon, R., Bayourthe, C., Chicoteau, P., 1999. Effects of yeast culture (Saccharomyces cerevisiae) on ruminal digestion in non-lactating dairy cows. Anim. Feed Sci. Tech. 76:195-206.
- Evans, J.D., Martin, S.A., 2000. Effects of thymol on ruminal microorganisms. Curr. Microbiol. 41:336-340.
- Grainger, C., Clarke, T., Auldist, M.J., Beauchemin, K.A., McGinn, S.M., Waghorn, G.C., Eckard, R.J., 2009. Mitigation of greenhouse gas emissions from dairy cows fed pasture and grain through supplementation with Acacia mearnsii tannins. Can. J. Anim. Sci. 89:241–251.
- Grainger, C., Beauchemin, K.A., 2011. Can enteric methane emissions from ruminants be lowered without lowering their production? Anim. Feed Sci. Tech. 166-167:308-320.
- Hart, K.J., Yáñes-Ruiz, D.R., Duval, S.M., McEwan, N.R., Newbold, C.J., 2008. Plant extract to manipulate rumen fermentation. Anim. Feed Sci. Tech. 147:8-35.
- Helander, I.M., Alakomi, H.L., Latva-Kala, K., Mattila-Sandholm, T., Pol, I., Smid, E.J., Gorris, L.G.M., Von Wright, A., 1998. Characterization of the action of selected essential oil components on gram-negative bacteria. J. Agr. Food Chem. 46:3590–3595.
- Hess, H.D., Monsalve, L.M., Lascano, C.E., Carulla, J.E., Diaz, T.E., Kreuzer, M., 2003. Supplementation of a tropical grass diet with forage legumes and Sapindus saponaria fruits: effects on in vitro ruminal nitrogen turnover and methanogenesis. Aust. J. Agr. Res. 54:703–713.
- Italian Regulation, 1992. Application of the Council Directive (EEC) No. 86/609 regarding the protection of animals used for experimental and other scientific purposes. LD 116/1992. In: Official Journal No. 294, 18/2/1992, pp 5-24.
- Jayanegara, A., 2009. Ruminal methane production on simple phenolic acids addition in in vitro gas production method. MEDIA PETERNAKAN-J. Anim. Sci. Tech. 32:53-62.
- Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. J. Anim. Sci. 73:2483–2492.

- Lila, Z.A., Mohammed, N., Kanda, S., Kamada, T., Itabashi, H., 2003. Effect of sarsaponin on ruminal fermentation with particular reference to methane production in vitro. J. Dairy Sci. 86:3330-3336.
- Lopez-Soto, M.A., Valdes-Garcia, Y.S., Plascencia, A., Barreras, A., Castro-Perez, B.I., Estrada-Angulo, A., Rios, F.G., Gomez-Vazquez, A., Corona, L., Zinn, R.A., 2013. Influence of feeding live yeast on microbial protein synthesis and nutrient digestibility in steers fed a steam-flaked corn-based diet. Acta Agr. Scand. A-An. 63:39-43.
- Lynch, H.A., Martin, S.A., 2002. Effects of Saccharomyces cerevisiae culture and Saccharomyces cerevisiae live cells on in vitro mixed ruminal microorganism fermentation. J. Dairy Sci. 85:2603-2608.
- Macheboeuf, D., Morgavi, D.P., Papon, Y., Mousset, J.L., Arturo-Schaan, M., 2008. Dose–response effects of essential oils on in vitro fermentation activity of the rumen microbial population. Anim. Feed Sci. Tech. 145:335–350.
- Magyar, J., Sxentandrassy, N., Banyasz, T., Fulop, L., Varro´, A., Nanasi, P.P., 2004. Effects of terpenoid phenol derivatives on calcium current in canine and human ventricular cardiomyocytes. Eur. J. Pharmacol. 487:29–36.
- Martin, C., Morgavi, D.P., Doreau, M., 2010. Methane mitigation in ruminants: from microbe to the farm scale. Animal 4-3:351-365.
- Martinez, S., Madrid, J., Hernandez, F., Megias, M.D., Sotomayor, J.A., Jordan, M.J., 2006. Effect of thyme essential oils (Thymus hyemalis and Thymus zygis) and monensin on in vitro ruminal degradation and volatile fatty acid production. J. Agric. Food Chem. 54:6598–6602.
- McAllister, T.A., Newbold, C.J., 2008. Redirecting rumen fermentation to reduce methanogenesis. Aust. J. Exp. Agr. 48:7–13.
- Mertens, D.R., 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds using refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85:1217-1240.
- Misharina, T.A., 2002. Sorption of monoterpenoids and sesquiterpenoids by natural polysaccharides. Appl. Biochem. Microbiol. 38:480–486.
- Moore J.A., Poore, M.H., Luginbuhl, J.M., 2002. By-product feeds for meat goats: Effects on digestibility, ruminal environment, and carcass characteristics. J. Anim. Sci. 80:1752-1758.
- Moss, R.A., Jounany, J.P.M., Newbold, C.J., 2000. Methane production by ruminants: its contribution to global warming. Ann. Zootech. 49:231-253.
- National Research Council. 2001. Protein and amino acids. In: Nutrient requirements of dairy cattle 7th rev. ed., pp.43-104. National Academy of Sciences, Washington DC.
- Newbold, C.J., Rode, L.M., 2006. Dietary additives to control methanogenesis in the rumen. In: Soliva CR, Takahashi J, Kreuzer M. (eds.), Greenhouse gases and animal agriculture: an update, pp 138–147. Elsevier International Congress Series 1293, Elsevier, Amsterdam, The Netherlands.

- Ogino, A., Orito, H., Shimadad, K., Hirooka, H., 2007. Evaluating environmental impacts of the Japanese beef cow–calf system by the life cycle assessment method. J. Anim. Sci. 78:424–432.
- Patra, A.K., Yu, Z., 2013. Effects of vanillin, quillaja saponin, and essential oils on in vitro fermentation and protein-degrading microorganisms of the rumen. Appl. Microbiol. Biot. 2013, doi: 10.1007/s00253-013-4930-x.
- Pirondini, M., Malagutti, L., Colombini, S., Amodeo, P., Crovetto, G.M., 2012. Methane yield from dry and lactating cows diets in the Po plain (Italy) using an in vitro gas production technique. Ital. J. Anim. Sci. 11-e61, 330-335.
- Reynolds, C.K., Mills, J.A.N., Crompton, L.A., Givens, D.I., Bannink, A., 2010. Ruminant nutrition regimes to reduce greenhouse gas emissions in dairy cows. In: Crovetto GM (ed), Energy and protein metabolism and nutrition. EAAP publication No. 127, pp 427-437. Wageningen Academic Publishers, Wageningen, the Netherlands.
- Rosazza, J.P.N., Huang, Z., Dostal, L., Volm, T., Rousseau, B., 1995. Review: Biocatalytic transformations of ferulic acid: An abundant aromatic natural product. J. Ind. Microbiol. 15:457-471.
- Santoso, B., Mwenya, B., Sar, C., Gamo, Y., Kobayashi, T., Morikawa, R., Kimura, K., Mizukoshi, H., Takahashi, J., 2004. Effects of supplementing galacto-oligosaccharides, Yucca schidigera or nisin on rumen methanogenesis, nitrogen and energy metabolism in sheep. Livest. Prod. Sci. 91:209–217.
- SAS Institute. 2000. SAS/STAT User's Guide, Release 8.1. SAS Inst., Inc., Cary, NC, USA.
- Shibata, M., Terada, F., 2010. Factors affecting methane production and mitigation in ruminants. Anim. Sci. J. 81:2-10.
- Tamburini, A., Colombini, S., Penati, C., Zucali, M., Roveda, P., Rapetti, L., Crovetto, G.M., 2010. Methane emission in livestock and diet characteristics. In: Crovetto GM, (ed), Energy and protein metabolism and nutrition. EAAP publication No. 127, pp 465-466. Wageningen Academic Publishers, Wageningen, the Netherlands.
- Tavendale, M.H., Meagher, L.P., Pacheco, D., Walker, N., Attwood, G.T., Sivakumaran, S., 2005. Methane production from in vitro rumen incubations with Lotus pedunculatus and Medicago sativa, and effects of extractable condensed tannin fractions on methanogenesis. Anim. Feed Sci. Tech. 123-124:403-419.
- Tekippe, J.A., Hristov, A.N., Heyler, K.S., Cassidy, T.W., Zheljazkov, V.D., Ferreira, J.F., Karnati, S.K., Varga, G.A., 2011. Rumen fermentation and production effects of Origanum vulgare L. leaves in lactating dairy cows. J. Dairy Sci. 10:5065-5079.
- Theodorou, M.K., Williams, B.A., Dhanoa, M.S., McAllan, A.B., France, J., 1994. A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. Anim. Feed Sci. Tech. 48:185-197.
- Ultee, A., Bennik, M.H.J., Moezelaar, R., 2002. The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen Bacillus cereus. Appl. Environ. Microb. 68:1561-1568.

- Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods of dietary fiber, neutral detergent fiber and non-polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597.
- Wallace, R.J., Arthaud, L., Newbold, J.C., 1994. Influence of Yucca schidigera extract on ruminal ammonia concentrations and ruminal microorganisms. Appl. Environ. Microb. 60:1762–1767.
- Wallace, R.J., McEwan, N.R., McIntosh, F.M., Teferedegne, B., Newbold, C.J., 2002. Natural products as manipulators of rumen fermentation. Asian Austral. J. Anim. Sci. 15:1458–1468.
- Walsh, S.E., Maillard, J.Y., Russell, A.D., Catrenich, C.E., Charbonneau, D.L., Bartolo, R.G., 2003. Activity and mechanisms of action of selected biocidal agents on gram-positive and -negative bacteria. J. Appl. Microbiol. 94:240–427.
- World Medical Association (WMA). 2008. WMA Declaration of Helsinki - Ethical Principles for Medical Research Involving Human Subjects. Available from URL: http://www.wma.net/en/30publications/10policies/b3/index.html.

4 METHANE EMISSIONS OF LACTATING DAIRY COWS FED DIETS WITH DIFFERENT STARCH CONTENT SUPPLEMENTED WITH FISH OIL

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4.1 ABSTRACT

The aim of this study was to evaluate the effects of diets with different starch contents and fish oil (FO) supplementation on lactation performance, in vivo total-tract nutrient digestibility, N balance and methane (CH_4) production in lactating dairy cows.

The experiment was conducted as a 4×4 Latin square design with a 2×2 factorial arrangement: two levels of dietary starch content (low vs high), the presence or absence of FO supplement (0.80% DM) and their interaction were evaluated. Four Italian Friesian cows were fed one of the following four diets in four consecutive 26-d periods: 1) low starch (LS), 2) low starch plus FO (LSO), 3) high starch (HS) and 4) high starch plus FO (HSO). The diets contained the same amount of forages (corn silage, alfalfa and meadow hay). The starch content was balanced using different proportions of corn meal and soybean hulls. The cows were housed in metabolic stalls inside open-circuit respiration chambers to allow the measurement of CH_4 production and the collection of separate urine and feces samples.

No differences among treatments were observed for dry matter intake: neither FO nor the use of soybean hulls negatively influenced this parameter (22.9 kg/d, on average). A trend was observed for the effect of FO on milk yield ($P=0.10$), with a higher milk production compared with the diets without FO (29.2 vs 27.5 kg/d on average, respectively). Moreover, FO supplementation favorably decreased the n-6:n-3 ratio of the milk polyunsaturated fatty acids (PUFA). Consistent with the literature, the milk protein percentage was negatively influenced by FO ($P<0.01$): 3.37% on average for the LSO and HSO diets vs 3.67% for the LS and HS diets. With regard to digestibility, the HS diet negatively influenced all the parameters measured, with starch as the only exception. The percentage of N intake excreted with feces was influenced by both the FO ($P=0.09$) and starch content ($P=0.04$): the HS diet showed the highest value, followed by the HSO and LS diets and finally by the LSO diet. With regard to methanogenesis, there was a trend ($P=0.08$) of lower CH_4 emissions (g/d) with the HS and HSO diets compared with the LS and LSO diets (396 vs 415 on average, respectively). Fish oil supplementation did not reduce methanogenesis. Previous in vitro studies have demonstrated a reduction in CH_4 production as a consequence of FO supplementation; the decrease in CH_4 production is generally accompanied by marked reductions in dry matter and fiber digestibility, which were not observed in the present work.

Key words: methane, starch, fish oil, dairy cow

4.2 INTRODUCTION

There is growing interest in decreasing the potential for global warming by reducing emissions of greenhouse gases (GHG) into the atmosphere (Cassandro et al., 2013). Methane (CH_4) is a potent GHG, and its release into the atmosphere is partially linked to animal husbandry, particularly ruminant production systems. CH_4 emissions result from the microbial fermentation of feed within the rumen and represent a loss of energy for the animal (Beauchemin and McGinn, 2005). Energy losses in the form of enteric CH_4 in mature cattle range from 2% to nearly 12% of gross energy (GE) intake (Johnson and Johnson, 1995), depending mainly on the level of dry matter intake (DMI) and the composition of the diet (Johnson and Johnson, 1995; Moss et al., 2000; Benchaar et al., 2001). Therefore, researchers are exploring various nutritional strategies to mitigate CH_4 emissions while maintaining or improving ruminant productivity (Patra and Yu, 2013). The most successful mitigation strategies will be those that lead to a profitable increase in milk or meat production while achieving the desired decreases in emissions (Moate et al., 2013). The proportion of energy concentrate in the diet has been reported to be negatively correlated with CH_4 emissions (Yan et al., 2000). Studies have investigated the effect of starch-based and fiber-based concentrates on enteric CH_4 production. Lovett et al. (2005) demonstrated that increased fibrous concentrate use at pastures (5.24 kg DM/d) reduced enteric CH_4 emissions (-16.8%) per kg of fat-corrected milk compared to a lower concentrate supplementation (0.87 kg DM/d). A positive response to diets including high levels of starch-based concentrates on CH_4 reduction has also been reported by Beauchemin and McGinn (2005) during an *in vivo* trial involving finishing beef cattle. A higher proportion of starch in the diet changes the pattern of rumen VFA concentrations, lowering acetate and increasing propionate production and thereby limiting the supply of hydrogen for methanogenesis. Furthermore, the rapid degradation of readily fermentable carbohydrates, such as starch, is associated with lower rumen pH values and is less favorable for methanogens, which prefer a pH of 6.0-6.4 (Johnson and Johnson, 1995; Jarvis et al., 2000; Lee et al., 2000). Benchaar et al. (2001) used a modeling approach to assess the effectiveness of different nutritional strategies to reduce CH_4 production, including variations of DMI, different forage:concentrate ratios, different types of concentrate (fibrous vs starch-based) and different types of starch (slowly vs rapidly degraded). CH_4 production as a percentage of GE intake was reduced by increasing DMI from 9 to 17 kg/d (-15.9%, on average) and the proportion of concentrate in the diet from 0 to 70% DM (-30.5%, on average). However, increased levels of concentrates may result in health problems such as rumen acidosis and high feeding costs, particularly in the case of low input systems such as pastures; hence, it is important to evaluate other dietary strategies such as the addition

of fats to ruminant diets. It has long been known that fat supplementation can reduce CH_4 production (Andrew et al., 1991; Tyrrell et al., 1991), and more recently, many studies have focused on the effects of different supplemental dietary fats on CH_4 emissions (Beauchemin et al., 2008; Martin et al., 2010).

Several *in vitro* and *in vivo* experiments have demonstrated marked reductions in methanogenesis as the level of dietary ether extract is increased. The effects vary according to the characteristics of the basal ration (Iqbal et al., 2008), type of fat, physical form of the supplement (Martin et al., 2008) and level of its inclusion in the diet (Beauchemin et al., 2008; Martin et al., 2010). The effects of supplemental fat on CH_4 production are multifactorial (Reynolds et al., 2010), as there are several mechanisms involved in reducing CH_4 losses, including the reduction of rumen fermentation, the biohydrogenation of unsaturated fatty acids, changes in VFA patterns (McGinn et al., 2004) and the inhibition of microorganisms (Johnson and Johnson, 1995). Fat supplies digestible energy that is not fermented in the rumen, increasing the energy density of diets and improving the energy status of high-yielding lactating dairy cows, thereby reducing the amount of CH_4 emitted relative to DMI or energy intake (Eugene et al., 2008; Martin et al., 2010; Grainger and Beauchemin, 2011). In practice, supplemental fats and oils are also fed to dairy cows to manipulate milk fatty acid profiles, increasing the concentrations of ω-3 polyunsaturated fatty acids and conjugated linoleic acids, which have potential beneficial effects on human health (Mele, 2009). The use of hydrogen in the rumen biohydrogenation of unsaturated fatty acids acts as an alternative to CH_4 production as a sink for hydrogen. Finally, the inhibitory effects of specific fatty acids on rumen bacteria and fiber fermentation, and associated changes in fermentation dynamics (Liu et al., 2012), can have pronounced inhibitory effects on rumen methanogenesis (Reynolds et al., 2010). Recent research has focused on the CH_4-suppressing effects of the medium-chain fatty acids due to their ability to suppress methanogenesis with lesser negative influences on DMI and animal performance than longer-chain polyunsaturated fatty acids (Reynolds et al., 2010). Beauchemin et al. (2008) have demonstrated large reductions in CH_4 emissions for diets supplemented with C12:0- and C14:0-rich oils, which have anti-microbial properties against both protozoa and methanogen communities (Machmuller, 2006). Unfortunately, in a trial with lactating dairy cows (Crompton et al., 2010), decreases in CH_4 emissions with supplemental coconut oils rich in medium-chain fatty acids (approximately 80%; Patra and Yu, 2013) were obtained only with concomitant decreases in DMI.

Recent studies suggest that dietary fats containing high concentrations of long-chain polyunsaturated fatty acids, such as linoleic acid and alpha-linolenic acid, are particularly effective in suppressing CH_4 emissions (Martin et al., 2010). Very long-chain

unsaturated fatty acids present in fish oil (FO) or in certain algae species also have negative effects on methanogenesis (Martin et al., 2010). Hexadecatrienoic acid (C16:3), eicosapentaenoic acid (EPA; C20:5) and docosahexaenoic acid (DHA; C22:6) exhibited strong CH_4-suppressing effects when tested *in vitro* (Fievez et al., 2003; 2007; Castro-Montoya et al., 2012). This CH_4-suppressing effect may relate to the degree of unsaturation of these fatty acids as they undergo biohydrogenation in the rumen, their reactivity in the rumen and their effects on specific rumen microorganisms (e.g., cellulolytic bacteria and protozoa). Nevertheless, Moate et al. (2013), during an *in vivo* trial with lactating dairy cows, did not find a reduction in methanogenesis when supplementing the alfalfa hay-based diet with different doses of algal meal rich in DHA. Although interesting, to the best of our knowledge the existing experimental data on the effects of specific long-chain polyunsaturated fatty acids on rumen fermentation and CH_4 production are scarce and are primarily based on *in vitro* procedures. A greater understanding of the effects of these oils on the rumen microbiome may contribute to the development of effective feeding strategies to decrease ruminal CH_4 emissions and to improve ruminant product quality. Hence, there is a need for further *in vivo* research on the effects of these lipid sources on methanogenesis and animal productive performance.

The aim of the present study was to evaluate the effects of diets with different starch contents supplemented with fish oil and of the interaction between these two factors on the productive performances and methanogenesis of lactating dairy cows.

4.3 MATERIALS AND METHODS

Animals, Experimental Design, and Diets

The experiment was conducted at the Research Center of the Department of Agricultural and Environmental Sciences, University of Milan, Italy. Trial animals were handled as outlined by the guidelines of the Italian law on animal welfare for experimental animals (Italian Ministry of Health, 1992) and of the University of Milan Ethic Committee for animal use and care. Four lactating secondiparous Italian Friesian cows with mean BW of 617 kg (±18), 177 DIM (±46) and producing an average of 30.3 kg of milk/d (±3.43) at the start of the trial were used. The experiment was conducted as a 4×4 Latin square design with a 2×2 factorial arrangement: treatments were arranged to evaluate the main effects of two levels of dietary starch content (low vs high), the presence or absence of fish oil supplement and their interaction. Each cow was fed the four diets in four consecutive experimental periods of 26 d, including 21 d of adaptation and 5 d of sample collection and data registration. The four dietary treatments were as follows: 1) low-starch diet (LS), 2) low-starch diet supplemented with FO (LSO), 3) high-

starch diet (HS) and 4) high-starch diet supplemented with FO (HSO). The FO supplement (Danish Fish Oil HF, Magri Otello SRL, San Cesario sul Panaro, MO, Italy) was mixed manually with the other ingredients and added to the LSO and HSO diets to provide a theoretical level of 0.80% inclusion on a DM basis.

The diets (Tables 4.1 and 4.2) were formulated using the CNCPS model (version 6.1) and balanced to provide adequate metabolizable protein (MP) and energy for 677 kg lactating dairy cows producing 35 kg of milk/d containing 3.70% fat and 3.10% protein. All diets contained, on average, a content of 10.2% MP DM and were based (on a DM basis) on 29.4% corn silage (36.1% DM, 35.3% NDF and 37.7% starch on a DM basis), 11.4% alfalfa hay (17.9% CP, 46.1% NDF, 39.8% ADF and 10.4% ADL on a DM basis), 1.8% first-cut meadow hay (9.51% CP, 67.0% NDF, 42.5% ADF and 7.71% ADL on a DM basis), 9.1% second cut meadow hay (11.4% CP, 58.0% NDF, 37.7% ADF and 7.28% ADL on a DM basis), 6.7% soybean meal, 6.2% canola meal and 1.9% cane molasses. To balance the different starch levels, corn meal and pelleted soybean hulls (12.3% CP, 62.1% NDF, 47.2% ADF, and 5.65% ADL on a DM basis) were included in the experimental diets in different proportions. In the two experimental diets supplemented with FO (0.80% DM), the same amount of corn meal was replaced by the fat supplement.

During the entire experiment, the cows had free access to water and were individually *ad libitum* offered feed twice daily (0730 and 1800 h); orts were recorded once daily, and the feeding rate was adjusted to yield orts on the basis of at least 5% of the amount supplied (on an as-fed basis). During the adaptation periods, the cows were housed in individual tie-stalls fitted with rubber mattresses and bedded with chopped straw.

During the sample collection periods, the cows were moved to metabolic stalls inside individual open-circuit respiration chambers to enable the measurement of CH_4 production over four 24 h cycles through the use of Uras 4 gas analyzer (Hartmann & Braunn, ABB spa, Process Automation Division, Sesto San Giovanni, Italy) and urine and feces were collected separately. Each respiration chamber, equipped with a feeder, contained a 2.5×1.5-m stanchion that allowed the animal to stand or lie down. During the collection period, feces produced daily were measured as follows: feces left the chamber through openings on the floor in the back of the stanchion and were collected in tanks located underneath the floor of the chambers, as reported by Colombini et al. (2012). Urine was collected in plastic canisters through the use of Foley urinary catheters (model 1855H24, C.R. Bard Inc. Covington, GA, USA). The pH of urine was maintained below 2.5 (to avoid ammonia loss) through the addition of adequate volumes of sulfuric acid 25% (vol/vol). During each of the 4 collection periods, urine and feces were weighed daily, sampled (2% of the total weight) and pooled per cow. All the samples were stored at -20°C.

Before analysis, fecal samples were thawed and oven dried at 55°C until constant weight and ground through a 1-mm screen (Pulverisette 19, Fritsch, Idar-Oberstein, Germany). A fresh subsample was used for the N analysis.

Nitrogen balance was determined considering the N content of the condensed water produced daily by the animals in the respiration chambers.

During each collection period, TMR and ort samples were collected daily to obtain a composite sample and stored at -20°C. Twice weekly, first- and second-cut meadow hay, alfalfa hay, corn meal, pelleted soybean hulls, protein supplements and FO were also sampled for subsequent chemical analyses. Samples (with the exception of FO) were dried in a ventilated oven at 55°C for 48 h. After drying, the feed samples and orts were ground through a 1-mm screen (Pulverisette 19, Fritsch, Idar-Oberstein, Germany).

The cows were milked twice daily at 07:30 a.m. and 06:30 p.m., and milk production was recorded at each milking. Milk samples were collected daily (2% of total weight) with the addition of potassium dichromate as a preservative and stored at -20°C before analyses. During each of the two milkings on days 3 and 5, individual milk samples were collected for lactose and MUN determination. In addition, during each of the two milkings on days 3, 4 and 5, individual composite milk samples (100 mL) without preservative were frozen at -20°C until analysis of the milk fatty acids profile.

Rumen fluid was sampled from cows at the end of each collection period using an esophageal probe. Samples were taken immediately before the morning feeding to measure ruminal fermentation characteristics such as pH, ammonia nitrogen (NH_3-N) and VFA profile. Approximately 0.6 L of rumen fluid was strained through two layers of cheesecloth, and the pH of the filtered rumen fluid was immediately measured. Fifty milliliters of the filtered rumen fluid was added to 4 mL of 25% (vol/vol) sulfuric acid, and samples from each animal were retained for NH_3-N determination. Another 50 mL of the filtered rumen fluid was retained for VFA determination. All the samples were stored at -20°C until analysis.

Chemical Analyses

Corn silage and other feed components, orts and feces were analyzed for the contents of DM (method 945.15; AOAC, 1995); ash (method 942.05; AOAC, 1995); CP (method 984.13; AOAC, 1995); ether extract (EE) (method 920.29; AOAC 1995); starch (method 996.11; AOAC, 1998); NDF, corrected for insoluble ash and with the addition of α-amylase (aNDFom; Mertens, 2002), ADF and ADL (Van Soest et al., 1991), using the Ankom 200 fiber apparatus (Ankom Technology Corp., Fairport, NY); and GE using an adiabatic bomb calorimeter (IKA 4000; IKA Werke GmbH & Co. KG, Staufen, Germany). Fish oil was analyzed for EE (method 920.29; AOAC 1995), and the fatty acid profile of

the diets fed to animals was determined according to the methods reported by Mele et al. (2008).

Milk samples were pooled by cow and period and analyzed for total N (method 991.20; AOAC 1995), non-protein N (method 991.21; AOAC 1995), casein (method 927.03; AOAC 1995), and fat (method 2446; ISO, 1976) at the end of each experimental period. Fat- and protein-corrected milk production (FPCM; 4.0% fat and 3.3% protein) was calculated according to Gerber et al. (2010). Lactose content was analyzed using a Fourier transform infrared (FTIR) analyzer (MilkoScan FT6000; Foss Analytical A/S). Milk urea nitrogen content was determined with a differential pH technique (method 14637; ISO 2006).

Fat from milk samples was extracted according to Mele et al. (2008). Methyl esters of fatty acids were prepared by the alkali catalyzed trans-methylation procedure described by Christie (1982), with C19:0 methyl ester (Sigma Chemical Co., St. Louis, MO) as the internal standard. Milk fatty acid compositions were analyzed according to Buccioni et al. (2012). The identification of individual fatty acid methyl esters was based on a standard mixture of 52 Component FAME Mix (Nu-Chek Prep. Inc., Elysian, MN, USA), and the identification of C18:1 isomers was based on a commercial standard mixture (Supelco, Bellefonte, PA, USA) and on chromatograms published by Kramer et al. (2008). For each fatty acid, the response factors to flame ionization detector and inter- and intra-assay coefficients of variation were calculated by using a reference standard butter (CRM 164, Community Bureau of Reference, Brussels, Belgium). Intra-assay coefficients of variation ranged from 0.5 to 1.5%, whereas inter-assay coefficients of variation ranged from 1.5 to 2.5%.

The rumen fluid N-NH_3 concentration was determined through direct distillation and titration using a Kjeltec 2300 analyzer (Foss Analytical A/S, Hillerød, Denmark), and VFA determination was carried out through gas-chromatographic assay as described by Pirondini et al. (2012).

Statistical Analysis

Statistical analysis was performed using the Mixed procedure of SAS (SAS Institute, 2001). Data were analyzed with the following model:

$$Y=\mu + A_i + P_j + F_k + St_l + F \times St + e_{ijkl}$$

where Y is the dependent variable, μ is the overall mean, A_i is the animal effect (i=1, 4), P_j is the period effect (j=1, 4), F_k is the fish oil supplement effect (k=1, 2), St_l is the dietary starch level effect (l=1, 2) and e_{ijkl} is the residual error.

Least squares mean estimates are reported; the separation of least square means was conducted at α=0.05. For all statistical analyses, significance was declared at P≤0.05 and trends at P≤0.10.

4.4 RESULTS AND DISCUSSION

Experimental Diets

The composition of the experimental diets as well as their chemical analysis and fatty acid composition are reported in Tables 4.1 and 4.2. The diets were formulated to be iso-nitrogenous; however, the CP content of high-starch diets was slightly lower than that of low-starch diets because of the higher inclusion of corn meal in the former, which has a lower CP content in comparison with soybean hulls (9.30 vs 12.3% DM, respectively). The same tendency can be observed for ash because of the higher ash content of soybean hulls compared to corn meal (5.87 vs 1.39% DM, respectively). The ether extract content was higher for the LSO and HSO diets because they were supplemented with FO. The starch content was higher for the HS and HSO treatments, and as a consequence, fiber fractions exhibited the opposite trend. As a result, the starch:NDF ratios of the diets with low and high starch were equal to 0.68 and 0.89, respectively. According to NRC (2001), soybean hulls contain 60.3% NDF and 44.6% ADF on a DM basis; these values are similar to those observed in the present experiment (62.1 and 47.2% DM, respectively), although a wide variability is reported for soybean hull chemical composition (Ipharraguerre and Clark, 2003).

Table 4.1: Composition of the experimental diets (% of DM)

Composition	Diet[1]			
	LS	HS	LSO	HSO
Ingredient				
Corn Silage	29.4	29.4	29.4	29.4
Alfalfa hay	11.4	11.4	11.4	11.4
Meadow hay 2nd cut	9.1	9.1	9.1	9.1
Meadow hay 1st cut	1.8	1.8	1.8	1.8
Corn meal	18.2	24.7	17.4	23.9
Soybean hulls	13.5	7.0	13.5	7.0
Soybean meal (47.5% CP)	6.7	6.7	6.7	6.7
Canola meal	6.2	6.2	6.2	6.2
Salts[2]	1.4	1.4	1.4	1.4
Vitamin-mineral mix[3]	0.3	0.3	0.3	0.3
Nutri®-Met 50% Coated[4]	0.1	0.1	0.1	0.1
Fish oil	-	-	0.8	0.8
Cane molasses	1.9	1.9	1.9	1.9

[1]LS = low starch; HS = high starch; LSO = low starch supplemented with fish oil; HSO = high starch supplemented with fish oil.

[2]Salts: 41% sodium bicarbonate, 35% calcium carbonate, 10% magnesium oxide, 8% monocalcium phosphate, 6% sodium chloride.

[3]Provided (per kilogram): 720 mg Fe, 11100 mg Zn, 165 mg Cu, 55 mg Mn, 91 mg Se, 20 mg Co, 140 mg I, 1300 KIU vitamin A, 80 KIU vitamin D, and 9000 IU vitamin E.

[4]Rumen protected methionine (Nutriad Italia).

Table 4.2: Chemical analysis (% of DM, unless otherwise stated) and fatty acid profile (g/100 g of fatty acids) of the experimental diets

Item	Diet[1]			
	LS	HS	LSO	HSO
Chemical composition				
DM, %	62.4	62.3	62.5	62.4
Ash	7.1	6.8	7.1	6.8
CP	14.7	14.5	14.7	14.4
MP[2]	10.2	10.3	10.2	10.2
EE[3]	2.4	2.5	3.1	3.3
NDF[4]	34.7	31.2	34.5	31.1
ADF	24.7	21.8	24.6	21.7
ADL	5.5	5.2	5.5	5.2
Starch	23.8	28.0	23.2	27.4
Starch:NDF	0.7	0.9	0.7	0.9
ME[2] Mcal/kg DM	2.35	2.44	2.38	2.48
Fatty acid composition (g/100 g fatty acids)				
14:0	0.2	0.2	1.5	1.4
16:0	15.9	15.6	15.6	15.5
16:1 c9	0.6	0.5	1.9	1.8
18:0	2.8	2.5	2.9	2.7
18:1c9	22.2	22.6	23.8	24.0
18:1 c11	1.0	0.9	1.6	1.5
18:2 n-6	48.8	49.9	39.8	41.1
18:3 n-3	7.8	7.1	7.1	6.5
20:0	0.7	0.7	0.5	0.5
20:5 n-3	0.0	0.0	2.0	1.9
22:5 n-3	0.0	0.0	0.7	0.7
22:6 n-3	0.0	0.0	2.5	2.4

[1]LS = low starch; HS = high starch; LSO = low starch supplemented with fish oil; HSO = high starch supplemented with fish oil.
[2]Metabolisable protein and energy calculated according to the Cornell Net Carbohydrate and Protein System (CNCPS) version 6.1.
[3]Ether extract.
[4]NDF, corrected for insoluble ash and with the addition of α-amylase.

Milk Production

The results of DMI, milk yield and composition are reported in Table 4.3.

No differences among treatments were observed for DMI. Neither FO nor the utilization of soybean hulls negatively influenced DMI. The results of the present study are consistent with those reported by Keady and Mayne (1999), who found only a numerical decrease (P>0.05) in DMI when increasing the level of FO supplementation (from 0 to 450 g FO/head/d) in mature steer diets. In lactating cows, Keady et al. (2000) found a

negative effect of FO supplementation on DMI only at dosages higher than 300 g/d. It has often been proposed that the negative effect of lipid supplementation on DMI is mainly due to a depressive effect on ruminal fermentation or to a low palatability of fat supplements. However, this effect was not observed in the present work. Doreau and Chilliard (1997) found a decrease in feed intake as a result of the ruminal infusion of 300 mL/d of FO. In a second trial, the same authors reported a lower DMI when FO was administered to lactating dairy cows at a dosage of 400 mL/d but not at a supplementation of 200 mL/d (Doreau and Chilliard, 1997). Donovan et al. (2000) reported a similar DMI when dietary FO content was between 0 and 1% on DM in lactating cows, whereas higher levels (from 1 to 3%) significantly decreased DMI. The FO content of the diets used in our study was 0.8%, lower than the threshold value (1%) that caused negative feedback on DMI reported by Donovan et al. (2000). Based on the above results, it can be hypothesized that there is a dose-dependent effect of FO supplementation on DMI. Considering the use of soybean hulls instead of corn meal, the diets with the higher inclusion of soybean hulls had a higher NDF content, and a negative effect on DMI could be expected, as previous experiments reported a variety of DMI responses to the substitution of nonforage fiber for starch. In agreement with the present findings, DMI was not reduced by increasing levels of soybean hulls in the studies by Sarwar et al. (1992), Mansfield and Stern (1994) and Ipharraguerre et al. (2002b). Conversely, DMI was negatively affected by increasing the levels of fibrous by-products (soybean hulls and dried distiller grains with solubles) according to Ipharraguerre et al. (2002a), Aikman et al. (2006) and Ranathunga et al. (2010). Nakamura and Owen (1989) hypothesized that the dietary inclusion of soybean hulls may improve the digestion, the passage rate or the combination of these factors, thus counterbalancing the increase in NDF (rumen fill) because dietary fiber content is inversely and strongly correlated with DMI (Mertens, 1994). DMI and NDF correlate negatively when forages are the main or sole source of dietary NDF; moreover, soybean hulls contain a pool of potentially degradable NDF greater than that of most forages (Ranathunga et al., 2010) and have a high digestion rate (0.08/h) (Lanzas et al., 2007). Finally, the smaller particle size and higher specific gravity of soybean hulls can double the rumen passage rate compared with other forages (Ipharraguerre and Clark, 2003).

A linear trend was observed for an effect of FO supplement (P=0.10) on milk yield; in particular, FO led to a higher milk production compared with the diets without the supplement (29.2 vs 27.5 kg/d on average, respectively). Chilliard and Doreau (1997) did not find a negative effect of FO supplementation on milk yield, whereas Keady et al. (2000) found a higher milk yield as the level of FO in the diet increased. In our study, the dietary EE content was higher for the FO diet, and a recent meta-analysis (Rabiie et al.,

2012) showed that in different experiments (with different fat supplementation), milk production and milk fat percentage and yield increased as a response to fat feeding.
Milk production was not significantly affected by starch replacement with soybean hulls, which is consistent with Ipharraguerre et al. (2002a,b), Hindrichsen et al. (2005) and Ranathunga et al. (2010).
FPCM was affected (P=0.03) by the interaction between the factors tested: the LSO diet resulted in the highest FPCM production (31.3 kg/d), whereas the LS and HSO diets had the lowest value (28.7 kg/d on average). The FPCM production with the HS diet (29.6 kg/d) was not different from that of the LSO, LS and HSO diets. With regard to the percentage of milk fat, a significant interaction (P=0.05) between FO supplement (P=0.36) and starch content (P=0.09) was observed; the LSO and HS diets showed the highest percentages, followed by the LS and HSO diets. In contrast, Chilliard and Doreau (1997), Donovan et al. (2000) and Keady et al. (2000) found a significantly lower milk fat percentage as a consequence of FO supplementation, but the dosage used was higher than that used in the present experiment. These results, together with milk production, influenced fat yield (kg/d), which was significantly affected by both the starch content parameter (P=0.02) and the interaction (P<0.01); the LSO diet showed the highest value, followed by the HS, LS and finally by the HSO diets. Both milk fat percentage and yield were higher in the case of diets with the inclusion of soybean hulls: 4.45 vs 4.15% (P=0.09) and 1.21 vs 1.14 kg/d (P=0.02) on average, respectively. These data are consistent with Nakamura and Owen (1989), Sarwar et al. (1992) and Ipharraguerre et al. (2002a); in particular, milk fat percentage and yield normally increase when soybean hulls replace more than 30% of corn in high-grain diets. In addition, Ipharraguerre and Clark (2003) reported that the NDF from soybean hulls is effective for maintaining or slightly increasing milk fat percentage when soybean hulls are used to replace grain in cow diets.
In contrast, the percentage of milk protein was negatively influenced by FO (P<0.01); supplementation with FO resulted in a lower percentage of milk protein, on average, compared to the other diets (3.37%, on average, for the LSO and HSO diets vs 3.67% for the LS and HS diets). The present data are consistent with those reported by Chilliard and Doreau (1997) and Keady et al. (2000). The lower protein percentage was not related to a decrease in casein, as proposed by Keady et al. (2000). With regard to protein yield (kg/d), dairy efficiency (milk:DMI ratio) and the percentage of lactose, no differences among treatments were observed. The lack of dietary effects of soybean hull utilization in terms of protein and lactose, in terms of either percentage or yield, is consistent with previous research (Ranathunga et al., 2010). In contrast, a tendency for a higher lactose production (kg/d) (P=0.08) in the case of diets supplemented with FO

was observed. These data confirm the higher lactose yield resulting from FO supplementation reported by Keady et al. (2000). Milk urea nitrogen was numerically higher (P=0.11) for low-starch diets than for the HS and HSO treatments. This finding can be attributed to the slightly lower CP content and the concomitantly higher starch content of the HS and HSO diets in comparison with the other diets; in the HS and HSO diets, less ammonia was released in the rumen and more energy was available from rapidly fermented carbohydrates to convert NH_3-N into microbial protein N.

Table 4.3: Dry matter intake and milk yield of the cows fed the experimental diets

Item	Diet[1]				SE	P-value starch effect	P-value oil effect	P-value starch x oil effect
	LS	HS	LSO	HSO				
DMI, kg/d	22.8	22.7	23.7	22.2	0.96	0.34	0.79	0.44
Milk yield, kg/d	27.0	27.9	29.5	28.9	1.03	0.86	0.10	0.41
FPCM,[2] kg/d	28.7 ^{B}	29.6 ^{AB}	31.3 ^{A}	28.7 ^{B}	0.72	0.22	0.20	0.03
Milk/DMI	1.19	1.24	1.25	1.31	0.04	0.18	0.11	0.94
Fat, %	4.34 ^{AB}	4.40 ^{A}	4.55 ^{A}	3.90 ^{B}	0.17	0.09	0.36	0.05
Protein, %	3.77 ^{A}	3.57 ^{AB}	3.29 ^{C}	3.46 ^{BC}	0.07	0.81	<0.01	0.02
Lactose, %	4.92	4.92	4.98	4.96	0.07	0.83	0.44	0.88
Fat yield, kg/d	1.12 ^{BC}	1.19 ^{B}	1.30 ^{A}	1.09 ^{C}	0.02	0.02	0.12	<0.01
Protein yield, kg/d	1.02	1.00	0.97	0.99	0.04	0.91	0.43	0.51
Lactose yield, kg/d	1.29	1.33	1.43	1.39	0.05	0.97	0.08	0.40
Casein N, % total N	76.9	76.0	75.4	76.1	0.93	0.87	0.44	0.36
MUN, mg/dL	10.1	9.51	10.3	8.96	0.59	0.11	0.75	0.47

^{A,B,C} Least squares means within a row with different superscripts are significantly different (P<0.05). The Least squares means comparisons were tested when the interaction between the main effects was significant (P<0.05).

[1]LS=low starch; HS=high starch; LSO=low starch supplemented with fish oil; HSO=high starch supplemented with fish oil.

[2]Fat- and protein-corrected milk (4.0% fat and 3.3% protein) according to Gerber et al. (2010).

Milk fatty acid composition

The effects of dietary treatments on milk fatty acid composition are reported in Table 4.4.

As expected, the inclusion of FO led to an increase in trans fatty acids, which nearly doubled regardless of the level of starch in the diet and led to a significant increase in very long-chain PUFA n-3 (Table 4.4). However, with regard to omega-3 fatty acids, the level of enrichment was negligible compared with previous studies, which reported higher amounts of FO in the diet of dairy cattle. Abughazaleh et al. (2002) found levels of 20:5 n-3 and 22:6 n-3 exceeding 0.2 g/100 g of milk fatty acids when fish oil was

added to the diet at 2% of DM. In the present study, the intake of FO was nearly 190 g/d and 180 g/d for the LSO and HSO diets, respectively. The levels of 20:5 n-3 and 22:6 n-3 in milk fat were significantly higher than those found in milk from non-supplemented diets, which were lower than 0.06 g/100 milk fat (Table 4.4), similar to the levels reported by Abughazaleh et al. (2009) for milk samples obtained from cows fed 150 g/d of FO. Taking into consideration the content of 20:5 n-3 and 22:6 n-3 in FO (6.75 and 8.25 g/100 g oil, respectively) and the daily milk fat yield (Table 4.3), the average apparent transfer of these fatty acids from the diet to milk ranged from 2.45% for 22:6 n-3 in cows fed the HSO diet to 4.73% for 20:5 n-3 in cows fed the LSO diets. The transfer efficiency from diet to milk is usually low for 20:5 n-3 and 22:6 n-3 because of the high rate of rumen biohydrogenation and the preferential incorporation of these fatty acids into plasma phospholipids and cholesterol esters (Chilliard et al., 2007).

The increase in 18:1 t11 in milk fat also induced an increase in conjugated linoleic acid (CLA), i.e., milk c9, t11 CLA, which is mainly endogenously produced by the mammary desaturation of t11 18:1 by stearoyl-CoA desaturase enzyme (Bauman and Griinari, 2003). The content of c9, t11 CLA, in fact, nearly doubled in milk from cows fed LSO and HSO diets irrespective of the level of starch in the diet (Table 4.4). Previous research demonstrated that the stimulatory effect of fish oil on milk c9, t11 CLA is a consequence of the inhibition of 18:1 t11 biohydrogenation in the rumen (Shingfield et al., 2003). However, the level of the CLA enrichment was lower in the present study than that reported in previous trials, likely due to the lower amount of FO supplemented in the present trial. In a recent review, Shingfield et al. (2013) reported that the amount of CLA in milk fat may exceed 2% when FO is added at 200-300 g/d or when fish oil is supplemented in a blend with vegetable oils.

Dietary FO also resulted in an increase in 18:1 t10 in milk fat, whereas CLA t10, c12, the ruminant precursor of 18:1 t10 during biohydrogenation, was detected only in milk from cows fed the HSO diet (in a very small amount). Previous studies reported small or negligible increases in trans-10, cis-12 CLA with diets containing marine oils or high amounts of fermentable starch (Shingfield et al., 2013). In many cases, reductions in milk fat secretion have consistently been associated with an increase in milk CLA t10, c12 and, in some cases, in milk 18:1 t10. In the present experiment, a significant interaction effect between starch level and fish oil addition was observed on milk fat yield and content. The percentage decrease in milk fat yield in HSO diet compared to HS diet was close to the expected value obtained by applying the regression equation proposed by Shingfield et al. (2010) to explain the inhibitory effect of CLA t10, c12 on milk fat yield (8.4% and 10%, respectively). This result confirmed that, in dairy cows, CLA

t10, c12 is a potent inhibitor of milk fat synthesis and is effective in small amounts (Bauman and Griinari, 2003).

The content of saturated fatty acids (SFA) was negatively affected by FO supplementation, but in this case, a significant interaction effect with dietary starch was observed. In particular, the highest level of SFA was found in milk fat from the LS diet, whereas the level of SFA in milk from the HS diet did not differ from that from the LSO and HSO diets (Table 4.4). In particular, this trend was observed for 16:0, which is the main saturated fatty acid in milk, and, to a minor extent, for 8:0 and 10:0. Previous research reported similar effects of dietary FO on milk fatty acid composition but using higher amounts of fish oil in the diet (Abughazaleh et al., 2002).

The level of starch in the diet significantly affected the content of branched-chain fatty acids. In particular, the levels of iso 15:0 and iso 16:0 were higher in milk from diets with lower levels of starch (Table 4). Vlaeminck et al. (2006) reported that diets rich in starch reduced iso 14:0, iso 15:0 and iso 16:0 levels in milk fat. Recent studies highlighted that iso fatty acids are positively related to CH_4 emissions (Castro-Montoya et al., 2011). Iso fatty acids, in fact, are more abundant in cellulolytic bacteria (Vlaeminck et al., 2006), which in turn are usually related to higher CH_4 production.

Table 4.4: Milk fatty acid composition (g/100 g of milk fat) as affected by fish oil supplementation and dietary starch content

Item	Diet[1]				SE	P-value starch effect	P-value oil effect	P-value starch x oil effect
	LS	HS	LSO	HSO				
4:0	3.12	2.99	3.21	3.15	0.045	0.49	0.05	0.10
5:0	0.03	0.04	0.03	0.03	0.003	0.29	0.12	0.61
6:0	2.30	2.17	2.27	2.17	0.034	0.69	0.71	0.06
7:0	0.04	0.04	0.03	0.03	0.002	0.66	0.01	0.31
8:0	1.52 ^{A}	1.37 ^{A}	1.42 ^{AB}	1.33 ^{B}	0.031	0.11	0.35	0.01
10:0	3.84 ^{AB}	3.54 ^{A}	3.47 ^{AB}	3.18 ^{B}	0.075	0.89	0.01	0.01
10:1c9	0.27	0.29	0.24	0.21	0.016	0.74	0.04	0.21
11:0	0.09	0.10	0.06	0.06	0.007	0.46	0.01	0.52
12:0	4.16	4.49	3.93	3.74	0.127	0.62	0.01	0.12
13-iso	0.02	0.02	0.02	0.03	0.001	0.48	0.71	0.15
13-anteiso	0.05	0.04	0.05	0.05	0.008	0.87	0.97	0.99
12:1c9	0.06	0.08	0.05	0.05	0.006	0.53	0.04	0.23
13:0	0.11	0.11	0.08	0.08	0.004	0.77	0.01	0.88
14-iso	0.09	0.08	0.08	0.08	0.003	0.20	0.39	0.08
14:0	11.5	11.0	11.3	11.0	0.169	0.12	0.69	0.66

	LS	HS	LSO	HSO	SE	Starch	Fish Oil	S X FO
15-iso	0.25	0.21	0.24	0.21	0.005	0.01	0.69	0.51
15-anteiso	0.45	0.43	0.43	0.41	0.017	0.36	0.54	0.97
14:1c9	0.80	0.82	0.74	0.75	0.059	0.79	0.33	0.95
15:0	1.16	1.06	0.98	0.99	0.048	0.39	0.06	0.34
16-iso	0.20	0.17	0.21	0.20	0.006	0.04	0.03	0.20
16:0	31.4 A	26.6 B	27.1 B	26.8 B	0.535	0.01	0.01	0.01
other 16:1 trans	0.02	0.03	0.05	0.07	0.013	0.42	0.05	0.67
16:1t9	0.05	0.04	0.09	0.09	0.019	0.83	0.07	0.76
17iso	0.50	0.49	0.55	0.52	0.025	0.39	0.18	0.71
16:1 c9	1.17	1.27	1.05	1.12	0.074	0.33	0.15	0.87
17-anteiso	0.45	0.44	0.46	0.42	0.016	0.23	0.96	0.46
17:0	0.56	0.52	0.55	0.53	0.008	0.02	0.94	0.22
18-iso	0.04	0.04	0.05	0.05	0.004	0.86	0.18	0.96
17:1 c9	0.15	0.19	0.14	0.15	0.015	0.08	0.05	0.19
18:0	8.75	8.82	9.16	8.81	0.946	0.78	0.69	0.69
18:1 t4	0.02	0.02	0.03	0.03	0.004	0.86	0.03	0.62
18:1t5	0.01	0.02	0.03	0.03	0.004	0.99	0.01	0.59
18:1 t6-8	0.26	0.31	0.53	0.59	0.07	0.39	0.01	0.91
18:1 t9	0.19	0.23	0.41	0.44	0.047	0.48	<0.01	0.90
18:1 t10	0.34	0.47	0.72	1.01	0.173	0.28	0.05	0.67
18:1 t11	0.59	1.03	2.09	1.95	0.397	0.73	0.03	0.52
18:1 t12+t13+t14	0.37	0.40	0.80	0.82	0.079	0.72	<0.01	0.98
18:1 t15	0.36	0.45	0.56	0.56	0.054	0.44	0.03	0.42
18:1c9	14.9	17.6	14.8	15.3	0.748	0.12	0.23	0.27
18:1 c11	0.56	0.54	0.61	0.68	0.123	0.84	0.50	0.75
18:1 c12	0.28	0.32	0.40	0.36	0.02	0.89	0.01	0.10
18:2 t9,t12	0.20	0.21	0.25	0.23	0.02	0.91	0.14	0.51
18:2 t11,c15	0.03	0.04	0.11	0.13	0.025	0.49	0.02	0.84
18:2n-6	2.02 B	2.44 A	1.92 B	1.93 B	0.077	<0.01	<0.01	0.01
20:0	0.16	0.15	0.37	0.33	0.032	0.31	<0.01	0.62
18:3n-6	0.03	0.03	0.02	0.02	0.003	0.78	0.01	0.26
18:3 n-3	0.40 A	0.43 A	0.41 A	0.35 B	0.027	0.41	0.16	0.05
CLA 9,11 c/t	0.34	0.48	0.75	0.76	0.153	0.59	0.04	0.64
CLA 10,12 t/c	ND	ND	ND	0.01	0.001	9.45	<0.01	0.60
CLA 11,13 c/t	ND	ND	0.01	0.01	0.001	0.93	<0.01	0.64
21:0	0.03	0.02	0.03	0.03	0.003	0.13	<0.02	0.06

	LS	HS	LSO	HSO	SE	Starch	Fish Oil	S X FO
18:4n-3	0.02	0.02	0.02	0.02	0.002	0.91	0.32	0.26
20:2n-6	0.03	0.03	0.04	0.05	0.005	0.10	0.01	0.21
22:0	0.04	0.03	0.10	0.10	0.008	0.64	<0.01	0.53
20:3 n-6	0.11	0.14	0.09	0.09	0.008	0.10	0.01	0.21
20:3 n-3	0.00	0.00	0.04	0.04	0.005	0.37	<0.01	0.67
20:4 n-6	0.14	0.17	0.11	0.12	0.014	0.15	0.01	0.46
22:1 c9	0.01	0.00	0.03	0.03	0.004	0.59	<0.01	0.77
23:0	0.03	0.02	0.02	0.03	0.004	0.44	0.95	0.35
20:5 n.3	0.03 [B]	0.04 [AB]	0.05 [A]	0.04 [AB]	0.003	0.31	<0.01	0.03
22:4 n-3	0.02	0.03	0.02	0.02	0.004	0.23	0.04	0.72
22:5 n-3	0.06	0.07	0.08	0.09	0.007	0.16	0.01	0.53
22:6 n-3	0.02	0.01	0.03	0.03	0.003	0.99	0.01	0.47
SFA [2]	68.2 [A]	63.8 [B]	64.1 [B]	62.5 [B]	0.648	<0.01	<0.01	0.01
MUFA cis [3]	18.2	21.1	18.1	18.7	0.926	0.13	0.26	0.29
MUFA trans [4]	2.20	2.99	5.31	5.62	0.774	0.50	0.01	0.76
PUFA [5]	3.43 [B]	4.14 [A]	3.95 [AB]	3.95 [AB]	0.163	0.02	0.18	0.02
PUFA n-6 [6]	2.32 [B]	2.81 [A]	2.17 [B]	2.21 [B]	0.078	<0.01	<0.01	0.01
PUFA n-3 [7]	0.54	0.60	0.65	0.60	0.035	0.80	0.07	0.06
n-6:n-3 ratio	4.36	4.74	3.40	3.71	0.221	0.10	<0.01	0.83
BCFA [8]	2.05	1.92	2.10	1.97	0.124	0.04	0.31	0.99
BCFA iso [9]	1.10	1.01	1.16	1.09	0.062	0.03	0.07	0.68
BCFA ante [10]	0.94	0.91	0.94	0.89	0.064	0.16	0.71	0.62

[A, B]Least squares means within a row with different superscripts are significantly different (P<0.05). The Least squares means comparisons were tested when the interaction between the main effects was significant (P<0,05).

[1]LS=low starch; HS=high starch; LSO=low starch supplemented with fish oil; HSO=high starch supplemented with fish oil.

[2]Sum of saturated linear chain fatty acids from 4 to 23 carbon atoms.

[3]Sum of cis monounsaturated fatty acid from 10 to 22 carbon atoms.

[4]Sum of trans monounsaturated fatty acid from 16 to 18 carbon atoms.

[5]Sum of polyunsaturated fatty acids.

[6]Sum of omega-6 polyunsaturated fatty acids.

[7]Sum of omega-3 polyunsaturated fatty acids.

[8]Sum of branched-chain fatty acids.

[9]Sum of iso branched-chain fatty acids.

[10]Sum of anteiso branched-chain fatty acids.

Total-Tract Nutrients Digestibility

Nutrient digestibility is reported in Table 4.5. Dry matter, OM and energy digestibility values were significantly affected by both the main factors tested (starch content and FO), with the HS diet exhibiting the lowest values in comparison with the other dietary

treatments. This finding might be ascribed to the excellent quality of soybean hull fiber. Spanghero et al. (2010) reported values of NDF digestibility for soybean hulls of 74 and 90% after 30 and 48 h of *in vitro* incubation, respectively. This relation was confirmed by the review of Ipharraguerre and Clark (2003), who reported that the replacement of cereal grains with soybean hulls normally increases total-tract NDF digestibility, as the fiber fraction of soybean hulls is poorly lignified and has low concentrations of ferulic and p-coumaric acids, which are the primary phenolic monomers involved in the cross-linking between lignin and hemicelluloses (Garleb et al., 1988). Furthermore, the observed greater apparent total-tract nutrient digestibilities for the LS diet in comparison with the HS diet in the present study supports the review of Firkins (1997) and the study of Gencoglu et al. (2010), which reported that this effect was likely caused by reduced negative effects of starch on ruminal fermentation (Firkins, 1997). Overall, as summarized by Shaver (2008), lactation performance was reduced for diets with a starch content of 18% and 20% formulated using beet pulp and citrus pulp, respectively, to partially replace corn grain, whereas lactation performance was not reduced for 16%-17% starch diets formulated using soybean hulls to partially replace corn grain.

With regard to CP digestibility, the LSO and LS treatments showed the highest digestibility values because the starch content parameter significantly affected this variable. Fish oil supplementation revealed a trend ($P=0.09$) of a higher CP digestibility compared to diets without the presence of additional fat. Ether extract digestibility was significantly influenced ($P<0.01$) only by FO supplementation: the LSO and HSO treatments showed the highest EE digestibility values (on average, 67.1 vs 53.7% for the diets with or without fish oil, respectively). These results are consistent with those reported by Doreau and Chilliard (1997). As in the case of DM, OM and energy, NDF digestibility was influenced by both the factors studied; in particular, the LSO diet showed the highest value, followed by the LS and HSO diets and finally by the HS treatment. Starch digestibility was not influenced by either of the factors tested.

In general, the HS diet, characterized by the highest starch content, negatively influenced all the digestibility parameters measured, with starch as the only exception. The results of the present study are consistent with those reported by Doreau and Chilliard (1997), who found a trend for a higher (at 200 mL FO/d) and significantly higher (at 300 and 400 mL FO/d) DM, OM, NDF, ADF and EE digestibility values in dairy cows. The same results were also obtained by Keady et al. (2000), who reported a trend for a higher (at 150 g/d) and a significantly higher (at 300 g/d) DM and OM digestibility in dairy cows. The increase in digestibility resulting from the addition of lipids to ruminant diets is somewhat surprising, especially in case of fiber; generally, lipid supplementation decreases or does not modify digestibility values, as in case of the data reported by

Keady and Mayne (1999), who did not find a reduction in *in situ* DM, ADF or NDF digestibility of hay after 12 and 24 h of incubation. The absence of detrimental effects of FO treatment on *in situ* DM and fiber disappearance (Keady and Mayne, 1999) or total tract digestibility illustrates that the levels of FO supplementation commonly tested (up to 450 g/d) have little or no effect on bacteria and protozoa growth. No comprehensive explanation has been proposed for the increase in digestibility values consequent to FO supplementation. It can be hypothesized that one or more fatty acids present in FO (but not in other fat sources) might have a specific action on the metabolism of rumen microbes, particularly cell wall-degrading microorganisms, as proposed by Keady et al. (2000), or that FO does not modify rumen fermentation while increasing post-rumen (lower digestive tract) digestibility values.

Table 4.5: Total tract digestibility (%) of the experimental diets

Item	Diet[1]				SE	P-value starch effect	P-value oil effect	P-value starch x oil effect
	LS	HS	LSO	HSO				
DM	67.9	64.9	69.4	68.1	0.80	0.02	0.02	0.25
OM	69.6	66.4	71.1	69.7	0.80	0.02	0.02	0.24
CP	61.0	57.8	62.7	60.4	1.21	0.04	0.09	0.66
EE[2]	55.0	52.3	67.3	66.9	2.37	0.47	<0.01	0.60
NDF[3]	47.3	36.5	49.6	42.6	1.67	<0.01	0.03	0.22
Starch	96.3	96.7	95.9	96.8	0.38	0.10	0.60	0.48
Energy	67.3	63.9	69.4	67.7	0.89	0.02	0.01	0.32

[1]LS=low starch; HS=high starch; LSO=low starch supplemented with fish oil; HSO=high starch supplemented with fish oil.
[2]Ether extract.
[3]NDF, corrected for insoluble ash and with the addition of α-amylase.

Nitrogen Utilization and Excretion

The effects of dietary factors on variables related to N utilization and excretion are reported in Table 4.6. As expected, N intake was not different among diets, as they were formulated to be iso-nitrogenous. With regard to fecal excretion variables, no differences among treatments were observed in case of feces produced (kg DM/d) or total N excreted daily. In contrast, the percentage of N intake excreted with feces was influenced by both the presence of FO (P=0.09) and starch content factors (P=0.04): the HS diet showed the highest value, followed by the HSO and LS treatments and finally by the LSO diet, which was characterized by the highest N digestibility value.

Total excretion of urine (kg/d) was significantly influenced (P=0.01) by the starch content parameter; in particular, the experimental treatments richer in starch (HS and HSO) resulted in a lower daily urine production compared to the LS and LSO diets (18.8 vs 20.5 kg/d, on average, respectively). As expected, the same trend (P=0.06) was also

observed for the quantity of N excreted with urine (g/d). Dietary CP concentrations have been shown to affect urine excretion (Sannes et al., 2002; Broderick, 2003; Wattiaux and Karg, 2004) because a greater urine volume is required for excreting the excess N consumed by cows (Holter et al., 1982). The same holds true for the dietary ash content, as well as K concentration of diets: the mineral load that must be excreted largely determines the volume of urine, and a higher mineral concentration of the diet is generally associated with a higher urine production (Bannink et al., 1999). These three factors, a lower CP (14.4 vs 14.7% of DM, respectively), a lower dietary K (1.48 vs 1.54% of DM estimated through the CNCPS version 6.1, respectively) and ash content (6.8 vs 7.1% of DM, respectively), of the diets rich in starch (HS and HSO) in comparison with the diets low in starch (LS and LSO) are likely the reason for the lower urine yield and urinary N excretion of the cows fed the high-starch diets. No significant differences were observed for all the other variables related to N metabolism.

Table 4.6: Nitrogen balance of the cows fed the experimental diets

Item	Diet[1]				SE	P-value starch effect	P-value oil effect	P-value starch x oil effect
	LS	HS	LSO	HSO				
N intake, g/d	533	517	548	508	24.9	0.24	0.89	0.59
Fecal excretion								
DM, kg/d	7.35	8.00	7.26	7.10	0.34	0.42	0.14	0.22
Total N, g/d	207	219	203	202	11.3	0.60	0.31	0.50
Total N, % N intake	39.0	42.2	37.3	39.6	1.21	0.04	0.09	0.66
Urinary excretion								
Urine, kg/d	20.2	18.5	20.8	19.0	0.46	0.01	0.24	0.85
Total N, g/d	168	153	170	152	8.50	0.06	0.93	0.79
Total N, % N intake	31.8	29.8	31.8	30.1	1.48	0.21	0.90	0.90
Manure excretion								
Total N, g/d	375	372	373	353	16.4	0.44	0.50	0.55
Total N, % N intake	70.2	71.6	68.6	69.2	2.08	0.59	0.30	0.84
Milk excretion								
Total N, g/d	160	156	153	156	5.88	0.92	0.43	0.50
Total N, % N intake	29.9	30.3	27.7	30.5	1.05	0.13	0.31	0.24
N balance								
N retained, g/d	-2	-11	22	0	15.1	0.28	0.23	0.63
N retained, % N intake	-0.6	-2.3	3.2	-0.2	2.31	0.23	0.19	0.68

[1]LS = low starch; HS = high starch; LSO = low starch supplemented with fish oil; HSO = high starch supplemented with fish oil.

Methane Production

Dietary effects related to methanogenesis are reported in Table 4.7. The absolute values of daily CH_4 production are higher than those reported by Colombini et al. (2013) (405 vs 341 g/d, on average, respectively) because of a higher DMI recorded in the present experiment (22.9 vs 19.4 kg/d, on average, respectively). As expected, there was a trend (P=0.08) for a lower CH_4 emissions (expressed as g/d) with the HS and HSO diets compared with the LS and LSO diets (396 vs 415, on average, respectively). These data confirm the lower CH_4 production (Mcal/d) reported by Benchaar et al. (2001) resulting from the replacement of beet pulp with a starch concentrate (barley grain). In contrast, FO supplementation did not reduce methanogenesis. During an *in vitro* trial, Patra and Yu (2013) reported a reduction in CH_4 production as a consequence of FO supplementation within the fermentation *inoculum* at both doses tested (3.1 and 6.2 mL/L); these data agree with those from previous *in vitro* studies (e.g., Fievez et al., 2003; Jordan et al., 2006). However, this suppression of CH_4 production was accompanied by a marked reduction of DM and NDF digestibility values of feed substrate, which did not occur in the present work. To the best of our knowledge, no *in vivo* data are available on the effect of FO on methanogenesis. Among the *in vitro* experiments, Fievez et al. (2003) reported a reduction in CH_4 emissions (16%) as a consequence of FO injection in the rumen of cannulated wethers of 4.2% (w/w) on a dietary basis; this dose is 5.3 times higher than the supplementation used in the present work. Furthermore rumen CH_4 inhibition is positively related to the amount of FO supplementation with no further reductions at levels above a certain threshold (Fievez et al., 2003).

In the present study, the lack of a positive effect of FO on rumen CH_4 can be ascribed to the dose used and to the similar DMI among treatments. A recent meta-analysis study (Eugene et al., 2008) showed that cows fed lipid-supplemented diets had a lower CH_4 production than cows fed a control diet, mainly due to a decrease in DMI observed with lipid supplementation, whereas CH_4 production as a proportion of DMI was not affected by lipid supplementation.

Considering CH_4 emissions per kg of milk produced, a trend for a lower methanogenesis (P=0.09) relative to high-starch diets was also observed. This trend results from the lower milk yield consequent to LS diet utilization. In contrast, CH_4 production per kg of FPCM was significantly influenced (P<0.01) by the interaction between the main factors tested in the study: methanogenesis was higher for the LS and HSO diets in comparison with the HS and LSO treatments. This pattern is due to the fact that the former treatments resulted in a significantly lower FPCM production. In particular, although not significant, the LS diet resulted in a lower milk yield compared with the other dietary

treatments tested. In contrast, the HSO diet resulted in a lower percentage of both milk fat and protein. CH_4 production per kg of DMI or as a percentage of GE intake was not influenced by either of the factors tested in the present study. The present data regarding CH_4 emission as a percentage of GE intake are similar to the results of a previous work with lactating dairy cows (Colombini et al., 2013). Both CH_4 production per kg of NDF and digestible NDF ingested were significantly affected by the starch content factor: the HS and HSO treatments showed higher values compared with the LS and LSO diets. This finding must be ascribed to the fact that soybean hull supplementation, in comparison with high-starch diets, resulted in significantly higher levels of fiber (8.04 vs 7.02 kg/d, respectively; P=0.01) and digestible NDF intake (3.89 vs 2.78 kg/d, respectively; P=0.01). Although the LS and LSO treatments tended to produce more CH_4 in terms of absolute value (+4.6%) because soybean hulls produce more CH_4 than corn meal (Lee et al., 2003), this higher CH_4 emissions level was counterbalanced by higher levels of fiber and digestible NDF consumed (+12.7 and +28.5%, respectively). The same results were also obtained by Hindrichsen et al. (2004, 2005), as digestible fiber is considered to be the major contributor to methanogenesis in terms of absolute values.

Overall, there was a clear trend for a decrease in CH_4 emissions with an increasing DMI (R^2=0.68; P<0.001) and milk yield (R^2=0.69; P<0.001) (Figure 13).

Table 4.7: Methane production from the cows fed the experimental diets

Item	Diet[1]				SE	P-value starch effect	P-value oil effect	P-value starch x oil effect
	LS	HS	LSO	HSO				
CH_4, g/d	415	392	415	400	10.6	0.08	0.67	0.67
CH_4, g/kg DMI	18.3	17.4	17.9	18.3	0.58	0.54	0.63	0.23
CH_4, g/kg milk	15.4	14.1	14.3	14.1	0.45	0.09	0.21	0.20
CH_4, g/kg FPCM[2]	14.5 [A]	13.3 [B]	13.5 [B]	14.2 [A]	0.19	0.15	0.80	<0.01
CH_4, % GE Intake	5.64	5.33	5.46	5.55	0.18	0.48	0.88	0.23
CH_4, g/kg NDF Intake[3]	53.4	55.7	52.2	58.9	2.07	0.05	0.58	0.26
CH_4, g/kg dNDF Intake[4]	109	156	106	140	10.8	0.01	0.32	0.51

[A,B] Least squares means within a row with different superscripts are significantly different (P<0.05). The Least squares means comparisons were tested when the interaction between the main effects was significant (P<0.05).

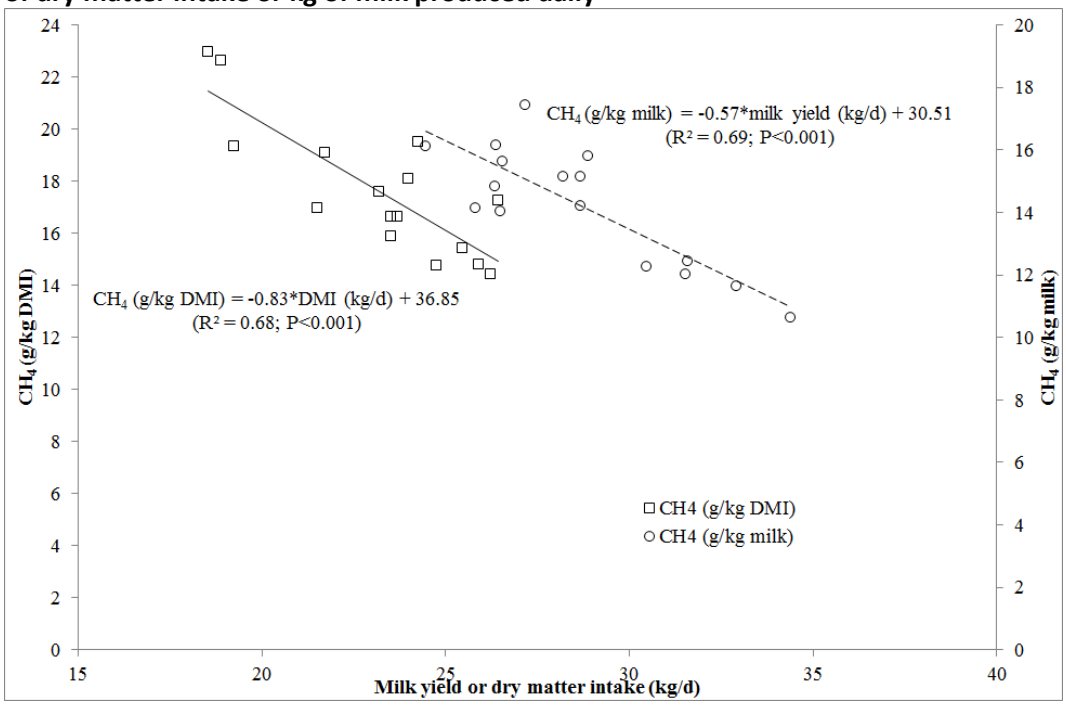
[1]LS=low starch; HS=high starch; LSO=low starch supplemented with fish oil; HSO=high starch supplemented with fish oil.

[2]Fat- and protein-corrected milk (4.0% fat and 3.3% protein) according to Gerber et al. (2010).

[3]NDF, corrected for insoluble ash and with the addition of α-amylase.

[4]Digestible NDF.

Figure 13: Relationship between milk yield or dry matter intake (kg/d) and methane emissions per kg of dry matter intake or kg of milk produced daily



Rumen fermentation parameters

Data regarding rumen fermentation parameters are reported in Table 4.8. With regard to total VFA production, a numerical (P=0.06) reduction associated with the HS and LSO dietary treatments compared to the LS and HSO diets can be observed. This finding can be attributed primarily to the numerically lower concentration of either acetate, propionate and butyrate associated with the HS and LSO diets. Acetate, propionate and butyrate, expressed as a percentage of total VFA, as well as the acetate:propionate ratio and rumen pH, were not significantly affected by starch content or the presence of FO. Hindrichsen et al. (2004) did not find differences in terms of acetate and propionate proportions between diets based on soybean hulls (richer in fiber) or wheat (with a higher starch content) during an *in vitro* trial. In contrast, in a review on the effects of soybean hulls used as an alternative feed for lactating dairy cows, Ipharraguerre and Clark (2003) reported that the substitution of soybean hulls for corn diminished the molar proportion of propionate and butyrate and enhanced that of acetate, with consequences for the acetate:propionate ratio. The same results were also obtained by Ipharraguerre et al. (2002b). Despite this shift in the rumen fermentation pattern, the use of soybean hulls in place of grains usually failed to affect the pH of rumen fluid (Ipharraguerre and Clark, 2003). Consistent with the present results, Doreau and Chilliard (1997), Keady and Mayne (1999) and Fievez et al. (2003) did not find pH or total VFA variations related to FO supplementation. However, Doreau and Chilliard (1997) and Fievez et al. (2003) reported a lower acetate proportion and a higher propionate

concentration in diets supplemented with fish oil, with lower acetate:propionate ratios in the case of FO utilization. The same results were also obtained in a second trial, with the shift in acetate and propionate percentages significant with a 400 mL supplementation, and a numerical tendency was found with a 200 mL supplementation (Doreau and Chilliard, 1997). In contrast, Keady and Mayne (1999) reported that FO utilization had no effect on the composition of volatile fatty acids in the rumen liquor in terms of acetate, propionate, butyrate or the acetate:propionate ratio. Keady and Mayne (1999) suggested that the absence of any effect of fish oil on rumen VFA may be associated with the feeding management used during the experiment. In the present study, the fish oil was offered in two equal amounts per day, reducing total oil intake at any one period and consequently reducing potential changes in rumen fermentation pattern.

The ammonia nitrogen concentration (mmol/L) observed in the present study was, on average, higher than the values reported by Keady and Mayne (1999) in the case of diets supplemented with FO and higher than the values reported by and Ipharraguerre et al. (2002b) in the case of soybean hull replacement for corn grain. Ammonia concentration was affected (P=0.05) by the presence of FO: the LSO and HSO treatments resulted in higher concentrations than the diets without additional fat supplementation (16.3 vs 11.2 mmol/L, on average, respectively). We have no adequate explanation for this finding, but the data are consistent with those reported by Keady and Mayne (1999). In contrast, the effect of soybean hulls fed to dairy cows in place of grains on rumen fluid N-NH_3 concentration is inconsistent; various authors have reported a decrease (Mansfield and Stern, 1994), an increase (Ipharraguerre et al., 2002b) or no difference (Elliott et al., 1995).

Table 4.8: Rumen fermentation parameters

Item	Diet[1]				SE	P-value starch effect	P-value oil effect	P-value starch x oil effect
	LS	HS	LSO	HSO				
Acetate, mmol/L	66.2	52.4	48.0	63.5	8.42	0.91	0.64	0.09
Propionate, mmol/L	18.7	13.9	12.8	16.4	2.41	0.80	0.45	0.09
Isobutyric acid, mmol/L	1.03	0.85	0.70	0.94	0.22	0.90	0.54	0.32
Butyrate, mmol/L	10.7	9.86	8.37	10.8	1.01	0.38	0.46	0.11
Isovaleric acid, mmol/L	1.56	1.32	1.53	2.07	0.32	0.60	0.24	0.21
n-valeric acid, mmol/L	1.06	0.99	0.82	1.36	0.24	0.30	0.76	0.19
VFA, mmol/L	99.2	79.3	72.2	95.1	10.8	0.88	0.56	0.06
Acetate, % VFA	67.6	65.6	66.7	67.1	2.65	0.72	0.88	0.61
Propionate, % VFA	17.8	17.8	18.0	17.5	1.85	0.88	0.99	0.91
Isobutyric acid, % VFA	1.12	1.02	0.86	0.94	0.23	0.96	0.40	0.65
Butyrate, % VFA	10.5	12.7	11.4	10.9	1.48	0.52	0.73	0.32
Isovaleric acid, % VFA	1.79	1.73	2.02	2.18	0.50	0.91	0.46	0.80
n-valeric acid, % VFA	1.20	1.25	1.04	1.36	0.27	0.45	0.91	0.55
Acetate:Propionate	3.90	3.89	3.86	3.88	0.42	0.97	0.95	0.97
pH	6.96	7.04	7.01	7.01	0.09	0.67	0.87	0.60
Ammonia N, mmol/L	9.91	12.5	14.7	18.0	2.44	0.20	0.05	0.86

[1]LS = low starch; HS = high starch; LSO = low starch supplemented with fish oil; HSO = high starch supplemented with fish oil.

4.5 CONCLUSIONS

Formulating diets with a fairly high starch content can be effective in reducing methane emissions from lactating dairy cows both in absolute values and per kg of milk produced. Fish oil, at the dosage tested (0.8% of DMI), enhanced milk yield and positively decreased the n-6:n-3 ratio of the milk PUFA but did not reduce methane emissions. In general, our findings confirm that diet manipulation is less effective in reducing CH_4 emissions per kg milk compared with the reduction obtainable by increasing individual milk yield.

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4.7 REFERENCES

- AbuGhazaleh, A.A., Potu, R.B., Ibrahim, S., 2009. Short communication: The effect of substituting fish oil in dairy cow diets with docosahexaenoic acid-microalgae on milk composition and fatty acids profile. J. Dairy Sci. 92:6156–6159.
- AbuGhazaleh, A.A., Schingoethe, D.J., Hippen, A.R., Kalscheur, K.F., Whitlockm L.A., 2002. Fatty acid profiles of milk and rumen digesta from cows fed fish oil, extruded soybeans or their blend. J. Dairy Sci. 85:2266–2276.
- Aikman, P.C., Beever, D.E., Humphries, D.J., 2006. The effect of incremental replacement of wheat with soya hulls in diets for Jersey cows on lactational performance, diet digestibility and feeding behaviour. Livest. Sci. 104:23–32.
- AOAC (Association of Official Analytical Chemists) 1995. Official Methods of Analysis. 15th ed. AOAC, Washington, DC.
- AOAC (Association of Official Analytical Chemists) 1998. Official Methods of Analysis. 16th ed. Supplement 1998. AOAC, Washington, DC.
- Andrew, S.M., Tyrrell H.F., Reynolds, C.K., Erdman, R.A., 1991. Net energy value for lactation of a dietary fat, calcium salts of long chain fatty acids, for cows fed silage based diets. J. Dairy Sci. 74:2588-2600.
- Bannink, A., Valk, H. Van Vuuren, A.M., 1999. Intake and excretion od sodium, potassium, and nitrogen and the effects on urine production by lactating dairy cows. J. Dairy Sci. 82:1008-1018.
- Bauman, D.E., Griinari, J.M., 2003. Nutritional regulation of milk fat synthesis. Annu. Rev. Nutr. 23:203–227.
- Beauchemin, K.A., McGinn, S.M., 2005. Methane emissions from feedlot cattle fed barley or corn diets. J Anim. Sci. 83:653–661.
- Beauchemin, K.A., Kreuzer, M., O'Mara, F., McAllister, T.A., 2008. Nutritional management for enteric methane abatement: a review. Aust. J. Ep. Agric. 48:21–27.
- Benchaar, C., Pomar, C., Chiquette, J., 2001. Evaluation of dietary strategies to reduce methane production in ruminants: a modeling approach. Can. J. Anim. Sci. 81:563–574.
- Buccioni, A., Minieri, S., Conte, G., Benvenuti, D., Pezzati, A., Antongiovanni, M., Rapaccini, S., Mele, M., 2012. Changes in conjugated linoleic acid and C18:1 isomers profile during the ripening of Pecorino Toscano cheese produced with raw milk. Ital. J. Anim. Sci. 11: 426-430.
- Cassandro M., Mele M., Stefanon B., 2013. Genetic aspects of enteric methane emission in ruminants livestock. Ital. J. Anim. Sci. 12: 450-458.
- Castro-Montoya, J.M., Bhagwat, A.M., Peiren, N., De Campeneere, S., De Baets, B., Fievez, V., 2011. Relationship between odd- and branched-chain fatty acid profiles in milk and calculated enteric methane proportion for lactating dairy. Anim. Feed Sci. Technol. 166–167: 596–602.
- Castro-Montoya, J., De Campeneere, S., Van Ranst, G., Fievez, V., 2012. Interactions between methane mitigation additives and basal substrates on in vitro methane and VFA production. Anim. Feed Sci. Technol. 176:47–60.

- Chilliard, Y., Doreau, M., 1997. Effects of ruminal or postruminal fish oil supply on cow milk yield and composition. Reprod. Nutr. Dev. 37:338-339.
- Chilliard, Y., Glasser, F., Ferlay, A., Bernard, L., Rouel, J., Doreau, M., 2007. Diet, rumen biohydrogenation, cow and goat milk fat nutritional quality: A review. Eur. J. Lipid Sci. Technol. 109:828–855.
- Christie, W.W., 1982. A simple procedure of rapid transmethylation of glycerolipids and cholesteryl esters. J. Lipid Res. 23:1072-1075.
- Colombini, S., Galassi, G. Crovetto, G.M., Rapetti, L., 2012. Milk production, nitrogen balance, and fiber digestibility prediction of corn, whole plant grain sorghum, and forage sorghum silages in the dairy cow. J. Dairy Sci. 96:4457-4467.
- Colombini, S., Rapetti, L., Galassi, G., Malagutti, L., Pirondini, M., Crovetto G.M., 2013. Methane emission from lactating cows fed diets with different forage base. Pages 499-500 in Energy and protein metabolism and nutrition in sustainable animal production. EAAP publication No. 134. Wageningen Academic Publishers, Wageningen, The Netherlands.
- Crompton, L.A., Mills, J.A.N., Reynolds, C.K., 2010. Effect of feeding frequency and replacing calcium salts of palm oil with crushed rapeseed or coconut oil on methane emissions in lactating dairy cows. Page E329 in Proc. Nutr. Soc.
- Donovan, D.C., Schingoethe, D.J., Baer, R.J., Ryali, J., Hippen, A.R., Franklin, S.T., 2000. Influence on dietary fish oil on conjugated linoleic acid and other fatty acids in milk fat from lactating dairy cows. J. Dairy Sci. 83:2620-2628.
- Doreau, M., Chilliard, Y., 1997. Effects of ruminal or postruminal fish oil supplementation on intake and digestion in dairy cows. Reprod. Nutr. Dev. 37:113-124.
- Elliott, J.P., Drackley, J.K., Fahey, Jr. G.C., Shanks, R.D., 1995. Utilization of supplemental fat by dairy cows fed diets varying in content of nonstructural carbohydrates. J. Dairy Sci. 78:1512–1525.
- Eugene, M., Masse, D., Chiquette, J., Benchaar, C., 2008. Short communication: Meta-analysis on the effects of lipid supplementation on methane production in lactating dairy cows. Can. J. Anim. Sci. 88:331-334.
- Fievez, V., Boeckaert, C., Vlaeminck, B., Mestdagh, J., Demeyer, D., 2007. In vitro examination of DHA-edible micro-algae 2. Effect on rumen methane production and apparent degradability of hay. Anim. Feed Sci. Technol.136:80–95.
- Fievez, V., Dohme, F., Danneels, M., Raes, K., Demeyer, D., 2003. Fish oils as potent rumen methane inhibitors and associated effects on rumen fermentation in vitro and in vivo. Anim. Feed Sci. Technol. 104:41-58.
- Firkins, J.L., 1997. Effects of feeding nonforage fiber sources on site of fiber digestion. J. Dairy Sci. 80:1426–1437.
- Garleb, K.A., Fahey, Jr. G.C., Lewis, S.M., Kerley, M.S., Montgomery, L., 1988. Chemical composition and digestibility of fiber fractions of certain by-product feedstuffs fed to ruminants. J. Anim. Sci. 66:2650–2662.
- Gencoglu, H., Shaver, R.D., Steinberg, W., Ensink, J., Ferraretto, L.F., Bertics, S.J., Lopes, J.C., Akins, M.S., 2010. Effect of feeding a reduced-starch diet with or

without amylase addition on lactation performance in dairy cows. J. Dairy Sci. 93:723-732.
- Gerber, P., Vellinga, T., Opio, C., Henderson, B., Steinfeld, H., 2010. Greenhouse gas emissions from the dairy sector, a Life Cycle Assessment. FAO Food and Agriculture Organization of the United Nations, Animal Production and Health Division, Rome.
- Grainger, C., Beauchemin, K.A., 2011. Can enteric methane emissions from ruminants be lowered without lowering their production? Anim. Feed Sci. Technol. 166-167:308-320.
- Hindrichsen, I.K., Wettstein, H.R., Machmuller, A., Jorg, B., Kreuzer, M., 2005. Effect of the carbohydrate composition of feed concentratates on methane emission from dairy cows and their slurry. Environ. Monit. Assess. 107:329-350.
- Hindrichsen, I.K., Wettstein, H.R., Machmuller, A., Soliva, C.R., Bach Knudsen, K.E., Madsen, J., Kreuzer, M., 2004. Effects of feed carbohydrates with contrasting properties on rumen fermentation and methane release in vitro. Can. J. Anim. Sci. 84:265-276.
- Holter, J.B., Byrne, J.A., Schwab, C.G., 1982. Crude protein for high milk production. J. Dairy Sci. 65:1175–1188.
- Ipharraguerre, I.R., Clark, J.H., 2003. Soyhulls as an alternative feed for lactating dairy cows: A review. J. Dairy Sci. 86:1052–1073.
- Ipharraguerre, I.R., Ipharraguerre, R.R., Clark, J.H., 2002a. Performance of lactating dairy cows fed varying amounts. J. Dairy Sci. 85:2905–2912.
- Ipharraguerre, I.R., Shabi, Z., Clark, J.H., Freeman, D.E., 2002b. Ruminal fermentation and nutrient digestion by dairy cows fed varying amounts of soyhulls as a replacement for corn grain. J. Dairy Sci. 85:2890–2904.
- Iqbal, M.F., Cheng, Y.F., Zhu, W.Y., Zeshan, B., 2008. Mitigation of ruminant methane production: current strategies, constraints and future options. World J. Microbiol. Biotechnol. 24:2747–2755.
- Italian Ministry of Health. 1992. Protezione degli animali utilizzati a fini sperimentali o ad altri fini scientifici. D.Lgs 116/92. Gazzetta Ufficiale 40:5-25. (Rome, Italy).
- ISO (International Organization for Standardization) 1976. Milk: determination of fat content (Gerber method). ISO, Geneva, Switzerland.
- ISO (International Organization for Standardization) 2006. Milk: Determination of urea content. Enzymatic method using difference in pH (Reference method). ISO, Geneva, Switzerland.
- Jarvis, G.N., Strompi, C., Burgess, D.M., Skillman, L.C., Moore, E.R.B., Joblin, K.N., 2000. Isolation and identification of ruminal methanogens from grazing cattle. Curr. Microbiol. 40:327-332.
- Johnson, K.A., Johnson, D.E., 1995. Methane emissions from cattle. J. Anim. Sci. 73:2483–2492.
- Jordan, E., Lovett, D.K., Monahan, F.J., Callan, J., Flynn, B., O'Mara F.P., 2006. Effect of refined coconut oil or copra meal on methane output and on intake and performance of beef heifers. J. Anim. Sci. 84:162–170

- Keady, T.W.J., Mayne, C.S., 1999. The effects of level of fish oil inclusion in the diet on rumen digestion and fermentation parameters in cattle offered grass silage based diets. Anim. Feed Sci. Technol. 81:57-68.
- Keady, T.W.J., Mayne, C.S., Fitzpatrick, D.A., 2000. Effects of supplementation of dairy cattle with fish oil on silage intake, milk yield and milk composition. J. Dairy Res. 67:137-153.
- Kramer, J.K., Hernandez, M., Hernandez, C.C., Kraft, J., Dugan, M.E., 2008. Combining results of two GC separations partly achieves determination of all cis and trans 16:1, 18:1, 18:2 and 18:3 except CLA isomers of milk fat as demonstrated using Ag-ion SPE fractionation. Lipids 43: 259-273.
- Lanzas, C., Sniffen, C.J., Seo, S., Tedeschi, L.O., Fox, D.G., 2007. A revised CNCPS feed carbohydrate fractionation scheme for formulating rations for ruminants. Anim. Feed Sci. Technol. 136:167-190.
- Lee, H.J., Lee, S.C., Kim, J.D., Oh, Y.G., Kim, B.K., Kim, C.W., Kim, K.J., 2003. Methane production potential of feed ingredients as measured by in vitro gas test. Asian-Aust. J. Anim. Sci. 16:1143-1150.
- Lee, S.C., Lee, H.J., Oh, Y.K., 2000. Methane production from enteric fermentation in ruminants. J. Anim. Sci. 13 (Special issue):171-181.
- Liu, S.J., Bu, D.P., Wang, J.Q., Liu, L., Liang, S., Wei, H.Y., Zhou, L.Y., Li, D., Loor, J.J., 2012. Effect of incremental levels of fish oil supplementation on specific bacterial populations in bovine ruminal fluid. J. Anim. Physiol. Anim. Nutr. 96:9–16.
- Lovett, D.K., Stack, L.J., Lovell, S., Callan J., Flynn, B., Hawkins, M., O'Mara, F.P., 2005. Manipulating enteric methane emissions and animal performance of late-lactation dairy cows through concentrate supplementation at pasture. J Dairy Sci 88:2836–2842.
- Machmuller, A., 2006. Medium chain fatty acids and their potential to reduce methanogenesis in domestic ruminants. Agr. Ecosyst. Environ. 112:107-114.
- Mansfield, H.R., Stern, M.D., 1994. Effects of soybean hulls and lignosulfonate-treated soybean meal on ruminal fermentation in lactating dairy cows. J. Dairy Sci. 77:1070–1083.
- Martin, C., Rouel, J., Jouany, J.P., Doreau, M., Chilliard, Y., 2008. Methane output and diet digestibility in response to feeding dairy cows crude linseed, extruded linseed, or linessed oil. J. Anim. Sci. 86:2642-2650.
- Martin, C., Morgavi, D.P., Doreau, M., 2010. Methane mitigation in ruminants: from microbe to the farm scale. Animal 4-3:351-365.
- McGinn, S.M., Beauchemin, K.A., Coates, T., Colombatto, D., 2004. Methane emissions from beef cattle: Effects of monensin, sunflower oil, enzymes, yeast, and fumaric acid. J. Anim. Sci. 82:3346–3356.
- Mele, M., Serra, A., Buccioni, A., Conte, G., Pollicardo, A., Secchiari, P., 2008. Effect of soybean oil supplementation on milk fatty acid composition from Saanen goats fed diets with different forage:concentrate ratios. Ital. J. Anim. Sci. 7:297-311.

- Mele, M., 2009. Designing milk fat to improve healthfulness and functional properties of dairy products: from feeding strategies to a genetic approach. Ital. J. Anim. Sci. 8:365-373.
- Mertens, D.R., 1994. Regulation of feed intake. Pages 450–493 in Forage Quality, Evaluation, and Utilization. G. C. Fahey, ed. American Society of Agronomy Inc., Madison, WI.
- Mertens, D.R., 2002. Gravimetric determination of amylase-treated neutral detergent fiber in feeds using refluxing in beakers or crucibles: collaborative study. J. AOAC Int. 85:1217-1240.
- Moate, P.J., Williams, S.R.O., Hannah, M.C., Eckard, R.J., Auldist, M.J., Ribaux, B.E., Jacobs, J.L., Wales, W.J., 2013. Effects of feeding algal meal high in docosahexaenoic acid on feed intake, milk production, and methane emissions in dairy cows. J. Dairy Sci. 96:3177-3188.
- Moss, A.R., Jouany, J.P., Newbold, J., 2000. Methane production by ruminants: its contribution to global warming. Ann. Zootech. 49:231-253.
- Nakamura, T., Owen, F.G., 1989. High amounts of soyhulls for pelleted concentrate diets. J. Dairy Sci. 72:988–994.
- National Research Council. 2001. Nutrient Requirements of Dairy Cattle. 7th rev. ed. Natl. Acad. Sci., Washington, DC.
- Patra, A.K., Yu, Z., 2013. Effects of coconut and fish oils on ruminal methanogenesis, fermentation, and abundance and diversity of microbial populations in vitro. J. Dairy Sci. 96:1782–1792.
- Pirondini, M., Malagutti, L., Colombini, S., Amodeo, P., Crovetto, G.M., 2012. Methane yield from dry and lactating cows diets in the Po Plain (Italy) using an in vitro gas production technique. Ital. J. Anim. Sci. 11-e61:330-335.
- Rabiee, A. R., Breinhid, K., Scott, W., Golder, H.M., Block, E., Lean, I.J., 2012. Effect of fat additions to diets of dairy cattle on milk production and components: A meta-analysis and meta-regression. J. Dairy Sci. 95:3225-3247.
- Ranathunga, S.D., Kalscheur, K.F., Hippen, A.R., Schingoethe, D.J., 2010. Replacement of starch from corn with nonforage fiber from distillers grains and soyhulls in diets of lactating dairy cows. J. Dairy Sci. 93:1086–1097.
- Reynolds, C.K., Mills, J.A.N., Crompton, L.A., Givens, D.I., Bannink, A., 2010. Ruminant nutrition regimes to reduce greenhouse gas emissions in dairy cows. Pages 427-437 in Energy and protein metabolism and nutrition. EAAP publication No. 127. G. M. Crovetto, ed. Wageningen Academic Publishers, Wageningen, the Netherlands.
- Sarwar, M., Firkins, J.L., Eastridge, M.L., 1992. Effects of varying forage or concentrate carbohydrates on nutrient digestibilities and milk production by dairy cows. J. Dairy Sci. 75:1533–1542.
- SAS Institute. 2001. User's Guide: Statistics. Release 8.01. SAS Inst. Inc., Cary, NC.
- Shaver, R.D., 2008. Coping with high corn prices: Low starch diets and lactation performance by dairy cows. Pages 128–133 in Proc. 6th Mid-Atlantic Nutr. Conf., Timonium, MD.

- Shingfield, K.J., Ahvenjärvi, S., Toivonen, V., Ärölä, A., Nurmela, K.V.V., Huhtanen, P., Griinari, J.M., 2003. Effect of dietary fish oil on biohydrogenation of fatty acids and milk fatty acid content in cows. Anim. Sci. 77:165-179.
- Shingfield, K.J, Bernard, L., Leroux, C., Chilliard, Y., 2010. Role of trans fatty acids in the nutritional regulation of mammary lipogenesis in ruminants. Animal. 4:1140–1166.
- Shingfield, K.J., Bonnet, M., Scollan, N.D., 2013. Recent developments in altering the fatty acid composition of ruminant-derived foods. Animal. 7:132-162.
- Spanghero, M., Berzaghi, P., Fortina, R., Masoero, F., Rapetti, L., Zanfi, C., Tassone, S., Gallo, A., Colombini, S., Ferlito, J.C., 2010. Technical note: Precision and accuracy of in vitro digestion of neutral detergent fiber and predicted net energy of lactation content of fibrous feeds. J. Dairy Sci. 93: 4855-4859.
- Tyrrell, H.F., Reynolds, C.K., Baxter, H.D., 1991. Energy metabolism of Jersey and Holstein cows fed total mixed diets with or without whole cottonseed. J. Dairy Sci. 74 (Suppl.2):192.
- Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods of dietary fiber, neutral detergent fiber and non-polysaccharides in relation to animal nutrition. J. Dairy Sci. 74:3583-3597.
- Vlaeminck, B., Fievez, V., Cabrita, A.R.J., Fonseca, A.J.M., Dewhurst, R.J., 2006. Factors affecting odd- and branched-chain fatty acids in milk: a review. Anim. Feed Sci. Technol. 131:389–417.
- Yan, T, Agnew, R.E., Gordon, F.J., Porter, M.G., 2000 Prediction of methane energy output in dairy and beef cattle offered grass silage based diets. Livest. Prod. Sci. 64:253–263.

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