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NUTRITIONAL STRATEGIES TO REDUCE METHANE EMISSIONS IN DAIRY COWS USING *IN VITRO* TECHNIQUE

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ABBREVIATIONS

ADF = acid detergent fibre

aNDF = amylase-treated neutral detergent fibre

CH_4 = methane

CO_2 = carbon dioxide

CP = crude protein

DM = dry matter

DMI = dry matter intake

EPA = Environmental Protection Agency of United States

FAO = Food and Agriculture Organization of the United Nations

FM = weight as fed

GC = gas chromatography

GHG= greenhouse gases

GP = gas production

H_2 = hydrogen

IVGPT = *in vitro* gas production technique

NDF = neutral detergent fibre

NDFd = neutral detergent fibre degradability

$N\text{-}NH_3$ = ammonia nitrogen

NO_2 = nitrous oxide

OM= organic matter

RUSITEC = RUmen Simulation TEChnique

SF_6 = sulphur hexafluoride

SS = sostanza secca

TDMd = true dry matter degradability

VFA = volatile fatty acid

RIASSUNTO

Il recente scenario del riscaldamento climatico globale, ha portato ad un maggiore interesse dell'opinione pubblica riguardo ai gas che causano l'effetto serra, ponendo particolare attenzione al gas metano (CH_4) e alla sua relazione con il settore zootecnico. Per questa ragione, numerosi gruppi di ricerca hanno proposto delle strategie nutrizionali per ridurre le emissioni di CH_4 da parte dei ruminanti.

Nella presente tesi sono stati sviluppati due obiettivi principali. Il primo riguardava lo studio dei fattori metodologici che influenzano la produzione *in vitro* del CH_4 e lo sviluppo di un nuovo approccio per la misurazione dello stesso gas prodotto durante delle fermentazioni *in vitro*. In secondo luogo, si è inteso valutare l'efficacia di alcune strategie nutrizionali sulla riduzione della produzione di CH_4. Tali obiettivi sono stati raggiunti attraverso cinque diverse attività di ricerca.

Nel primo lavoro è stato valutato l'effetto che specifici fattori metodologici possono avere sulla produzione *in vitro* di gas e di CH_4 (espresso sia in termini di produzione che di proporzione). Questa analisi è stata effettuata attraverso un approccio di meta-analisi utilizzando 274 osservazioni, che rappresentavano i trattamenti di controllo di 39 articoli scientifici pubblicati negli ultimi 12 anni. I fattori considerati erano: la pressione che si forma nello spazio di testa dello strumento utilizzato per le fermentazioni *in vitro* (costante o incrementale), il tempo di incubazione (24 o ≥48 ore), il momento di raccolta del liquido ruminale (prima o dopo la somministrazione del pasto agli animali donatori), la presenza di azoto nella composizione della saliva artificiale (presenza o assenza), e il rapporto tra la miscela di liquido ruminale e saliva artificiale sul campione alimentare incubato (130 or 130-140 or >140 ml/g DM). Questi cinque fattori sono stati considerati come variabili discrete, invece il contenuto di NDF del campione alimentare incubato (392 ± 175.3 g/kg) è stato considerato come variabile continua. I risultati hanno mostrato che la produzione totale di gas è principalmente influenzata dal tempo di incubazione, mentre la produzione di CH_4 è influenzata soprattutto dal rapporto tra la miscela di liquido ruminale e saliva artificiale sul campione alimentare incubato. Quando invece il CH_4 viene espresso in termini di proporzione sul gas totale prodotto, i valori sono principalmente influenzati dal momento di raccolta del liquido ruminale. Inoltre, questa meta-analisi dimostra che i fattori considerati spiegavano una parte considerevole (circa il 65 %) della variabilità dei dati di gas e CH_4 prodotti durante le fermentazioni *in vitro*. Quindi, sarebbe desiderabile una

maggiore standardizzazione dei protocolli metodologici internazionali, in modo da facilitare il confronto di dati ottenuti in diversi sperimentazioni.

Il secondo contributo sperimentale ha inteso mettere a punto una procedura di raccolta e di misurazione del CH_4 prodotto, utilizzando due diverse tipologie di fermentazione *in vitro*: i) un "sistema aperto", con sfiato regolare del gas all'interno di un sacchetto connesso alle bottiglie di fermentazione, dal quale viene prelevato il campione di gas per l'analisi del CH_4; ii) un "sistema chiuso", con accumulo progressivo dei gas di fermentazione nello spazio di testa delle bottiglie, dal quale viene prelevato il campione per l'analisi del CH_4. Per le fermentazioni sono stati usati cinque alimenti singoli utilizzati nell'alimentazione dei ruminanti (fieno polifita, loietto, farina di mais, panello di lino e polpe di bietola). Il disegno sperimentale prevedeva: 2 incubazioni × 5 alimenti × 3 replicazioni per alimento × 2 tecniche di campionamento del gas, più 4 bianchi (bottiglie incubate senza campione alimentare), per un totale di 64 bottiglie incubate. Metà delle bottiglie non venivano sfiatate, mentre le rimanenti venivano sfiatate a pressione fissa e il gas era raccolto in un sacchetto a tenuta connesso ad ogni bottiglia. Alla fine di ogni incubazione, il gas veniva campionato dallo spazio di testa delle bottiglie utilizzate per il sistema chiuso o dallo spazio di testa e dal sacchetto delle bottiglie utilizzate per il sistema aperto. Tutti i campioni sono stati poi analizzati per quantificare la concentrazione di CH_4. I valori di gas prodotto venivano o meno corretti per la quantità di CO_2 che si era disciolta nel liquido di fermentazione. I valori di produzione (ml CH_4/g DM) e di proporzione (ml CH_4/100 ml gas) di CH_4 sono stati calcolati utilizzando i valori corretti o non corretti di produzione di gas totale. Il sistema chiuso ha mostrato una produzione di gas totale non corretto inferiore (-18%) rispetto al sistema aperto, mentre la correzione del gas ha ridotto ma non rimosso le differenze tra le due tecniche. Le bottiglie chiuse hanno mostrato una proporzione di CH_4 non corretto superiore (+23%) rispetto al sistema aperto, mentre la correzione ha ridotto ma non rimosso le differenze tra le due tecniche. La produzione di CH_4 corretto non è stata influenzata dalla tecnica utilizzata. Concludendo, il sistema chiuso non offre buone misurazioni della produzione del gas, mentre il sistema aperto consente una valutazione attendibile sia del gas che del CH_4 prodotto.

Nel terzo contributo sperimentale è stato valutato l'effetto che le variazioni quantitative dei principali componenti chimici (rapporto amido:ADF, contenuto proteico e contenuto lipidico) di diete per vacche da latte, posso avere sulla produzione di gas e CH_4. Le variazioni sono state fatte

tenendo conto degli intervalli di fibra, amido, proteina e lipidi effettivamente utilizzate negli allevamenti intensivi del Nord Italia. La dieta di riferimento utilizzata aveva la seguente composizione chimica: 273, 361, 158, e 33 g/kg SS di amido, NDF, CP, e lipidi, rispettivamente. Le altre 6 diete avevano un minore o maggiore rapporto di amido:ADF (0.40 or 1.77, rispettivamente), o di contenuto proteico (115 or 194 g/kg DM, rispettivamente), o di contenuto lipidico (26 or 61 g/kg DM, rispettivamente), rispetto alla dieta di riferimento. Il disegno sperimentale prevedeva: 4 incubazioni × 7 diete × 5 replicazioni per dieta, più 20 bianchi (bottiglie incubate senza campione alimentare), per un totale di 160 bottiglie incubate. Il gas prodotto veniva regolarmente sfiatato a pressione fissa e alla fine di ogni incubazione un campione di gas veniva raccolto dallo spazio di testa delle bottiglie e veniva analizzato per misurare la concentrazione di CH_4. La quantità di CH_4 perso durante lo sfiato del gas di fermentazione è stata stimata. I risultati mostrano che all'aumentare del rapporto amido:ADF si riduce la produzione di gas (per g di SS e per g di degradabilità "vera" della SS), aumenta la produzione di CH_4 (per g di SS), mentre la produzione di CH_4 espressa come g di degradabilità "vera" della SS non varia. All'aumentare del contenuto proteico si riduce la produzione di gas, mentre la proporzione di CH_4 è stata ridotta solo nella dieta ipoproteica. L'aumento del contenuto lipidico ha ridotto la produzione di gas (per g di SS), ma non ha influenzato i valori di CH_4. Si può quindi concludere che l'entità degli effetti, esercitati dalle variazioni quantitative dei principali componenti chimici delle diete sulla fermentazione *in vitro,* è stata quasi inesistente.

Il quarto lavoro ha inteso valutare l'effetto di quattro estratti puri di piante (allil-sulfide, cinnamaldeide, eugenolo e limonene) e di un composto sintetico (monensin), tutti con proprietà antimicrobiche, utilizzati come additivi di una dieta per vacche da latte, sulla produzione di gas e CH_4. Sono stati utilizzati due diversi dosaggi: 3 or 30 mg/g di per gli estratti puri di piante, 0.015 or 0.030 mg/g di dieta per il monensin. Le procedure di incubazioni utilizzate erano le stesse dell'esperimento precedente. Il disegno sperimentale prevedeva: 4 incubazioni × 5 additivi × 2 dosaggi × 3 replicazioni, più 12 bianchi (bottiglie incubate senza campione alimentare, 3 per incubazione), e 12 controlli (dieta incubata senza additivi, 3 per incubazione) per un totale di 144 bottiglie incubate. I risultati hanno mostrato che tutti i composti testati con il basso dosaggio non hanno mai influenzato la produzione *in vitro* di gas e CH_4. Rispetto al controllo, gli alti dosaggi di allil-sulfide, cinnamaldeide, eugenolo, limonene, e monensin hanno ridotto significativamente la

produzione *in vitro* di gas (ml/g DM; -16%, -12%, -9%, -38%, -12%, rispettivamente). La produzione *in vitro* di CH_4 è stata significativamente ridotta solo dell'alto dosaggio di allil-sulfide, cinnamaldeide, limonene, e monensin (-32%, -12%, -43%, -18%, rispetto al controllo). Solo gli alti dosaggi di allil-sulfide e limonene hanno ridotto significativamente anche la proporzione di CH_4 (-18% e -12% rispetto al controllo). I risultati più promettenti sono stati osservati per la cinnamaldeide, che ha depresso la produzione CH_4 senza influenzare negativamente gli altri parametri fermentativi.

Il quinto e ultimo contributo sperimentale ha valutato la possibilità di ridurre la produzione *in vitro* di gas e CH_4 attraverso la combinazione di un diretto inibitore del CH_4 (cloroformio) con due inibitori indiretti: i) i nitrati, che agiscono come accettori di idrogeno e ii) le saponine, dei noti agenti antiprotozoari. L'esperimento è stato condotto utilizzando un sistema di simulazione ruminale a flusso semi-continuo (RUSITEC®). Una dieta base è stata incubata singolarmente (dieta 1) o addizionata con 31.5 g/ kg di nitrati (dieta 2) o con 50 g/kg saponine ottenute dall'estratto d'edera (dieta 3). Queste tre diete sono state incubate senza o con l'aggiunta di cloroformio (2 µL/L), ottenendo un totale di 6 trattamenti testati. Ogni trattamento è stato incubato in quattro bottiglioni, per un totale di 24 bottiglioni. L'intera incubazione è durata 21 giorni. I risultati mostrano che la produzione totale di gas (in media 2.56 l/d) non è stata influenzata dalla dieta o dalla presenza del cloroformio. Quando utilizzati singolarmente, cloroformio, nitrati e saponine riducono la produzione giornaliera di CH_4 del 96, 66 e 22%, rispettivamente. Nonostante ciò, quando il cloroformio era combinato con inibitori indiretti, non si è evidenziato nessun effetto addizionale sulla riduzione di CH_4 prodotto. Concludendo, si può quindi affermare che nessun effetto sinergico è emerso tra l'inibitore diretto di CH_4 e i due inibitori indiretti.

SUMMARY

Due to the warming of the climate system, public opinion has increased its interest in greenhouse gas, with particular predilection for methane (CH_4) and their relationship with livestock sector. For this reason, several researches have proposed different nutritional strategies to reduce methane production in ruminants.

The two main aims of this thesis were: i) to study methodological factors affecting *in vitro* CH_4 production and to develop a new approach to measure *in vitro* CH_4 production; ii) to evaluate effectiveness of some nutritional strategies on *in vitro* CH_4 reduction. The objectives have been achieved through the research activities and reported in five experimental contributions.

The first contribution aimed to analyse specific factors affecting *in vitro* gas production (GP), CH_4 production and their ratio by a meta-analysis approach using 274 control treatments from 39 scientific papers published over 12 years. The common factors included in the analysis were: the pressure in the GP equipment (constant or increasing), the incubation time (24 or ≥48 h), the timing of rumen fluid collection (before or after feeding of donor animals), the presence of N in the buffer solution (presence or absence), and the ratio between amount of buffered rumen fluid and feed sample (<130 or 130-140 or >140 ml/g DM). The NDF content of feed sample incubated (392 ± 175.3 g/kg) was considered as a continuous variable. Results showed that GP measures were influenced mainly by the incubation time; values of CH_4 production were affected mainly by the ratio between amount of buffered rumen fluid and feed sample; values of CH_4 proportion (CH_4/GP) were mainly affected by timing of rumen fluid collection. Results suggest that factors considered in the meta-analysis explained a significant part (about 65%) of variability of GP and CH_4 measures obtained *in vitro* . Thus, a higher standardization of specific methodological protocols would be desirable in order to compare results of different trials.

The second contribution compared two *in vitro* techniques (closed or vented bottles) of sampling and measuring CH_4 production from ruminant feeds. Five feeds (meadow hay, ryegrass hay, corn grain, dry sugar beet pulp, and expeller flaxseed) were tested. The experimental design was: 2 incubations×5 feeds×3 replications×2 gas sampling techniques, plus 4 blanks (bottles without feed sample), for a total of 64 bottles incubated. Half of the bottles were not vented during the

incubation, whereas the remaining were vented at a fixed pressure and gas was collected into a tight bag connected to each bottle. At the end of each incubation, gas samples were sampled from the headspace of closed bottles or from headspace and bags of vented bottles and analyzed for CH_4 concentration. GP values were adjusted or not for the amount of CO_2 solubilized in the fermentation fluid. Values of CH_4 concentration (ml CH_4/100 ml gas) and production (ml CH_4/g DM) were computed using corrected or uncorrected GP values. Closed bottles showed lower uncorrected GP (−18%) compared with vented bottles, whereas the correction for dissolved gas reduced but did not remove differences between techniques. Closed bottles showed unadjusted CH_4 concentrations 23% greater than that of vented bottles, but the adjustment of measurements for solubilized CO_2 reduced but did not remove this difference. Adjusted CH_4 production was not influenced by technique. Closed bottles provide good measurements of CH_4 production but not of GP. Venting of bottles at low pressure permits a reliable evaluation of total GP and CH_4 production.

The third contribution evaluated the influence that changes in main dietary constituents (starch:ADF ratio, protein and lipid contents) could have on *in vitro* GP, CH_4 concentration and production. All changes have been made within ranges of diets used in intensive farms of the North Italy. A reference diet was used (273, 361, 158, and 33 g/kg DM of starch, aNDF, CP, and lipids, respectively). Other 6 diets had a smaller or a greater starch:ADF ratio (0.40 or 1.77, respectively), CP content (115 or 194 g/kg DM, respectively), or lipid content (26 or 61 g/kg DM, respectively), compared to the reference diet. The experimental design was: 4 incubations × 7 diets × 5 replications, plus 20 blanks (5 blanks/run), for a total of 160 bottles. Bottles were vented at fixed pressure and at the end of each incubation, gas samples were collected from the bottle headspace and analyzed for CH_4. The proportion of CH_4 lost with venting was estimated. An increasing starch:ADF ratio reduced GP per g DM and per g TDMd, increased the amount of CH_4 produced per g DM, whereas CH_4 produced per g TDMd was unchanged. The increase of CP content decreased GP, whereas CH_4 production was reduced in low protein diet only when expressed as proportion of GP. The increase of lipid content reduced GP per g DM, but no influences were observed on CH_4 values. Results of this study showed that the magnitude of effects exerted by the dietary changes on *in vitro* fermentation was small.

The fourth contribution aimed to explore effects of 4 pure plant extracts (allyl-sulfyde; cinnamaldehyde; eugenol; limonene) and 1 synthetic compound (monensin), all with antimicrobial properties, added to a commercial diet for dairy cows, on GP, CH_4 concentration and production. Two dosages were tested: 3 or 30 mg/g of diet for pure plant extracts, 0.015 or 0.030 mg/g of diet for monensin. Incubation procedures were the same as the previous experiment. The experimental design was: 4 incubations×5 additives×2 dosages×3 replications, plus 12 bottles as control (without additive; 3/run) and 12 bottles as blanks (3/run), for a total of 144 bottles. Results showed that low dosages of all compounds did not exert any effect on *in vitro* GP and CH_4 production. Compared to the control, high dosage of allyl-sulfyde, cinnamaldehyde, eugenol, limonene, and monensin significantly reduced *in vitro* GP (ml/g DM; -16%, -12%, -9%, -38%, -12%, respectively). *In vitro* CH_4 production was significantly reduced only by high dosage of allyl-sulfyde, cinnamaldehyde, limonene, and monensin (-32%, -12%, -43%, -18%, respectively, compared to the control). Only high dosage of allyl-sulfyde and limonene significantly reduce CH_4 proportion (-18%, -12% respectively, compared to the control). The most promising results were observed for cinnamaldehyde, as the depression of CH_4 production was not accompanied by a reduction of *in vitro* degradability.

The fifth contribution investigated the possibility to reduce *in vitro* gas and CH_4 production by combining a direct inhibitor of methanogenisis (chloroform) with two indirect inhibitors: i) nitrate, acting as a H_2 sink, and ii) saponins, acting as antiprotozoal agent. The experiment was conducted using a semi-continuous flow system (RUSITEC®). A diet, based on grass hay, was incubated alone (diet 1) or supplemented with 31.5 g/kg of nitrate (diet 2) or with 50 g/kg of saponins extracted from ivy extract (diet 3). Three diets were incubated without or with chloroform (2 µL/L), obtaining a total of 6 dietary treatments. Each of the 6 dietary treatments was incubated in four vessels, for a total of 24 vessels. Each vessel was inoculated with 800 ml of buffered rumen fluid. Whole incubation lasted 21 d. Total GP (on average 2.56 L/d) was not affected by diet or chloroform addition. When incubated alone, chloroform, nitrate and saponins reduced daily CH_4 production by 96, 66 and 22%, respectively. However, when chloroform was combined with indirect inhibitors, neither nitrate or saponins had any additional effect in chloroform treated vessels on *in vitro* CH_4 production. Results suggest that no synergistic effects emerged between the direct inhibitor and the two indirect inhibitors.

1. GENERAL INTRODUCTION

1.1 METHANE AS IMPACTING FACTOR OF LIVESTOCK SECTOR

Carbon dioxide (CO_2), methane (CH_4) and nitrous oxide (NO_2) are the prevalent greenhouse gases (GHG) keeping the infrared radiation emitted by Earth's surface, atmosphere and clouds causing detrimental effects to the environment. Some of these alterations are the increases in global average air and ocean temperatures, widespread melting of snow and ice and rising global average sea level.

Compared to CO_2, the presence of CH_4 in the atmosphere is lower (82.5 *vs* 9.5%, respectively; EPA, 2015), but its atmospheric lifetime is 12 years, and it is 25 times more effective than CO_2. Because of this, CH_4 maintains its negative effects for a period of 20 – 30 years, therefore it is very important to impose the aim of forcing the short-term reduction of CH_4 emissions (Herrero et al., 2011). This is an important purpose, especially considering the trend emission of CH_4 since before the Industrial Revolution to date. Concentrations of this gas large increase since 1750 (150%) and more recently, after a period of stability (decade of 1990s), atmospheric CH_4 measurements showed refreshed increases since 2007 (IPCC, 2014).

According to the Intergovernmental Panel for Climate Change (IPCC, 2007), agriculture accounts for 14% on GHG emissions and it is the fourth sector after energy supply, industry and forestry. Regard to the contribution of livestock to global GHG emissions there is a lack of unanimity among scientists: the FAO report of 2006 estimated that livestock production contributed for 18% on global emissions of GHG but Herrero et al. (2011) reported that, depending on different assessment, GHG emissions by livestock range from 8 to 51%. On opposite, the majority of scientists agreed that about 25% of GHG arise from enteric fermentation in livestock (O'Mara, 2011; EPA, 2015).

Alarmed by unequivocal warming of the climate system, since the first years of 21st century, public opinion has increased its interest in climate change caused by GHG and their relationship with livestock sector. For this reason, several researches evaluated and estimated the magnitude of the gases implied in the greenhouse effect, with particular interest on CH_4 emission.

Using a satellite data, Turner et al. (2015) showed a global distribution of CH_4 concentrations for June 2009–December 2011 and they assert that CH_4 concentrations were highest where livestock and fossil fuels contribute as large sources (Figure 1). The same authors estimated that global CH_4 emission derived mostly from wetlands (39%) and livestock (22%).

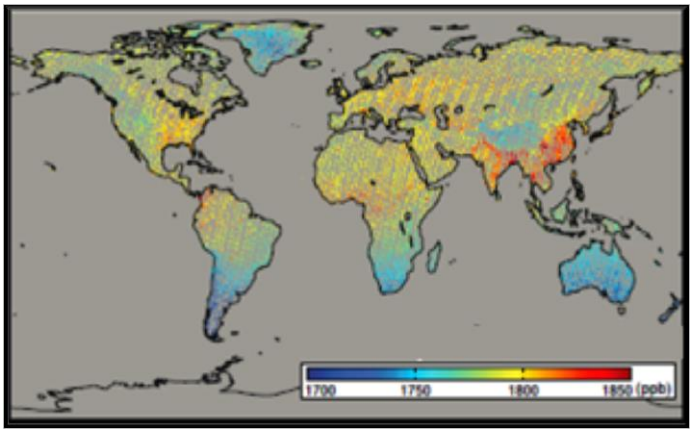

Figure 1: Global observations of methane concentration (dry-mole fraction, XCH_4) for June 2009 – December 2011. Source: Turner et al., 2015

The U.S. Environmental Protection Agency (EPA, 2015) estimated that enteric fermentation associated to domestic livestock sector is the main sources of CH_4 emission, followed by natural gas system and other sources (Figure 2). The authors of this inventory asserted that trend of enteric CH_4 emissions is strictly related to the patterns in cattle populations and the digestibility of their feed.

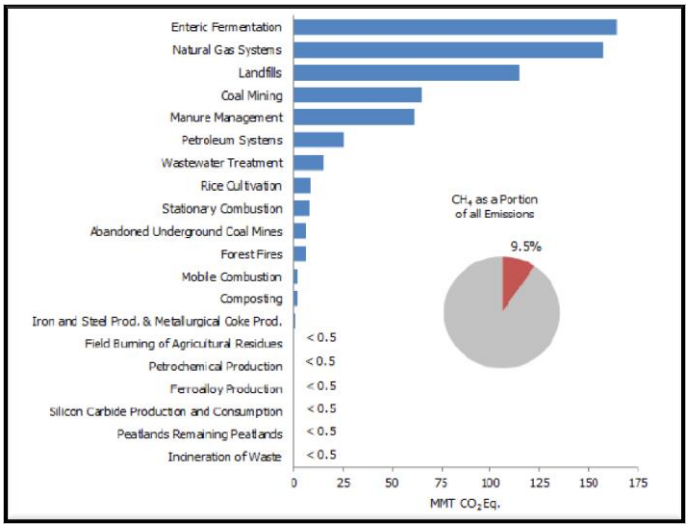

Figure 2: Sources of CH_4 emissions for 2013 (values are presented in CO_2 equivalent). Source: EPA, 2015

1.2 METHANOGENESIS IN RUMINANTS

Cattle, being big ruminants, are the most impacting family of this suborder that plays an important role in global warming. Furthermore, CH_4 production is also associated with notable energy losses from ruminants, which vary between 2 to 12% of gross energy intake (Johnson and Johnson, 1995), and it leads to decreasing energy gain and productivity.

The assessment of total emission from the global livestock sector, by main animal species, revealed that 67% of GHG were emitted from beef and dairy cattle (Hristov et al., 2013b). By a global life cycle assessment, the FAO asserted that a copious amount of total GHG emissions is from enteric CH_4 which contributes about 92 and 97% of the total CH_4 emissions in dairy and beef cattle (Figure 3; Opio et al., 2013).

Figure 3: Relative contribution of different processes to total GHG emissions from the global cattle sector. Source: Opio et al., 2013

In ruminants, CH_4 production occurs mainly in the reticulo-rumen: about 87% of total CH_4 production is produced in the rumen, whereas 13% is produced in the lower digestive tract (Murray et al., 1976). Rectal emissions are about 2-3% of the total CH_4 emissions in sheep and dairy cows (Muñoz et al., 2012); specifically 11% is excreted through the anus, while 89% is excreted through the lungs (Murray et al., 1976).

The rumen is an anaerobic fermenter in which nutritional components (carbohydrates, proteins and, in lesser extent, lipids) are degraded by rumen microbial community (bacterial, protozoal and fungal species) and transformed mainly into volatile fatty acids (acetate, propionate and butyrate) (Mitsumori and Sun, 2008). Moreover formate, ethanol, lactate, succinate and

branched chain volatile fatty acids are formed in a lower amount. In addition, ammonia, carbon dioxide and H_2 gas are produced (Janssen, 2010).

Rumen fermentation pathways have been intensively investigated and, to date, it is known that CO_2 and H_2 are the major precursors of CH_4 and that H_2 derives mainly from carbohydrates degradation. The H_2 gas must be eliminated to maintain the hydrogenase activity and to avoid negative feedback on microbial OM degradation (Wallace and Chesson, 2008). The removal of H_2 can be through methanogenesis (as reported in Reaction 1) and acetogenesis (as reported in Reaction 2) as described in the following pathways:

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

[Reaction 2] $2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O$

Another way to confiscate H_2 to the rumen is the stimulation of the organisms producing propionic acid (as reported in Reaction 4), while the acetate (as reported in Reaction 3) and butyrate (as reported in Reaction 5) productions are producers of H_2 in the fermentation pathway (Hegarty and Gerdes, 1998).

[Reaction 3] $C_6H_{12}O_6 + 2H_2O \rightarrow 2C_2H_4O_2 + 2CO_2 + 4H_2$

[Reaction 4] $C_6H_{12}O_6 + 2H_2 \rightarrow 2C_3H_6O_2 + 2H_2O$

[Reaction 5] $C_6H_{12}O_6 \rightarrow C_4H_8O_2 + 2CO_2 + 2H_2$

The H_2 and CO_2 formed are further used as energy sources by the methanogenic *Archaea* to produce CH_4 (Janssen, 2010). Indeed enteric CH_4 is produced in anaerobic conditions also by highly-specialized microbes known as *Archaea*. Even though it is not completely sure the phylogeny of the group, *Archaea* are, together with the Eukaryotes and Bacteria, one of the basic groups of living things (Figure 4). To date, about 40 species of *Archaea* were identified and they are divided into 3 subgroups: Halophiles, Thermophiles and Methanogens. The latter ones produce CH_4 as a by-product of their metabolism and they can be classified into 2 different physiology pathways of CH_4 production: hydrogenotrophic methanogens and hydrogen-requiring methylotrophic methanogens (Wedlock et al., 2013). The first converts one or four moles of H_2 to one mole of CH_4, whereas the second metabolises one mole of H_2 plus methanol (and other methyl compounds) to one of CH_4.

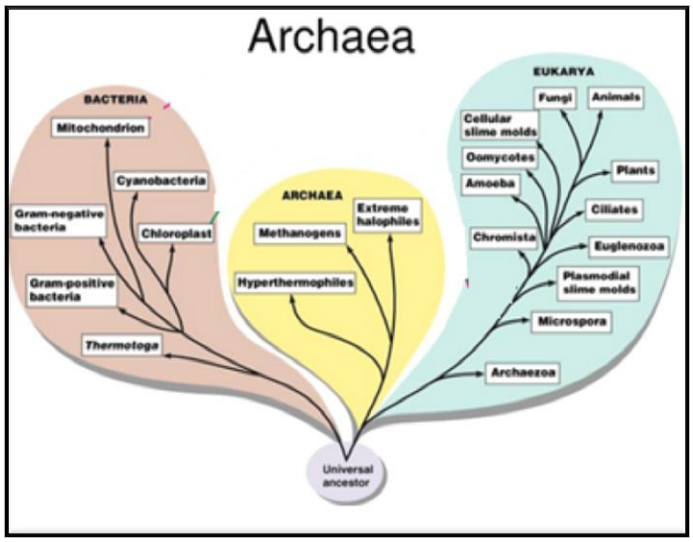

Figure 4: Phylogenetic groups and string of methanogens. Source: www.docstoc.com

Formate is also a precursor of CH_4 (Figure 5) and this way of formation represents about 15-20% of total CH_4 production in the rumen. (Mitsumori and Sun, 2008). Some species of *Archaea* utilized H_2/CO_2 and formate to produce CH_4.

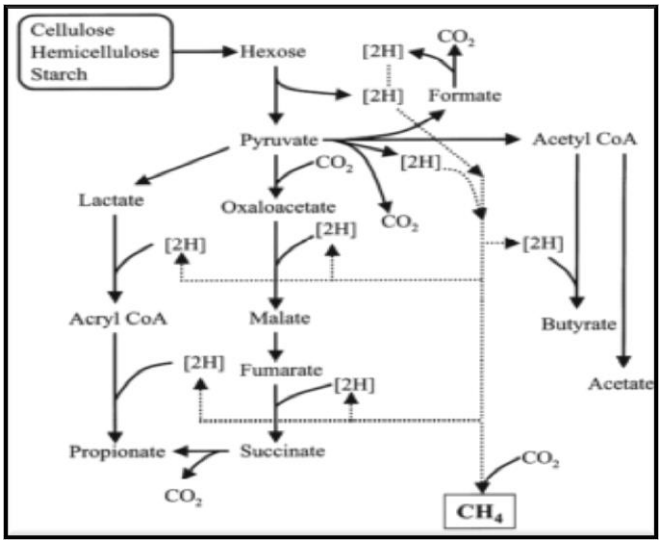

Figure 5: possible fermentation pathways of methane production in the rumen.
Source: Mitsumori and Sun, 2008

Therefore, CH_4 is a physiological end-product of microbial fermentation of carbohydrates in the rumen and it is an essential metabolic pathway for H_2 removal in the rumen.

1.3 QUANTIFICATION METHODS OF METHANE EMISSIONS

To develop suitable strategies to reduce CH_4 emissions, it must be possible to quantify cattle emissions under a wide range of circumstances. Therefore over the last three decades, different methods have been tested to quantify CH_4 emissions from ruminants, with a particular focus on cattle. These methods range from *in vivo* to *in vitro* techniques.

1.3.1 IN VIVO TECHNIQUES

One of the first systems used to study *in vivo* CH_4 emission is the use of respiration chambers (Figure 6). They consists in a system for open-circuit indirect calorimetry, where CH_4 emissions are determined by measuring the total air flow through the system and the difference in concentration between inspired and expired air (Johnson and Johnson, 1995). The chamber is structurally built with 5 thigh-gas walls (two side panels, a top one, a front one and a rear one) placed in a square and it is equipped with a analyser of the composition of the air input and output. In this way it is possible to measure continuously the difference in concentration of CH_4 and CO_2, and then the production rate of this gas by the animals that are singularly housed in the chamber for about 7 days.

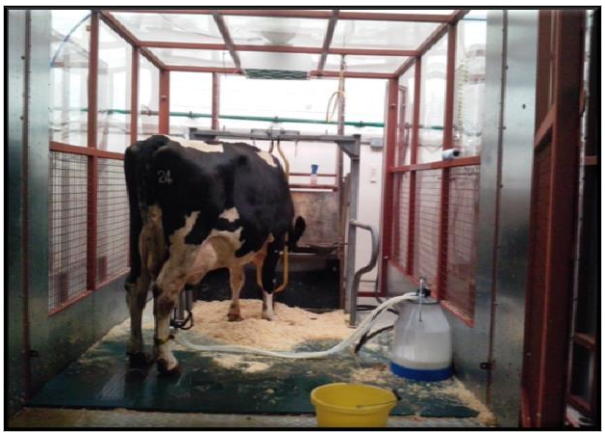

Figure 6: dairy cow in a respiration chamber during milking time.

Source: picture of my personal collection

It is the most accurate method to quantify and to study the methanogenesis and it is considered the reference method. However, this technique is very expensive because of the demand for sealing, climate regulation, and internal circulation of air and then it is not available for all research centres. Furthermore, the chambers traditionally built were not in transparent material

and had only small windows which may restrict normal social behaviour among animals (Storm et al., 2012). This restriction could change the behaviour of the animals also in terms of dry matter voluntary intake and production stress and then also the emissions of CH_4 could be distorted and unreliable. Moreover, building traditional respiration chambers is expensive and they allow to have a lot of data but for a very limited number of animals. Therefore, despite the accuracy of measurement, the use of this method is also limited for the expense of the manual operation needed to keep the animal during the measurements (Kebreab et al., 2006).

Another system to measure CH_4 *in vivo* is the use of polytunnel (Figure 7). It is an adaptation of CH_4 chamber that can be used as large dynamic chambers for small ruminants under semi-normal grazing condition. This construction can be moved in different patches when the group of animal changes place of pasture.

Figure 7: sheep in a polytunnel during grazing.

Source: www.southerninflatables.net

It is a structure built as a polythene-clad greenhouse modified to make possible the drawn of air through the tunnel with a speed-controllable fan (Murray et al., 1999) and it is much less expensive compared to the classic respiration chambers (Kebreab et al., 2006). The air is sampled inside and outside the tunnel, to quantify the amount of CH_4 and CO_2 ambient level using a laser system. The differences between inside and outside levels permit to determine the CH_4 and CO_2 emissions. The polytunnel allows free movement of animals (Kebreab et al., 2006) and the interaction between animals, thus the normal animal social behaviour is maintained. However, some fluctuations of CH_4 emission can occur due to changes in internal temperature, relative humidity and

grazing pattern of the animal confined into the tunnel during the days of measurements (Lockyer and Champion, 2001).

To measure directly and individually the enteric CH_4 emission of free ranging big ruminants, the sulphur hexafluoride (SF_6) tracer technique was developed (Johnson et al., 1994). The basic theory of this method is that CH_4 emission can be measured when the emission rate of a non-toxic, physiologically inert and stabile gas from the rumen is known (Storm et al., 2012; Figure 8a).

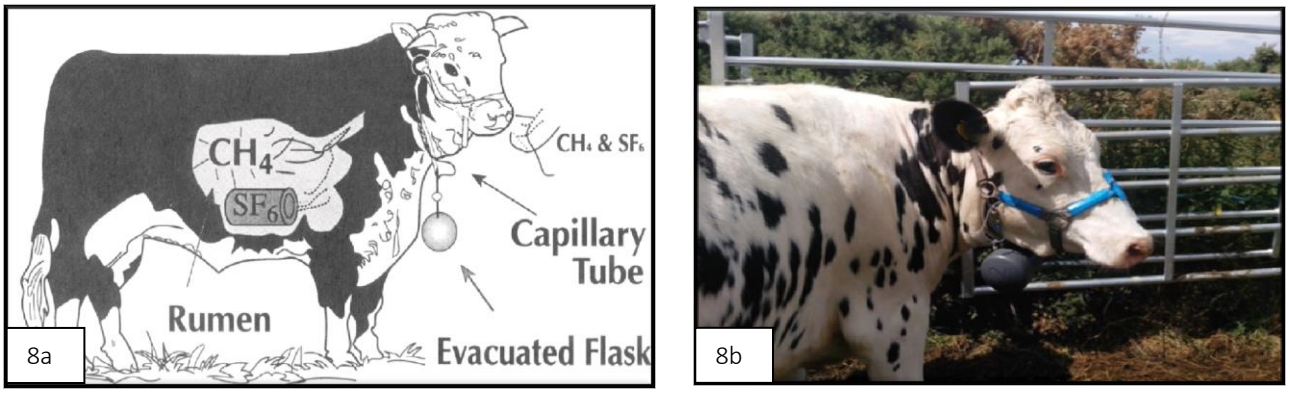

Figure 8: (8a) illustration of the SF6 tracer technique; (8b) cow with SF_6 worn.

Source: (8a) Storm et al., 2012; (8b) picture of my personal collection

The technique involves to place a permeation tube containing SF_6 in the rumen and to collect samples of gases eructed and respired from nose and mouth of animal (Johnson et al., 1994). To collect the animal breath, a capillary tube is placed on the animal's head and connected to a vacuum-sealed sampling canister (Johnson and Johnson, 1995; Figure 8b). At the end of the experiment, the canister is removed and the CH_4 and SF_6 concentrations are quantified by GC. Methane production is then calculated as CH_4/SF_6 ratio multiplied by the known release rate of SF_6 (Kebreab et al., 2006). However, this tracer technique had some shortcomings: it is necessary to train the animal to wear the canister, milk produced during the experiment have to be discarded, training is required in handling tracer gases, a constant release rate from permeation tubes is not always maintained, and hindgut CH_4 is not collected causing some inconsistencies between values determined in chambers and with SF_6 (Johnson and Johnson, 1995; Kebreab et al., 2006; Storm et al., 2012).

To study the whole farm emissions or the interactions between animals and landscape on CH_4 emission, micrometeorological techniques have been developed. This method is based on measurements of wind velocity and CH_4 concentration (Laubach et al., 2005; Storm et al., 2012). Measurements are performed using a motorized scanning unit infrared equipped with a diode lasers (Tomkins et al., 2011). Considering that the integrity of data collected with micrometeorological technique is highly dependent on environmental factors, the open path laser method needs further investigations.

A new method have been developed using CO_2 as a tracer gas (Madsen et al., 2010). Using this method, air samples are analyzed simultaneously for CH_4 and CO_2 with a gas analyser based on Fourier transform infrared (FTIR) detection, that uses CO_2 from the breath of cows as tracer gas (Lassen et al., 2012). The CH_4/CO_2 ratio in air of the animal is measured at regular intervals and combined with the calculated total daily CO_2 production of the animal (Storm et al., 2012). This equipment is portable and it can be easily mounted on an automated milking to measure individual CH_4 emission (Figure 9).

Figure 9: automated milking system equipped with FTIR.

Source: Storm et al., 2012

However, there are some disadvantages related to the fact that CO_2 production is influenced by size, activity and production of the animal. This may produce errors when the quantitative CH_4 production is measured on an individual animal (Storm et al., 2012). Moreover, the quantification of CH_4 and CO_2 concentrations in the cow's breath, is highly influenced by the distance from the head to the sampling unit (Lassen et al., 2012).

A newly commercial automatic feeding system measuring CH_4 and CO_2 is the Greenfeed® patented by Zimmerman and Zimmerman (2012; Figure 10a). This machinery acts as a concentrate feeder and measures repeatedly individual CH_4 emission for short-term (3-6 min) everytime the animal visits the Greenfeed to consume concentrate (Hegarty, 2013). Air is continuously aspirated through the apparatus to quantify CH_4 and CO_2 emitted during eating by a gas tracer apparatus (Storm et al., 2012). Possible applications are in conventional farms and for grazing animals fed supplements (Figure 10b), whereas a limitation of the technique is that that CH_4 emissions are quantified only when the animals are eating.

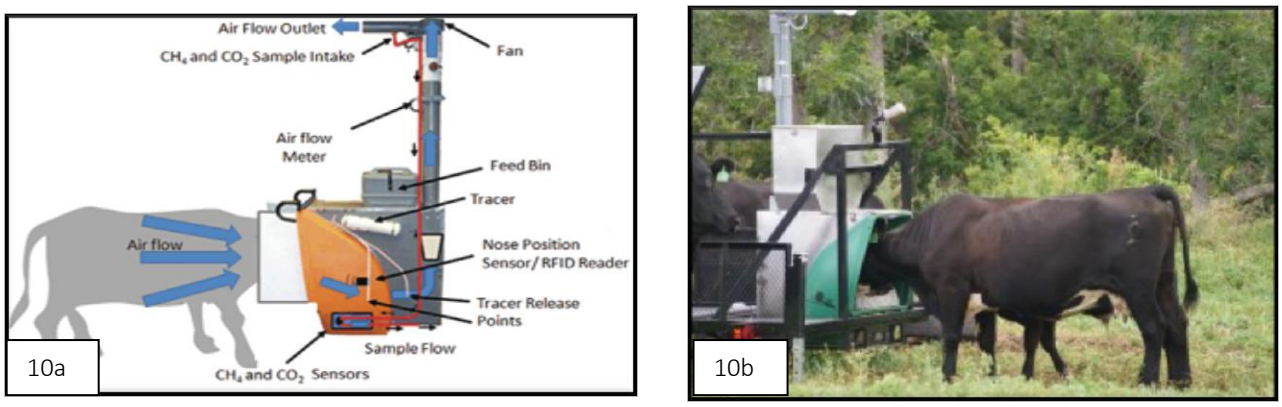

Figure 10: (10a) illustration of the Greenfeed® unit; (10b) cow using Greenfeed® unit during grazin.

Source: (10a) pasturedairy.kbs.msu.edu; (10b) www.c-lockinc.com

Considering each technique discussed above, it possible to conclude that none of these *in vivo* methods for measuring CH_4 emissions are impeccable and they require careful consideration of advantages and disadvantages before application. Moreover they appear laborious, time-consuming and expensive.

1.3.2 IN VITRO TECHNIQUES

High associated costs and hand-work of *in vivo* technique have led to the development of alternative *in vitro* techniques, which carried out some important advantages: i) they do not involve the direct use of animals; ii) they are less laborious and more suitable for a large-scale evaluation of ruminant feeds; iii) they are less time-consuming and less expensive. However, *in vitro* techniques have also disadvantages. Indeed, comparison among values of GP and CH_4 obtained from different *in vitro* trials is complicated. This is probably due to the confounding effects exerted by different

protocols used with different *in vitro* techniques. This point of view has been deepen in the first contribution of this thesis.

Due to the known close link between rumen fermentation and gas production, the *in vitro* gas production technique (IVGPT) has been used to simulate the ruminal fermentation of feedstuffs since 1940s (Getachew et al., 1998). More recently, with increasing public interest in GHG and their relationship with livestock sector, the traditional IVGPT has been modified to include the determination of gas composition, specifically CH_4 production (Pellikaan et al., 2011; Ramin and Huhtanen, 2012).

One of the first GP systems is represented by glass syringes working at atmospheric pressure (Menke et al., 1979; Figure 11). With this method the fermentations occurred in glass syringes with a piston lubricated with Vaseline and a needle connected with a short silicon rubber closed with a clip. Feed sample is firstly introduced into the syringe and then the buffered rumen fluid is pipetted with an automatic pump into each syringe warmed at 39°C. After the operations of filling, the position of each piston is recorded and each syringe is placed into the incubation apparatus with a rotor, which is rotated and warmed for whole incubation. At fixed time and the end of experiment the new position of each piston is recorded, and with a difference between initial and final position, the gas production of each feed samples is obtained.

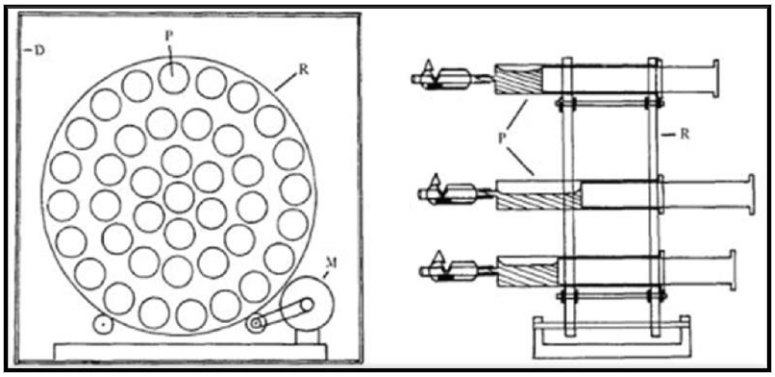

Figure 11: illustration of the glass syringes technique.

Source: Menke et al., 1979

The basic principle of new IVGPT is to ferment feedstuffs under controlled laboratory conditions in vessel or bottles used as fermenter units. These units are kept into an incubator or a

water-bath, to maintain the ideal temperature of rumen microbes (39°C). The feedstuffs are inoculated with a buffered rumen fluid and the anaerobic conditions are maintained, using a flux of CO_2 during the filling of the bottles. During the whole incubation time (typically 24, 48, or 72 h), pressure data are recorded and then converted in terms of volume of total gas produced. Gas composition can be analyzed, to obtain data on the *in vitro* CH_4 production.

One of the new IVGPT, is the commercial apparatus Ankom^{RF} Gas Production System (Ankom Technology, NY, USA; Figure 12a) consisting in a set of bottles equipped with pressure sensors and wireless connected to a computer (Figure 12b). During incubation the headspace pressure of each bottle is read with a frequency of 1 minute. Each bottle is equipped with an electromechanical valve that controls the release of gas: for each bottle the gas produced can be vented when a threshold pressure is reached (fixed pressure) or in a pre-defined sequence of times (fixed times) (Tagliapietra et al., 2011).

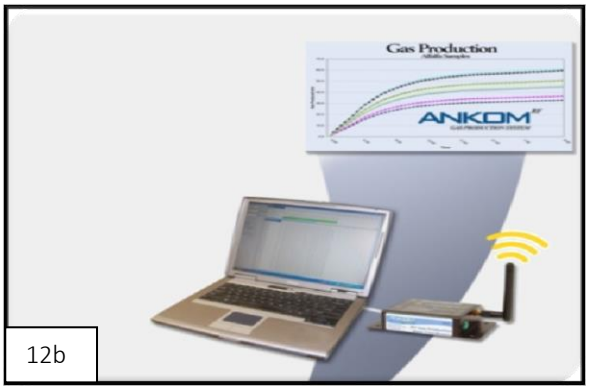

Figure 12: (12a) illustration of commercial apparatus Ankom^{RF}; (12b) wireless connection with computer. Source: (12a and 12b) www.ankom.com

The *in vitro* techniques can be classified as: closed and open systems. The effect on gas composition due to the type of system has been evaluated in the second contribution of this thesis.

1.3.2.1 CLOSED SYSTEMS

This category includes the techniques where gas produced is accumulated in fermentation vessels over the whole incubation time, without being vented (Pell and Schofield, 1993), thus it remains in the bottle headspace until the time of collection for CH_4 analysis (Pellikaan et al., 2011). Consequently, the gas pressure generated in the headspace will cause a partial dissolving of CO_2 in

the fermentation fluid, causing an underestimation of GP measured (Tagliapietra et al., 2010) and an alteration of the CH_4 concentration in the collected gas (Patra and Yu, 2013).

1.3.2.2 OPEN SYSTEMS

This category comprises the techniques equipped with devices for gas venting. Venting can occur at fixed times (Theodorou et al., 1994) or at fixed pressure (Davies et al., 2000). Gas venting is recommended to avoid pressure conditions that cause a partial dissolution of CO_2 in the fermentation fluid, with a consequent possible disturbance of microbial activity (Theodorou et al., 1994) and, thus, a possible alteration of CH_4 production.

1.4 NUTRITIONAL STRATEGIES TO REDUCE METHANE EMISSIONS

The potential of nutritional strategies to reduce enteric CH_4 emission has been extensively reviewed (Boadi et al., 2004; Benchaar et al., 2011; Bayat and Shingfield, 2012; Hristov et al., 2013a; Hristov et al., 2013b; Knapp et al., 2014; Kumar et al., 2014). The two main areas of intervention, resulting from these reviews and represented by the changes in the diet and the use of feed additives, are briefly discussed below.

1.4.1 DIETARY MANIPULATION

The modification of nutrient balance, increasing or decreasing the concentration of one dietary chemical component, will decrease or increase concentration of another of them. With this strategy, the potential effects on CH_4 emissions are often a consequence of changes in other dietary constituents (Hristov et al., 2013a) causing combined and confuse interpretation. In this thesis, the modification of nutrient balance is evaluated for its effectiveness in reducing CH_4 emissions from ruminants (third contribution).

The lowering of forage:concentrate ratio, due to the increase of concentrate inclusion in the diet, is one of the most studied dietary strategy. This kind of dietary manipulation reduces rumen pH and the acetate: propionate ratio and consequently decreases also the amount of CH_4 produced per unit of DMI (Beauchemin et al., 2008). However, the proportion of concentrates needed to cause this effect should be beyond 35 to 40% (Sauvant and Giger-Reverdin, 2009), while smaller concentrations or moderate changes seemed to not affect CH_4 emission (Hristov et al., 2013a).

Nevertheless, it must be underlined that high levels of concentrate are not desirable due to health consequences.

Another approach is the selection of better quality forages (with a low fibre and a high soluble carbohydrates content), since low-quality forages have greater proportion of CH_4 emitted per kg digestible OMI (Boadi and Wittenberg, 2002). Moreover, CH_4 reduction is correlated with greater digestibility of forage and with its maturity (Hristov et al., 2013a).

The increase of dietary fat content is another strategy that it has been proposed as promising to reduce CH_4 emissions from ruminants (Eugène et al., 2008, Rasmussen and Harrison, 2011). Specifically, it is estimated that CH_4 emissions can be reduced by 4–5% (g/kg DMI) for every 1% of increase in the fat content of the diet (Grainger and Beauchemin, 2011). However, dietary lipids inclusion beyond 6–7% on DMI can cause a negative reduction of feed intake and fibre digestibility (Jenkins, 1997).

1.4.2 FEED SUPPLEMENTS

Feed additives used to reduce CH_4 production are usually classified into different categories and on the basis of their respective mechanisms of action.

Inhibitors are chemical compounds that directly affect rumen *Archaea,* with potential anti-methanogenic activity (Moate et al., 2014). Within this category, the most successful compounds are the halogenated CH_4 analogues (i.e., bromochloro-methane, 2-bromoethane sulfonate, chloroform, and cyclodextrin). They can be used alone or combined with each other to stabilize their effect, in order to obtain a more pronounced reduction of enteric CH_4 emission (Kumar et al., 2014). Some of these compounds are potent CH_4 inhibitors both *in vitro* (Klein et al., 1988; Romero-Peréz et al., 2015) and *in vivo* (Knight et al., 2011; Abecia et al., 2012; Mitsumori et al., 2012), but the long-term effect is uncertain, suggesting a kind of adaptation by the rumen ecosystem (Hristov et al., 2013b). Moreover, the use of these compounds as feed supplement is not well accepted by public opinion, due to the possible risk to animal and human health (i.e, chloroform is known as carcinogens molecule and bromochloro-methane is a recently banned ozone-depleting molecule). Effects of chloroform on CH_4 production has been evaluated in this thesis (fifth contribution).

Electron receptors are compounds acting as alternative H_2 sinks in the rumen (Hristov et al., 2013b). Dicarboxylic acids (as fumarate and malate), nitrates, and sulphates are the most studied mitigating compounds belonging to this category (Ungerfeld et al., 2007; van Zijderveld et al., 2010; Hulsof et al., 2012; Pal et al., 2014). Malate and fumarate are precursors to propionate production in the rumen, consuming H_2 in the process (McAllister and Newbold, 2008). Nitrate and sulphates can substitute CO_2 forming ammonia as an alternative H_2 sink in the rumen (McAllister and Newbold, 2008). The use of nitrate as additive has largely been ignored, because of the potential toxic effects of intermediary products (nitrite) that are formed during the reduction of nitrate in the rumen. Recently, the toxicity of nitrite has been deeper studied and it has been learn that its production from nitrate in the rumen may be prevented by feeding management (Leng, 2008). In this thesis, the use of these products as alternative H_2 sinks to methanogenesis is explored (fifth contribution).

Ionophores are organic molecules, often antibiotics, that transport ions across the lipid bilayer of the cell membrane (Pressman, 1976). Monensin is one of the commonly used ionophores in ruminant nutrition, although ionophores are banned in the European Union (Hristov et al., 2013b). It shifts the acetate-to-propionate ratio in the rumen towards propionate, thereby reducing CH_4 production (Eckard et al., 2010). Moreover, it also decreases ruminal protozoal numbers (Beauchemin et al., 2008) and it has a long-term persistency (Odongo et al., 2007). However, the effect of monensin is dose-, feed intake- and diet composition dependent (Hristov et al., 2013b). Effects of this antibiotic on CH_4 production has been evaluated in this thesis (fourth contribution).

Plant bioactive compounds are variety of plant secondary compounds, specifically tannins, saponins, and essential oils and their active ingredients (Hristov et al., 2013b). Many of these compounds have been studied for their potential to reduce methanogenesis (Hu et al., 2005; Calsamiglia et al., 2007; Hart et al., 2008; Hristov et al., 2008; Macheboeuf et al., 2008; Rochfort et al., 2008; Spanghero et al., 2008; Holtshausen et al., 2009; Mao et al., 2010; Patra and Saxena, 2010; Soliva et al., 2011) and results of these studies are very promising. Saponins are a potent anti-protozoal agent and their effects on CH_4 reduction has been evaluated in this thesis (fifth contribution). The general mode of action of saponins is their interaction with cholesterol present in the membrane of protozoa causing breakdown of the membrane, cell lysis, and death (Cheeke,

2000). Essential oils with antimicrobial properties against bacterial and fungal act on control of rumen fermentation, gases and VFA production (Boadi et al., 2004). The *in vitro* effectiveness of some plant extracts, with known antimicrobial activity, able to lower CH_4 production (allyl-sulfide, cinnamaldehyde, eugenol, limonene) is investigated in fourth contribution of this thesis.

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2. OBJECTIVES

The general objective of the present thesis was to study different nutritional strategies for reducing CH_4 emission in cattle using *in vitro* techniques. This aim was pursued by a large survey of variation sources influencing *in vitro* total gas and CH_4 values by using a meta-analysis approach (first contribute). After the literature research, it has been developed a new *in vitro* method to collect and to measure CH_4 production (second contribute). Once that the methodological system was defined, the experiments evaluated the effect on *in vitro* CH_4 production using two different nutritional strategies applicable in intensive farming systems. Specifically, it was tested the effect of dietary modification in order to estimate the range of variation on CH_4 emission (third contribute). Secondly, it was verified the effect of addition of natural (pure extract compounds) and synthetic compounds with antimicrobial activity on CH_4 production (fourth contribute). Finally, in the Aberystwyth laboratory (Wales, UK), it was analyzed the effect of combination among additives on *in vitro* rumen fermentation, CH_4 emissions and modification of rumen bacteria, by the use of the RUSITEC (RUmen Simulation TEChnique) (fifth contribute).

3. FIRST CONTRIBUTION

Methodological factors affecting gas and methane production during in vitro rumen fermentation evaluated by meta-analysis approach

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3.1 ABSTRACT

A meta-analysis approach (274 experimental data, 39 scientific papers published over 12 years) was used to establish the magnitude of experimental factors affecting *in vitro* measures of gas production (GP, ml/g DM), methane (CH_4, ml/g DM), and CH_4/GP. Experimental factors considered were: the pressure in the GP equipment (PR; 0 = constant; 1 = increasing), the incubation time (IT; 0 = 24; 1 = ≥ 48 h), the timing of rumen fluid collection (CRF; 0 = before feeding of donor animals; 1 = after feeding of donor animals), the presence of N in the buffer solution (N; 0 = presence; 1 = absence), and the ratio between amount of buffered rumen fluid and feed sample (BRF/FS; 0 = < 130 ml/g DM; 1 = 130-140 ml/g DM; 2 = > 140 ml/g DM). The NDF content of feed sample incubated (NDF; 392 ± 175.3 g/kg) was considered as a continuous variable. To avoid over-parameterization of the model, a preliminary mixed model analysis was carried out on GP, CH_4 and their ratio considering the trial effect as random factor. Variables adjusted for the trial effect were analysed using a backward stepwise analysis including all the above mentioned variables. Results showed that GP measures were influenced by IT followed by PR, BRF/FS, N, NDF and CRF (R^2 of model = 0.74). Values of CH_4 production were affected by BRF/FS, IT, CRF, N, and NDF, in decreasing order of importance (R^2 of model = 0.55). The CH_4/GP ratio was influenced in order of magnitude by CRF, BRF/FS, N, and PR (R^2 of model = 0.68). As expected, increase of IT and reduction of NDF content led to an increase of GP and CH_4 values. Rumen fluid collected after feeding increased values of GP and CH_4 compared to CRF taken before feeding of donor animals (+ 6.5 and + 8.0 ml/g DM, for GP and CH_4, respectively). The use of an N-free buffer solution caused an increase of + 12.1 and + 9.2 ml/g DM for GP and CH_4, respectively. For each class of the BRF/FS ratio, measures of GP and CH_4 were increased of 7.4 and 6.2 ml/g DM, respectively. The GP equipment working at increasing pressure induced a reduction of GP values in the order of -13.2 ml/g DM, compared to GP systems operating at constant pressure. Results highlight that factors considered in this meta-analysis explained a significant part (about 65 % on average) of variability of *in vitro* GP and CH_4 measures. An effort to standardize the GP technique would be necessary in order to facilitate comparison between results of different experiments.

Keywords: Meta-analysis, *In vitro* rumen fermentation, Experimental factors, Gas production, Methane production

3.2 INTRODUCTION

In recent years *in vitro* gas production (GP) technique has been applied routinely to evaluate the nutritional value of ruminant feeds. The most diffused techniques were designed to measure GP from feed samples incubated in glass syringes at atmospheric pressure (Menke and Steingass, 1988) or in fermentation vessels where gas is measured over the whole incubation time (Pell and Schofield, 1993) or regularly vented at fixed times (Theodorou et al., 1994) or at fixed pressure (Cone et al., 1996; Davies et al., 2000). More recently, such equipment has been adapted to determine the composition of gases, particularly CH_4 produced from *in vitro* rumen fermentation (Navarro-Villa et al., 2011; Pellikaan et al., 2011; Ramin and Huhtanen, 2012). To date, *in vitro* GP measures are not easily comparable because of the poor degree of standardization of the methods and the presence of many confounding effects due to different factors. Among these factors, the most relevant are represented by procedures used to collect and to treat rumen fluid (Cornou et al., 2013), the composition of the buffer (Patra and Yu, 2013), the type of GP equipment (Gierus et al., 2008; Tagliapietra et al., 2010; Cattani et al., 2014) and the ratios between fermentation fluid and feed sample size (Ramin and Huhtanen, 2012). The current literature encompasses reviews which explored the magnitude of various factors influencing *in vitro* GP values (Getachew et al., 1998; Rymer et al., 2005); however, to our knowledge, no literature reviews have been produced on factors affecting CH_4 measures obtained *in vitro*. The objective of the present study was to evaluate factors affecting *in vitro* GP and CH_4 production by means of the meta-analysis approach. As a secondary objective, the study was addressed to quantifying the relative incidence of each factor affecting *in vitro* GP and CH_4 production through the use of a multiple regression method.

3.3 MATERIALS AND METHODS

3.3.1 Literature search

An as wide as possible literature search was conducted using search generators of public data (i.e. Web of knowledge, Google scholar, Science direct, and Scopus) and contacts with researchers working in this field, to find scientific papers reporting data of gas and CH_4 production obtained from *in vitro* fermentation of feeds or diets commonly used for ruminants. The web searches were conducted using the following keywords in different combinations: *in vitro* technique, rumen fermentation, gas production, methane production and ruminants.

3.3.2 Factors selected

The search strategy aimed at selecting articles focussing on the study of specific factors known to exert notable effects on *in vitro* gas and CH_4 production. Specifically, the following factors were considered: the pressure in the GP equipment used (PR), the incubation time (IT), the collection time of rumen fluid (CRF), the donor species (DS), the presence or absence of N in the buffer solution added to the rumen fluid (N), the NDF content of incubated feed samples (NDF), the amount of buffer solution (B), the amount of rumen fluid (RF), and the amount of feed sample incubated (FS). The correction of *in vitro* data for "blank" samples, despite its relevance, was not considered as most papers did not provide this information. Agitation (or not) of bottles during the incubation and the diet offered to donors of rumen fluid were not taken into account because these factors have been reported as having minor effects on *in vitro* GP (Rymer et al., 2005). Moreover, this information is absent in many of the considered manuscripts.

3.3.3 Inclusion and exclusion of literature for the study and building of the starting dataset

A total of 55 scientific papers were identified and initially screened for acceptability by checking if all publications reported the above mentioned information.

Table 1. List of references excluded from the meta-analysis.

Reference	Reason for exclusion
Rossi et al., 2001 Wallace et al., 2006 Wood et al., 2009 Becker and van Wikselaar, Castro-Montoya et al., 2012 Cao et al., 2012 Poulsen et al., 2012 O'Brien et al., 2013	Only methane production data were reported and not the whole gas production (GP) or their ratio
Hu et al., 2005	More than one important investigating factor was missing
Polyorach et al., 2014	Methane production data were indirectly predicted
Ungerfeld et al., 2007 Hart et al., 2008	Control data of feed sample treatment were missing
Wang et al., 1998 Soliva et al., 2011 Williams et al., 2011 Li et al., 2013	GP and methane data were obtained using continuous or semi-continuous apparatus

To be included in the database, papers had to report all values of GP, CH_4, and the CH_4/GP ratio, or at least two of them, so that the third variable could be calculated. Results of studies carried out by using continuous (i.e. dual flow) or semi-continuous (i.e. RUSITEC®) GP equipment were not considered in the study. Experiments conducted using alternative inocula (i.e. faeces) instead of rumen fluid were excluded from the database. After discarding 16 out of the 55 papers (Table 1), a starting dataset was built considering 39 articles published over the last 12 years, accounting for a total of 274 observations (Table 2). Such observations included only control treatments, defined as feeds or diets incubated alone. Observations referred to effects of additives on *in vitro* GP and CH_4 production were not considered, to avoid possible further confounding effects due to the presence of such compounds. In the dataset some unpublished data of completed studies were included (Cattani et al., 2014).

3.4 COMPUTATIONS AND STATISTICAL ANALYSIS

3.4.1 Data harmonization

Because of the heterogeneity in GP and CH_4 values reported among publications, data were adjusted to a uniform scale. All GP data were transformed to ml per gram of incubated DM. Likewise CH_4 values were converted and expressed in terms of total CH_4 production (ml per gram of incubated DM) and as a proportion of total GP (ml per 100 ml of total GP). When not otherwise specified, the weight of the sample was considered as fed (on a wet basis). To reconcile the weight of a feed sample into g of incubated DM, values were corrected using the DM content of each sample. When DM was not indicated, a value of DM equal to 920 g/kg was used, corresponding to the general DM mean content of feed samples included in the dataset. When papers presented GP and CH_4 values in terms of moles, a correction was adopted using Gay-Lussac's law, assuming that 1 mole was equivalent to 25.6 L of gas under atmospheric pressure and temperature conditions of GP equipment (39°C). In the case of Bodas et al. (2008), 1 mole of gas was considered to be equivalent to 25.4 L, as indicated by the authors. To convert values of GP and CH_4 expressed as ml per gram of OM, the DM and ash contents of feed samples were considered. When CH_4 values were expressed in mg/g, these values were converted into ml using Gay-Lussac's law and considering the molecular weight of CH_4. When CH_4 values were expressed as mmol per litre, they were reconciled considering Gay-Lussac's law and GP values; when CH_4 data were expressed as ml per litre, they were reconciled considering only values of GP.

3.4.2 Preliminary evaluation of variables

Variables such as the pressure in the GP equipment used (PR; constant vs. increasing; 125 vs. 149 observations, respectively), the incubation time (IT; 24 vs. ≥ 48 h; 170 vs. 75 observations, respectively), the collection time of rumen fluid (CRF; before or after feeding of donor animals; 138 vs. 105 observations, respectively), the donor species (DS; sheep vs. cattle; 45 vs. 229 observations, respectively), and the presence of N in the buffer solution (N; presence vs. absence; 232 vs. 42 observations, respectively) were all coded as dichotomous variables, i.e., 0 or 1 in respective order. Syringes and vented bottles were considered as equipment working at constant pressure, whereas closed bottles were considered as apparatus operating at increasing pressure. For CRF, data of rumen fluid obtained at slaughterhouse were considered as collected before feeding and, thus, coded as 0. The actual NDF content of feed sample (NDF, 392 ± 175.3 g/kg), the amount of buffer (B, 35.5 ± 13.93 ml), the amount of rumen fluid (RF, 12.9 ± 5.54 ml), and the amount of feed sample incubated (FS, 0.40 ± 0.160 g DM) were initially treated as possible continuous variables. The NDF content of feeds was the only chemical constituent considered because: i) it was the only analytical measure reported by all scientific papers taken into account; ii) the NDF fraction is commonly considered a good descriptor of fermentation properties of feeds and/or diets (Van Soest, 1994) and it is strictly related with gas and CH_4 production (Moe and Tyrrell, 1979).

Two preliminary analyses of data were carried out. The first aimed at investigating the best classification system for the IT variable. Two classes of IT (24 or ≥ 48 h) were chosen as the final outcome of the first preliminary investigation. The second analysis was carried out to test the possibility of treating B, RF, and FS (amounts of buffer, rumen fluid, and feed sample incubated, respectively) as continuous variables. Because of their low variability within experiment, the three variables were not run separately in the statistical model, but they were included as the ratio between the buffered rumen fluid (BRF) and the FS, here defined as the BRF/FS ratio. This choice was further motivated by the fact that the BRF/FS ratio has a relevant effect on *in vitro* GP and GP kinetics (Beuvink and Spoelstra, 1992; Rymer et al., 2005). In the present study the ratio was coded into three classes (< 130 vs. 130-140 vs. > 140 ml/g DM; 149, 46, and 79 observations, respectively) as 0 or 1 or 2 in respective order.

3.4.3 Data cleansing to obtain the final dataset

The final dataset submitted to the statistical analysis accounted for only 183 (corresponding to 15 papers) out of the 274 initial observations. The majority of observations were excluded because of: i) one explanatory variable was missing (42 observations); ii) the observation was taken from IT < 24 h (29 observations). To follow the indications suggested by St-Pierre (2001) for meta-analysis, other observations were discarded because: i) the continuous variable considered (NDF) was constant in the trial (9 observations); and ii) trials accounted for a single observation (8 observations). After this data cleansing, the resulting database included only 3 observations belonging to the class of sheep as donor species. For this reason, also these 3 data were discarded and, thus, the final dataset (n = 183) comprised only the observations referred to bovine as donor species.

3.4.4 Statistical analysis

The latter dataset was analyzed using a mixed model analysis accounting for the random trial effect, with the scope of eliminating possible confounding effects due to differences across studies, as suggested by St-Pierre (2001).

To overcome a possible over-parameterization of the mixed model, a first analysis accounting for the random trial effect via the PROC MIXED of SAS (SAS Institute Inc., 2007) was carried out, considering a variance component (TYPE=VC) covariance structure (St-Pierre, 2001). To take into account the different accuracies among studies as well, all dependent variables were weighed by the inverse of the squared standard error divided by the mean of all the squared standard errors, as suggested by St-Pierre (2001). At a later stage, all data of GP, CH_4 and CH_4/GP, adjusted for the heterogeneity due to different studies, i.e., trial effect (St-Pierre, 2001),, were analysed using the backward elimination technique (Oldick et al., 1999) of SAS (PROC REG; SAS Institute Inc., 2007). The exit level for each variable (5 discrete and 1 continuous) was set at $P > 0.10$.

3.5 RESULTS

3.5.1 Description of the starting dataset

The list of references excluded from the meta-analysis and the reasons for exclusion are given in Table 1. The references entering the starting dataset and the corresponding description of factors are

listed in Table 2. In most of the experiments (30 out of a total of 39), fermentations occurred in conditions of increasing pressure, and gas was accumulated into the GP system during the incubation; the remaining 9 studies were conducted at constant pressure, by a regular venting of fermentation gases. The majority of the *in vitro* experiments (22) were stopped at 24 h; in 6 studies, fermentations lasted less than 24 h, whereas 11 studies used an incubation time ≥ 48 h. In 5 researches, different incubation times were compared. Rumen fluid used for *in vitro* tests was preferentially collected from bovine (29 studies), and only 7 experiments used sheep as donors. In most of the cases rumen fluid was collected before feeding of donor animals (before feeding in 17 trials; at slaughterhouse in 3 trials); however, in a relevant number of cases (i.e., 13 trials) rumen fluid was collected after feeding of donors. Six publications did not indicate the timing of rumen fluid collection. In a large number of the experiments (27 on 39), rumen fluid was mixed with a buffer solution containing N. The NDF content of feed samples incubated showed a high variability, ranging from a very low (0 g/kg, for potato starch and corn starch) to an extremely high value (929 g/kg, for sugarcane bagasse). The amounts of buffer and rumen fluid used in the study presented no variability, whereas in six papers different amounts of feed sample were tested.

Table 2. List of preliminary references considered with their respective description of factors selected as possible sources of variation on total gas production (GP), methane (CH_4) and their ratio (CH_4/GP).

References	N°[1]	Pressure (PR)[2]	Incubation time (IT), h[3]	Donor species (DS)[4]	Collection time (CRF)[5]	N buffer[6]	NDF, g/kg[7]	B, mL[8]	RF, mL[9]	FS, g DM[10]
Lila et al., 2003	6	increasing	6; 24	bovine	before-feeding	yes	0-473	20	10	0.18
Lila et al., 2004	1	increasing	6	bovine	before-feeding	yes	466	20	10	0.18
Lovett et al., 2004	2	constant	8; 24	bovine	Unknown	yes	522	80	20	0.14
Getachew et al., 2005	28	constant	6; 24; 48; 72	bovine	post-feeding	yes	250-315	20	10	0.18
Tavendale et al., 2005	2	increasing	12	sheep	Unknown	no	256-340	13	3	0.50
Lovett et al., 2006	6	constant	72	bovine	Unknown	yes	364-412	80	20	0.46
Bodas et al., 2008	11	increasing	24	sheep	before-feeding	yes	450	40	10	0.55
Garcia-Gonzales et al.,	2	increasing	24	sheep	before-feeding	yes	440	40	10	0.45
Garcia-Gonzales et al.,	1	increasing	24	sheep	before-feeding	yes	386	40	10	0.52
Macheboeuf et al., 2008	8	increasing	16	sheep	before-feeding	no	262	25	15	0.37
Soliva et al., 2008	26	constant	24	bovine	before-feeding	yes	254-583	10	20	0.28
Holtshausen et al., 2009	1	increasing	24	bovine	post-feeding	no	347	15	5	0.50
Martínez et al., 2010	8	constant	8; 24	sheep	before-feeding	yes	374-499	32	8	0.37
Sallam et al., 2010	3	increasing	24	sheep	before-feeding	no	547-616	50	25	0.46
Xu et al., 2010	15	increasing	24	bovine	post-feeding	yes	126-749	42	8	0.55
Araujo et al., 2011	1	increasing	16	sheep	before-feeding	yes	203	50	25	0.46
Avila et al., 2011	1	increasing	48	bovine	post-feeding	no	385	18	6	0.50
Guglielmelli et al., 2011	5	increasing	48	bovine	Slaughtering	no	391-523	74	5	0.93
Lee et al., 2011	2	increasing	24	bovine	before-feeding	yes	116-451	40	10	0.43-0.45
Navarro-Villa et al., 2011a	27	increasing	24	bovine	before-feeding	yes	187-871	33-43	7-	0.28-0.64
Navarro-Villa et al., 2011b	4	increasing	24	bovine	before-feeding	no	396-498	40	10	0.46
Pellikaan et al., 2011	11	increasing	72	bovine	post-feeding	yes	25-648	40	20	0.45-0.48

Table continued on next page

References	N°[1]	Pressure (PR)[2]	Incubation time (IT), h[3]	Donor species (DS)[4]	Collection time (CRF)[5]	N buffer[6]	NDF, g/kg[7]	B, mL[8]	RF, mL[9]	FS, g DM[10]
Purcell et al., 2011	9	increasing	24	bovine	before-feeding	no	351-426	40	10	0.46
Sun et al., 2011	8	constant	24	sheep	Unknown	yes	281-499	48	12	0.62
Amaro et al., 2012	1	increasing	24	bovine	Slaughtering	yes	383	33	17	0.39
Blanco et al., 2012	3	increasing	24	bovine	Unknown	yes	196-323	40	10	0.36
Carrasco et al., 2012	1	increasing	17	bovine	Slaughtering	no	179	32	8	0.40
Garcia-Gonzales et al.,	1	increasing	12	sheep	post-feeding	yes	0	40	10	0.46
Hassanat et al., 2012	1	increasing	24	bovine	post-feeding	no	331	17	3	0.18
Pirondini et al., 2012	2	increasing	24	bovine	before-feeding	yes	321-492	20	10	0.23
Ramin and Huhtanen, 2012	4	constant	48	bovine	post-feeding	no	570	48	12	0.29-1.15
Hansen et al., 2013	1	constant	48	bovine	before-feeding	yes	465	60	30	0.46
Narvaez et al., 2013	3	increasing	48	bovine	post-feeding	no	372	27	13	0.46
Naumann et al., 2013	10	increasing	48	bovine	Unknown	yes	182-351	16	4	0.18
Patra and Yu, 2013a	1	increasing	24	bovine	post-feeding	yes	292	30	10	0.37
Patra and Yu., 2013b	2	increasing	24	bovine	post-feeding	yes	290-416	30	10	0.37
Ramin et al., 2013	32	constant	24; 48	bovine	post-feeding	yes	249-613	40	20	0.46
Tuyen et al., 2013	4	increasing	48	bovine	post-feeding	yes	714-929	40	20	0.42-0.52
Cattani et al., 2014	20	increasing	24	bovine	before-feeding	yes	106-591	40	20	0.36-0.38

[1]N° = number of observations per article
[2]Pressure = pressure produced in the GP equipment used
[3]Incubation time = duration of incubation
[4]Donor species = donor species of rumen fluid
[5]Collection time = origin of rumen fluid: if it was collected from fasted donors, from post-fed donors or from donors at slaughterhouse
[6]N buffer = presence of N in the buffer solution
[7]NDF, g/kg = actual NDF content of feed samples used
[8]B, mL = buffer incubated
[9]RF, mL = rumen fluid incubated
[10]FS, g DM= feed sample incubated

3.5.2 In vitro values of GP, methane production, and proportion

The mean and standard deviation (s.d.) values of *in vitro* GP, CH_4 production and proportion, obtained considering the entire analysed dataset (i.e., including 183 observations), are given in Table 3. *In vitro* GP ranged from a minimum of 95 to a maximum of 276 ml/g DM, with an s.d. value ranging from 7.7 to 77.6 ml/g DM. Values of CH_4 production ranged from 15 to 77 ml/g DM, with s.d. values included between 1.0 and 23.3 ml CH_4/g DM. When expressed as a proportion of the total GP, CH_4 values ranged from 10.6 to 40.6 % of total GP, with s.d. values included between 0.1 and 6.0.

Table 3. Means and standard deviation (s.d.) of total gas production (GP), methane (CH_4) and their ratio (CH_4/GP) on the 183 observations belonging to 15 references retained for the final analysis.

References	N°[1]	GP, mL/g DM		CH_4, mL/g DM		CH_4/GP, %	
		mean	s.d.	mean	s.d.	mean	s.d.
Lila et al., 2003	3	199.6	31.01	76.0	23.26	37.6	6.05
Getachew et al., 2005	21	234.7	20.86	53.8	15.65	22.6	5.20
Soliva et al., 2008	26	129.4	49.48	15.0	10.46	10.6	3.72
Xu et al., 2010	15	163.2	75.55	16.9	3.90	12.1	4.51
Guglielmelli et al., 2011	5	141.1	16.65	24.5	5.23	17.3	2.32
Lee et al., 2011	2	193.6	65.83	23.8	8.27	12.3	0.07
Navarro-Villa et al., 2011a	27	140.9	59.86	20.5	8.78	14.8	2.58
Navarro-Villa et al., 2011b	4	158.5	14.53	35.3	1.80	22.4	0.95
Pellikaan et al., 2011	11	275.6	70.69	47.3	9.65	17.5	2.22
Purcell et al., 2011	9	182.7	7.68	25.1	1.04	13.7	0.36
Pirondini et al., 2012	2	243.5	40.31	40.3	5.73	16.6	0.35
Patra and Yu., 2013b	2	191.5	12.52	77.5	4.60	40.6	5.09
Ramin et al., 2013	32	223.2	77.52	36.2	9.83	16.9	3.03
Tuyen et al., 2013	4	94.9	41.81	17.6	7.17	19.1	2.39
Cattani et al., 2014	20	191.6	77.59	23.0	8.03	12.3	1.28

[1] N° = number of observations per article

Table 4 shows the mean and s.d. values of *in vitro* GP (ml/g DM), CH_4 production (ml/g DM) and their ratio (% CH_4 on total GP) for the different possible sources of variation taken into account for the 15 literature papers considered in the meta-analysis. Values of *in vitro* GP were numerically greater when fermentations were carried out: i) at constant pressure, that is by venting GP system (+ 13.3 % compared to increasing pressure); ii) with incubation time ≥ 48 h (+ 36.9 % compared to 24 h); iii) using rumen fluid collected after feeding of donor animals (+ 36.6 % compared to before feeding); iv) when the buffer solution contained N (only + 3 % GP compared to when N was not included), and v) using a BRF/FS ratio included between 130 and 140 ml/g DM (+ 54.9 % and + 14.1 % compared to BRF/FS < 130 and BRF/FS > 140 ml/g DM, respectively). A similar pattern was observed for CH_4 production values,

which increased under constant pressure conditions (+ 17.2 % compared to increasing pressure), with incubation times ≥ 48 h (+ 79.1 % compared to 24 h), when rumen fluid was collected after the feeding of donors (+ 69.0 % compared to before feeding), and when N was present in the buffer solution (+ 0.4 % compared to the absence of N). Values of CH_4 production were numerically greater when the BRF/FS ratio was > 140 ml/g DM (+ 79.8 % and + 0.5 % compared to BRF/FS < 130 and 130 ≤ BRF/FS ≤ 140 ml/g DM, respectively). When CH_4 data were expressed in terms of proportion (% of the total GP), values resulted numerically greater at increasing incubation times (+ 33.3 % with time ≥ 48 h compared to 24 h), when collection of rumen fluid was performed after feeding (+ 28.2 % compared to before feeding), and when BRF/FS was > 140 ml/g DM (+ 31.4 % and + 7.6 %, compared to BRF/FS < 130 and 130 ≤ BRF/FS ≤ 140 ml/g DM, respectively). The use of vented GP systems (operating at constant pressure) and the presence of N in the buffer slightly reduced CH_4 proportion (- 3.1 % compared to increasing pressure and - 3.6 % compared to the absence of N, respectively).

Table 4. Descriptive statistics of total gas production (GP), methane (CH_4) production and their ratio (CH_4/GP) for the main sources of variation analysed in the multivariate stepwise analysis after correction for the trial effect (n = 183 observations).

Main factors	N°[1]	GP, mL/g DM		CH_4, mL/g DM		CH_4/GP, %	
		mean	s.d.[2]	mean	s.d.	Mean	s.d.
Pressure							
Constant	89	196.4	74.72	32.7	18.49	15.8	5.93
Increasing	94	173.3	71.53	27.9	16.68	16.3	6.49
Incubation time, h							
24	133	167.6	65.68	24.9	14.81	14.7	6.19
≥ 48	50	229.5	76.03	44.6	16.82	19.6	4.70
Collection time							
Fasted donors	98	157.7	60.92	22.9	13.93	14.2	5.60
Post-feeding	85	215.4	75.60	38.7	17.89	18.2	6.21
N in the buffer							
Presence	165	186.6	77.16	30.6	18.53	16.0	6.43
Absence	18	165.8	21.74	27.2	5.24	16.6	3.71
BRF/FS[3]							
<130 mL/g DM	86	149.5	56.42	21.3	13.07	14.0	5.93
130-140 mL/g	43	231.5	83.59	38.1	11.59	17.1	2.95
>140 mL/g DM	54	202.9	62.79	38.3	21.21	18.4	7.49

[1]N° = number of observations accounted in each class [3]BRF/FS = buffered rumen fluid and feed sample ratio
2 s.d. = standard deviation of means

Table 5 shows the relative magnitude (i.e., rank of factors) accounted in the backward multivariate stepwise analysis and the predictive equations for *in vitro* GP (mL/g DM; equation 1), CH_4 production (mL/g DM; equation 2) and their proportion (% CH_4 on total GP; equation 3). The 3 predictive equations were the following:

[equation 1] In vitro GP (mL/g DM) = 189.9 –13.2 × PR +28.3 × IT +6.5 × CT +12.1 × N +7.4 × BRF/FS – 0.02 × NDF;

[equation 2] In vitro CH_4 (mL/g DM) = 21.9 +12.7 × IT +8.0 × CT +9.2 × N +6.24 × BRF/FS –0.01 × NDF;

[equation 3] In vitro CH_4 (% on total GP) = 11.7 +2.0 × PR +2.4 × CT +3.7 × N +1.3 × BRF/FS.

where PR = pressure conditions in the GP system (0 = constant; 1 = increasing); IT = incubation time (0 = 24 h; 1 ≥ 48 h); CT = collection time of rumen fluid (0 = from fasted donors; 1 = after feeding of donors); N = nitrogen in the buffer (0 = presence; 1 = absence); BRF/FS = buffered rumen fluid and feed sample ratio (0 = <130 mL/g DM; 1 = 130-140 mL/g DM; 2 = >140 mL/g DM); and NDF = NDF content of feed sample incubated (g/kg DM).

For *in vitro* GP values, the greatest amount of variation was depended on the IT, followed by PR, BRF/FS, N, NDF and CT in decreasing order, as shown by the F distribution value (Table 5). A different rank was found for CH_4 production values, with the BRF/FS showing the greatest magnitude, followed by IT, CT, N, and NDF, in decreasing order (Table 5). When CH_4 data were expressed as proportion of the total GP, most of the variability depended on CT (F = 148), followed by BRF/FS (F = 119), N (F = 115), and PR (F = 101).

Multi-collinearity was analysed through the variance inflation factor (VIF, Table 5). For all analysed factors, the maximum VIF was lower than 10, which is the threshold value under which the multi-collinearity among predictor variables can be assumed not significantly inflated (Rawlings, 1988). Moreover, collinearity among explanatory variables included in the multivariate stepwise analysis, expressed as a maximum condition index (Table 5), showed few dependencies among the considered variables, ranging from 4.59 to 7.65, which are widely under the threshold value of 30 suggested by Belsley et al. (1980) as indicator of possible dependencies (i.e., common variance explained).

Table 5. Outcome of the backward stepwise multivariate regression analysis on predicted values obtained by correcting for the trial effect and adjusting raw data for different accuracies[1] the total gas production (GP), the methane (CH_4) production and their ratio (CH_4/GP).

	GP, mL/g DM			CH_4, mL/g DM			CH_4/GP, %		
	Estimate ± SE	F	P	Estimate ± SE	F	P	Estimate ± SE	F	P
Intercept	189.9±2.77	4685	<0.001	21.9±2.14	105	<0.001	11.7±0.21	3085	<0.001
Pressure, PR[2]	-13.2±1.93	47	<0.001	-	-	-	2.0±0.20	101	<0.001
Incubation time, IT[3]	28.3±2.43	136	<0.001	12.7±1.98	41	<0.001	-	-	-
Collection time, CRF[4]	6.5±2.25	8	0.004	8.0±1.83	19	<0.001	2.4±0.20	148	<0.001
N in the buffer, N[5]	12.1±3.42	13	0.001	9.2±2.72	12	<0.001	3.7±0.35	115	<0.001
BRF/FS[6]	7.4±1.12	43	<0.001	6.2±0.91	47	<0.001	1.3±0.12	119	<0.001
NDF, g/kg DM[7]	-0.02±0.005	9	0.003	-0.01±0.004	8	0.005	-	-	-
R^2	0.74			0.55			0.68		
Max VIF[8]	1.65			1.63			1.25		
Max condition index[9]	7.65			7.06			4.59		

[1]Adjustment for different accuracies of measurements in different trials was carried out by weighting raw data by the inverse of the squared standard error divided by the mean of all the squared standard errors (St-Pierre, 2001)
[2]class 0 = constant or class 1 = increasing pressure
[3]class 0 = 24 h; class 1 = ≥48 h of incubation
[4]class 0 = fasted animal; class 1 = post-fed donors
[5]class 0 = presence; class 1 = absence of N in the buffer
[6]BRF/FS = (buffered rumen fluid and feed sample ratio) class 0 = <130 mL/g DM; class 1 = 130-140 mL/g DM; class 2= >140 mL/g DM)
[7]actual NDF content of feed sample used: treated as continuous variable
[8]VIF= variance inflation index. When value is less than 10, the predictor variables show no significant multicollinearity
[9]collinearity index. When value is less than 30, the variables tested are independent

Predicted values of *in vitro* GP and CH_4 production showed a correlation of 0.88; the relationship obtained regressing *in vitro* predicted CH_4 production against *in vitro* predicted GP produced a slope lower than 1 and a negative intercept (Figure 1a). On the other hand, predicted values of *in vitro* GP were totally independent from predictions of CH_4 proportion (coefficient of determination, i.e., R^2 = 0.002) (Figure 1b).

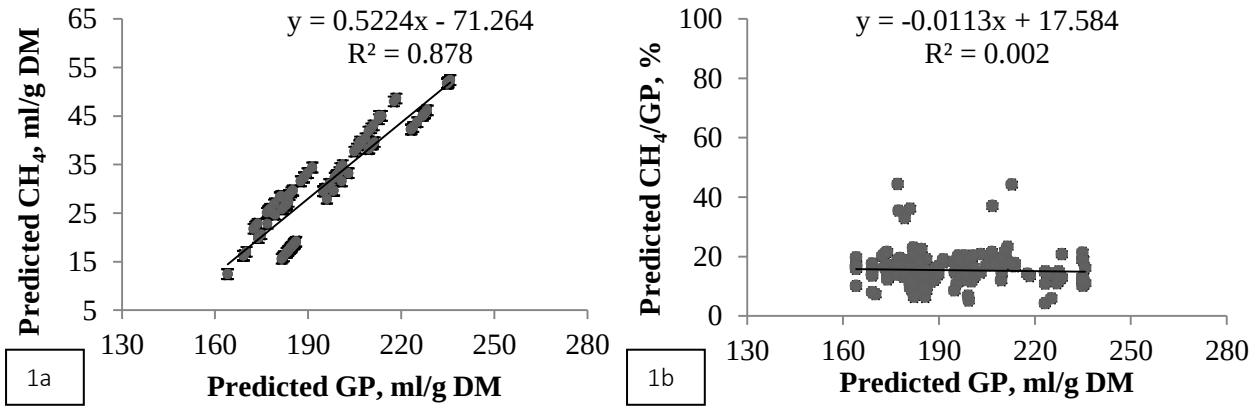

Figure 1: Relationship between gas production (GP) and methane (CH_4) (1a) or between gas production (GP) and their ratio (CH_4/GP) (1b) using the predicted values obtained from the mixed model analysis aimed at removing the trial effect (i.e., the heterogeneity of variance among studies) and considering also the correction of raw data for the different accuracies[1].

[1]Adjustment for different accuracies of measurements in different trials was carried out by weighting raw data by the inverse of the squared standard error divided by the mean of all the squared standard errors (St-Pierre, 2001

3.6 DISCUSSION

Over the last 10 years *in vitro* GP technique has been largely adopted to evaluate fermentation properties of single feeds and diets for ruminants, as it is a fast and cost-effective procedure (Rymer et al., 2005). However, this technique is still poorly standardized and several protocols of analysis are available, involving the use of different GP equipment, variable incubation times, methods of rumen fluid collection, and different analytical procedures. Results of this meta-analysis confirm that, to date, the *in vitro* GP technique has still a scarce degree of standardization and it is far from being possible to compare results obtained in many different studies.

3.6.1 Pressure conditions in GP equipment

When *in vitro* equipment is used to measure GP and CH_4, venting of gas is recommended to avoid overpressure conditions, which might disturb microbial activity (Theodorou et al., 1994) and cause a partial dissolution of CO_2 in the fermentation fluid, thus underestimating GP measures (Cattani et al., 2014). From this meta-analysis it results that GP equipment operating at increasing pressure (i.e., without gas venting) provide, on average, lower measures of GP compared to those working at constant pressure. In contrast, from this study it was found that values of CH_4 proportion increased significantly when GP systems operating at increasing pressure were used. In this regard, Patra and Yu (2013) hypothesized that the increase of CO_2 dissolved in the fermentation fluid, as result of overpressure conditions, would promote activity of methanogens. Additionally, when gas composition is analyzed, closed GP equipments are often preferred, to avoid complexity of collecting vented gas into proper devices (i.e., gas-proof bags). With this method, gas samples are collected from headspace of bottles at the end of incubation and analyzed for CH_4 concentration (Navarro-Villa et al., 2011; Pellikaan et al, 2011; Pirondini et al., 2012). These samples are considered to be representative and to provide reliable measurements of CH_4 because of its lower solubility in the fermentation fluid as compared to CO_2 (Navarro-Villa et al., 2011), hence measures are not affected by pressure changes in the bottles. According to this, the backward stepwise analysis did not highlight a significant effect of pressure on values of absolute CH_4 production (ml/g DM). Differently, the CH_4/GP ratio was significantly influenced by pressure conditions in the GP system, resulting in greater values for equipment working at increasing pressure (closed systems). As recently observed (Cattani et al., 2014), closed systems might underestimate *in vitro* GP, as a part of the CO_2 is dissolved in the fermentation fluid, leading to a possible overestimation of the CH_4 proportion on the total gas.

3.6.2 Incubation time

The positive correlation between incubation time and values of *in vitro* GP was expected and it is related to the progressive degradation of feed sample incubated over longer incubation times. Likewise, the significant increase of CH_4 production, in absolute terms, with the progress of *in vitro* fermentations is consistent with literature. For instance, Getachew et al. (2005) found that CH_4 production (ml/g DM) of seven commercial diets for dairy cows increased by 106.5 % passing from 6 to 72 h of incubation. Similar results have been reported by Lovett et al. (2004) and by Martínez et al.

(2010). Such tendency is explained by the fact that CH_4 formation is primarily related to fermentation of fibrous fraction that, as it is well known, has a slower degradation rate compared to other dietary components. However, results observed from the present meta-analysis might have been partially conditioned by the large predominance of roughages in the dataset considered. Nevertheless, it must be underlined that *in vitro* CH_4 production is often evaluated at a single incubation time, thus less information are provided about the kinetics of CH_4 formation *in vitro*. In this regard, only 3 of the 15 experiments considered in this meta-analysis measured CH_4 production at different incubation times.

3.6.3 Rumen fluid: collection time and donor species

Outcomes of this study showed that timing of rumen fluid collection had an impact on *in vitro* GP measures. More exactly, values of GP and CH_4 production were greater when rumen fluid was collected after feeding the donor animals. This result might be explained by the presence of feed particles suspended in the rumen fluid, which can lead to an overestimation of the actual GP. This problem could be overcome through the incubation of blanks (bottles containing only the buffered rumen fluid), where the GP of rumen fluid can be determined and then used to adjust values of GP provided by experimental treatments (Araujo et al., 2011). However, Cone (1998) observed that microbial turnover begins more rapidly in blanks, thus they have a different GP rate compared to other treatments. On this basis, Williams (2000) discouraged the adjustment of GP data by using blank values.

This meta-analysis also shows that the time of rumen fluid collection is, to date, one of the least standardized procedures of the *in vitro* GP technique. This evidence is supported by the fact that some protocols of analysis used worldwide involve the collection of rumen fluid before feeding of donor animals (Menke et al., 1979; Theodorou et al., 1994), whereas other authors suggest to feed animals before collection (Pell and Schofield, 1993; Cone et al., 1996). In this regard, Menke and Steingass (1988) indicated that rumen fluid used for *in vitro* tests should be collected before feeding of donor animals, as it has a less variable composition and, therefore, a more standardized effect on fermentations. However, the same authors (Menke and Steingass, 1988) specified that the interval time occurring between the feeding of donors and the collection of rumen fluid should not exceed 16 h, to ensure a sufficient microbial activity in the inoculum to sustain *in vitro* GP.

As previously described, the effect of donor species of rumen fluid (bovine or sheep) was not included in the final dataset. However, from the analysis of the starting database, it was clear that the

largest part of *in vitro* experiments had been carried out using rumen fluid collected from bovine (29 out of 39 references). Effects of donor species on *in vitro* GP are still uncertain, and a univocal ranking of various rumen fluids on the basis of GP is not possible (Rymer et al., 2005). In recent years, several studies have been conducted to compare bovine and sheep rumen fluid, but results were contrasting (Goncalves and Borba 1996; Bueno et al., 1999; Cone et al., 2002). However, GP values obtained using rumen fluid from different species might be reconciled by the appropriate use of blanks (Menke and Steingass, 1988), although not all authors are in agreement with the possible correction for blanks (Cone, 1998; William, 2000), as mentioned above. As a confirmation, in a ring test where rumen fluids collected from bovine or sheep were used (Cornou et al., 2013), correction of GP data for the relative blank samples gave a notable reduction of variability between laboratories.

3.6.4 Nitrogen in the buffer solution

The N in the buffer solution was found to be influential on *in vitro* GP and gas composition. More precisely, N-free buffers increased GP, CH_4 production and proportion. To our knowledge, there is no evidence from literature that the buffer composition might influence CH_4 measures obtained *in vitro*. The most experiments included in the dataset used a buffer solution containing N. In some cases the composition of buffer used for *in vitro* tests is related to incubated feeds (Rymer et al., 2005). For instance, some buffers are rich in N and poor in energy sources, in order to evaluate energy contribution of feed samples to fermentations (Menke and Steingass, 1988; Theodorou et al., 1994). On the opposite, other buffers are N-free, with the scope of evaluating the N contribution of high-protein feeds to *in vitro* fermentations (Cone et al., 2009). It is likely that the buffer solution alone cannot modify *in vitro* GP and CH_4 production in a significant way. More probably, some effects might appear when the mixture of buffer solution and feed sample is not balanced in terms of energy and N, thus microbial activity and growth might be impaired, with actual consequences on the various parameters of *in vitro* fermentation (Rymer et al., 2005).

3.6.5 Ratio between buffered rumen fluid and feed sample

Beuvink and Spoelstra (1992) indicated that BRF/FS ratio must not exceed the proportion of 60 ml of buffered rumen fluid with 0.4 g OM of feed sample, corresponding to about 136 ml/g DM. According to these authors, such ratio can avoid the exhaustion of buffer and the drop of pH under the threshold of 6.2, which causes a nonlinear relation between feed sample size and GP (Menke et al.,

1979). On a total of 15 papers (183 observations), only 9 papers reported pH values measured at the end of incubation (87 observations). Within these latter observations, 22 values were lower than the threshold of 6.2 (on average 5.96 ± 0.245; ranging from a minimum of 5.45 to a maximum of 6.19). However, in these experiments the drop of pH was not perforce related to a low BRF/FS ratio. This suggests that other factors (i.e., kind of buffer, kind of substrate, ratio between buffer solution and rumen fluid) could affect the pH trend during *in vitro* fermentation. Therefore, the actual effect of the BRF/FS ratio on GP and CH_4 values is difficult to comment on. Furthermore, considering the dataset of this meta-analysis, it is evident that BRF/FS is one of the least standardized parameters for *in vitro* GP technique. Indeed, only 4 authors out of 15 followed the indications of Beuvink and Spoelstra (1992), whereas 7 and 4 trials tested, respectively, lower (< 130 ml/g DM) and higher (> 140 ml/g DM) BRF/FS ratios. Results of the present meta-analysis show that the BRF/FS ratio was positively related to *in vitro* values of GP, CH_4 production and proportion. In this regard, it could be hypothesized that, when the BRF/FS ratio increases, the fermentation fluid could be more capable of buffering the VFA produced from feed degradation, promoting the release of CO_2 as indirect gas (Getachew et al., 1998). This process, in turn, would be expected to increase *in vitro* values of GP. Further, buffering action and maintenance of rumen pH would sustain the activity of methanogens, which are sensitive to acidification conditions (Russell, 1991), and thus the CH_4 production might be increased.

3.6.6 NDF content of feed samples

In line with our expectations, the NDF content of feeds incubated was one of the factors that affect *in vitro* measures of GP. More in detail, GP and NDF values were negatively correlated, as a high content of NDF in the feed is usually related to a reduced DM degradability (Hungate, 1966) and, thus, to a low GP. For the same reason, the fibrous content of feeds was also negatively correlated with the absolute amount of CH_4 produced *in vitro* (ml/g DM). It must be underlined that most of the data considered in this meta-analysis were referred to feed samples with a consistent amount of NDF (100 observations for roughages and 35 for diets), whereas only 48 observations were represented by concentrate feeds. Such data distribution is likely to have increased the incidence of the effect attributable to the NDF content. In this regard, the lower absolute CH_4 production (ml/g DM) of forages compared to concentrates, mainly due to the smaller extent of fermentation, was confirmed by several *in vitro* studies (Navarro-Villa et al., 2011; Pellikaan et al., 2011; Ramin et al., 2013; Cattani et al., 2014).

3.7 CONCLUSIONS

Results of the present meta-analysis highlight that about the 65 % of variability of *in vitro* gas and CH_4 values could be explained by the methodological factors taken into account and by the fibre content of feed substrates analyzed. Further, results show that *in vitro* GP technique is still plagued by a low degree of standardization. Clearly, this variability is partially conditioned by routine laboratory procedures and facilities, and to the specific aims of the various experiments. However, a greater level of standardization would be desirable to make possible the comparison among results obtained in different experiments.

3.8 ACKNOWLEDGMENTS

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APPENDIX 1. LIST OF THE PUBLICATIONS INCLUDED IN THE DATABASE

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Avila, J.S., Chaves, A.V., Hernandez-Calva, M., Beauchemin, K.A., McGinn, S.M., Wang, Y., Harstad, O.M., McAllister, T.A., 2011. Effects of replacing barley grain in feedlot diets with increasing levels of glycerol on *in vitro* fermentation and methane production. Anim. Feed Sci. Technol. 166-167, 265-628.

Blanco, C., Bodas, R., Prieto, N., Moran, L., Andres, S., Lopez, S., Giraldez, F.J., 2012. Vegetable oil soapstocks reduce methane production and modify ruminal fermentation. Anim. Feed Sci. Technol. 176, 40-46.

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4. SECOND CONTRIBUTION

Technical note: In vitro total gas and methane production measurements from closed or vented rumen batch culture systems

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4.1 ABSTRACT

This study compared measured gas production (GP) and computed CH_4 production values provided by closed or vented bottles connected to gas collection bags. Two forages and 3 concentrates were incubated. Two incubations were conducted, where the 5 feeds were tested in 3 replicates in closed or vented bottles, plus 4 blanks, for a total of 64 bottles. Half of the bottles were not vented, and the others were vented at a fixed pressure (6.8 kPa) and gas was collected into one gas collection bag connected to each bottle. Each bottle (317 mL) was filled with 0.4000 ± 0.0010 g of feed sample and 60 mL of buffered rumen fluid (headspace volume = 257 mL) and incubated at 39.0°C for 24 h. At 24 h, gas samples were collected from the headspace of closed bottles or from headspace and bags of vented bottles and analyzed for CH_4 concentration. Volumes of GP at 24 h were corrected for the gas dissolved in the fermentation fluid, according to Henry's law of gas solubility. Methane concentration (mL/100 mL of GP) was measured and CH_4 production (mL/g of incubated DM) was computed using corrected or uncorrected GP values. Data were analyzed for the effect of venting technique (T), feed (F), interaction between venting technique and feed (T × F), and incubation run as a random factor. Closed bottles provided lower uncorrected GP (−18%) compared with vented bottles, especially for concentrates. Correction for dissolved gas reduced but did not remove differences between techniques, and closed bottles (+25 mL of gas/g of incubated DM) had a greater magnitude of variation than did vented bottles (+1 mL of gas/g of incubated DM). Feeds differed in uncorrected and corrected GP, but the ranking was the same for the 2 techniques. The T × F interaction influenced uncorrected GP values, but this effect disappeared after correction. Closed bottles provided uncorrected CH_4 concentrations 23% greater than that of vented bottles. Correction reduced but did not remove this difference. Methane concentration was influenced by feed but not by the T × F interaction. Corrected CH_4 production was influenced by feed, but not by venting technique or the T × F interaction. Closed bottles provide good measurements of CH_4 production but not of GP. Venting of bottles at low pressure permits a reliable evaluation of total GP and CH_4 production.

Key words: *In vitro* gas production , Methane , Venting technique , Rumen fermentation

4.2 INTRODUCTION

Several batch culture systems are available to measure gas production (GP) during *in vitro* rumen fermentation. Many of these systems are equipped with devices for gas venting at fixed interval times (Theodorou et al., 1994) or at a fixed pressure (Cone et al., 1996; Davies et al., 2000; Calabrò et al., 2005; Tagliapietra et al., 2010). Gas venting is recommended to avoid pressure conditions that cause a partial dissolution of CO_2 in the fermentation fluid, with a consequent underestimation of total GP (Tagliapietra et al., 2010) and possible disturbance of microbial activity (Theodorou et al., 1994) and thereby CH_4 production. Vented GP systems are less frequently used to evaluate the composition of gas produced, because the vented gas must be collected in bags that are leak proof and are not permeable to CO_2 or CH_4. Closed GP systems are more commonly used for measurements of CH_4 production. With a closed system, the gas is not vented and remains in the bottle headspace until the time of collection for analysis (Pell and Schofield, 1993; Getachew et al., 2005; Pellikaan et al., 2011). However, the gas pressure generated in the headspace will cause a partial dissolving of CO_2 in the fermentation fluid that can alter the GP composition, so that a correction for solubilized CO_2 is required. The difference in CO_2 concentration in the headspace and the amount dissolved in the fluid can, in turn, alter the CH_4 concentration in the collected gas (Patra and Yu, 2013). The objective of the current study was to compare, also in term of repeatability, the effects of 2 systems of gas collection, one based on closed fermentation bottles and one based on vented bottles connected to tight plastic bags, on total GP, gas composition and CH_4 production computed from GP and gas composition measures.

4.3 MATERIALS AND METHODS

Two forages (meadow hay and ryegrass hay) and 3 concentrates (corn grain, dry sugar beet pulp, and expeller flaxseed) were incubated. The 5 feeds were selected to cover a large variability in chemical composition and to generate different pressure conditions in the bottle headspace during incubation. All feeds were provided by a dairy farm located in the province of Brescia (Italy). About 1 kg of each feed was ground by a hammer mill with a screen size of 1 mm. From each feed, 15 subsamples were prepared: 12 samples were used for the incubations (6 for each run) and the remaining 3 for chemical analysis. Feeds were analyzed in 3 replicates for proximate composition (AOAC International, 2012). The NDF, inclusive of residual ash, was determined with α-amylase and

sodium sulphite (Mertens, 2002) using the Ankom^{220} Fiber Analyzer (Ankom Technology, Macedon, NY). The ADF, inclusive of residual ash, and sulfuric acid lignin [lignin$_{(sa)}$] contents were sequentially determined according to Robertson and Van Soest (1981). Chemical composition of the 5 feeds is reported in Table 1.

Table 1. Dry matter (g/kg) contents and chemical composition (g/kg of DM) of the feeds (n=3)

	DM	NDF	ADF	Lignin$_{(sa)}$[1]	CP	EE	Ash	NSC[2]
Corn grain	900	106	23	-[3]	93	37	15	749
Dry sugar beet pulp	935	443	250	29	95	60	51	351
Flaxseed expeller	923	260	118	40	375	91	59	215
Meadow hay	893	435	305	60	90	15	86	374
Ryegrass hay	888	591	347	30	149	15	111	134

[1] Lignin$_{(sa)}$ = sulphuric acid lignin.
[2] NSC = non-structural carbohydrates, computed as: (100 – NDF – CP – EE – Ash).
[3] amount not measurable.

Incubations were conducted following the procedures detailed by Tagliapietra et al. (2012), according to a factorial design where the 5 feeds were simultaneously tested using the 2 techniques, closed or vented bottles, in each of 2 consecutive incubations. In each incubation, each feed was tested with 3 replications (using 3 bottles) plus 2 blanks containing only the buffered rumen fluid, for a total of 32 bottles. Rumen fluid was collected using an esophageal probe (Tagliapietra et al., 2012) from 3 intact, dry Holstein cows fed hay ad libitum and 2.5 kg/d of concentrates (0.5 kg of dry sugar beet pulp, 1 kg of corn grain, and 1 kg of sunflower meal) for 3 weeks preceding fluid collection. Differences between fluid collected with the probe instead of fluid collected from fistulated cows were not considered relevant for the comparative purposes of this work. The rumen fluid collected from the 3 cows was poured into 2 thermal flasks preheated to 39 ± 0.5°C and immediately transferred to the laboratory. The rumen fluid collected from the 3 cows was mixed, filtered through 3 layers of cheesecloth to eliminate feed particles, and then mixed with the buffer mineral solution in a 1:2 ratio (Menke and Steingass, 1988). All operations were conducted under anaerobic conditions by flushing with CO_2, and the time required for all operations was less than 30 min. Each bottle (317 mL) was filled with 0.4000 ± 0.0010 g of feed sample and 60 mL of buffered rumen fluid (Menke and Steingass, 1988), leaving a corresponding headspace volume of 257 mL. Bottles, placed into an incubator at 39.0 ± 0.5°C for 24 h, were not agitated during incubation. All bottles used in this experiment were from the A#^{RF} GP System (Ankom Technology), which was successfully tested for absence of gas pressure variation (70 kPa) over a period of 7 d. For the

purpose of the current study, half of the bottles were not vented during incubation, to simulate the closed system technique, and the others were connected with gas-proof Tygon tubing (Saint-Gobain Performance Plastics, Paris, France) to 1-L gas sample bags (standard FlexFoil, SKC Inc., Pennsylvania, PA) and automatically vented by an open-closed valve (model LHLX0502100BC, The Lee Co., Essex, CT) when headspace pressure reached 6.8 kPa. This pressure corresponded to 17 mL for a headspace volume of 257 mL. All gas sample bags were previously vacuum sealed to eliminate possible traces of air. The closed and vented bottles were equipped with pressure sensors (pressure range: –69 to +3,447 kPa; resolution: 0.27 kPa; accuracy: ±0.01 of measured value) that measured the pressure inside the bottles every 1 min. At the end of incubation (24 h), gas was collected with a 10-mL, gas-tight syringe (Artsana S.p.A., Como, Italy) from the headspace of each closed bottle, and from the headspace and gas-tight plastic bag for each vented bottle. At sampling, the syringe was flushed to ensure the collection of a homogeneous sample, which was immediately injected into a 9-mL Vacuette (Greiner Bio-One GmbH, Kremsmünster, Austria). From each Vacuette, an aliquot of 2.7 µL of gas was extracted with a gastight syringe (1701N, Hamilton, Bonaduz, Switzerland) and immediately analyzed for CH_4 concentration by GC with flame ionization detection (8000 TOP, Thermo Quest, Rodano, Milano, Italy) using a 30-m stainless steel column (GS-CarbonPLOT, Agilent Technologies, Milano, Italy) and H_2 as carrier gas (flow rate: 1.8 mL/min; isothermal oven temperature: 150°C). A 6-point standard curve was generated by mixing known volumes of CH_4 (99.5% pure, SAPIO s.r.l., Monza, Italy) with known volumes of air using the same graduated gas-tight syringe (1701N, Hamilton). The 6 gas mixtures used for calibration contained 10, 15, 20, 25, 50, and 100 mL of CH_4/L. The calibration regression had an R^2 >0.99.

4.4 COMPUTATIONS AND STATISTICAL ANALYSIS

Cumulative pressure values were calculated from measured pressure changes at given times, converted to units of volume (GP, mL) using the ideal gas law, and expressed per gram of incubated DM. Bottles with only rumen fluid and buffer (blanks) produced minimal amounts of gas (on average 7.2 ± 2.2 mL) and therefore were not used in further calculations. The GP volumes were corrected or not for the amount of dissolved gas computed according to Henry's law, from total gas pressure and CO_2 solubility, as described by Pell and Schofield (1993). Corrections for solubilized CO_2 are hereafter called adjustments. The total CH_4 production achieved from closed bottles was computed according to Lopez et al. (2007) as follows: CH_4 concentration in the headspace × [headspace

volume (257 mL) + total GP volume (mL)]. Methane production from vented bottles was computed as CH_4 concentration in the headspace × headspace volume (257 mL) + CH_4 concentration in the gas bag × total GP volume (mL). The CH_4 concentration and calculated production were both computed using adjusted and unadjusted total GP volumes. Data were analyzed using PROC MIXED of SAS Institute (2007) with a model that included, as fixed factors, the venting technique (T; 1 df), the feed (F; 4 df), the interaction between venting technique and feed (T × F; 4 df), and as a random variable the incubation run (1 df). A model considering all variables as random was used to estimate the variance components. The repeatability, defined as the value below which the absolute difference between 2 single measures obtained with the same technique and under the same conditions (same incubation run, same feed) is expected with a 95% probability (IOS, 1994), was computed as: $2\sqrt{2\sigma_e^2}$ where σ_e^2 is the residual variance.

4.5 RESULTS AND DISCUSSION

Closed bottles produced lower (approximately −18%; P<0.001) amounts of unadjusted GP compared with vented bottles (Table 2). The differences were particularly pronounced for highly fermentable substrates such as corn grain (−56 mL of gas/g of incubated DM; P<0.001) and dry sugar beet pulp (−54 mL of gas/g of incubated DM; P<0.001), whereas smaller, but still significant, differences were found for flaxseed expeller (−26 mL of gas/g of incubated DM; P<0.001), meadow hay (−26 mL of gas/g of incubated DM; P<0.001), and ryegrass hay (−17 mL of gas/g of incubated DM; P=0.005; data not shown). Values of repeatability were 5.9 and 8.9 mL of gas/g of incubated DM for closed and vented bottles, respectively. The T × F interaction (P<0.001) was found to influence the unadjusted values of GP, but this interaction effect was not significant when GP values were adjusted for dissolved gas (P=0.25). After adjustment for dissolved gas, the GP values provided by the 2 techniques continued to be different (P<0.001), even if this difference was numerically smaller. The magnitude of such adjustment was notable for closed bottles where a considerable increase of GP values (on average +25 mL of gas/g of incubated DM) was found, whereas the adjustment value was negligible for vented bottles (on average +1 mL of gas/g of incubated DM; Figure 1a and 1b). Feeds differed for unadjusted and adjusted GP (P<0.001), even though the ranking for GP was unaffected by venting. Values of repeatability for adjusted GP were 6.3 and 9.0 mL of gas/g of incubated DM for closed and vented bottles, respectively. The 2 techniques provided

different (P<0.001) unadjusted CH_4 concentration (mL/100 mL of GP), with values 23% greater for closed compared with vented bottles. The adjustment for dissolved GP reduced but did not completely remove this difference (P=0.002). Methane concentration was influenced by feed (P=0.005), but not by the T × F interaction (P=0.57). Unadjusted or adjusted CH_4 production (mL/ of incubated DM) was influenced by feed (P<0.001) but not by the venting technique or the T × F interaction.

Table 2. Effect of gas collection technique, feed, and their interaction on the unadjusted or adjusted measures of gas production (GP, mL/g of incubated DM), CH_4 concentration (mL/100 mL of GP), and CH_4 production (mL/g of incubated DM)

	GP		CH_4 concentration		CH_4 production	
	Unadjusted	Adjusted[1]	Unadjusted GP	Adjusted GP[1]	Unadjusted GP	Adjusted GP[1]
Technique						
Closed bottles	168	193	14.2	12.4	23.0	23.6
Vented bottles	204	205	11.5	11.2	22.9	23.0
SEM	7.3	7.7	0.63	0.23	0.43	0.44
Feed						
Corn grain	287	306	11.7	10.8	32.3	33.0
Dry sugar beet pulp	254	271	12.6	11.6	31.2	31.9
Flaxseed expeller	154	165	14.1	13.0	21.5	21.8
Meadow hay	137	147	12.8	11.7	17.1	17.3
Ryegrass hay	98	106	13.1	12.0	12.6	12.7
SEM	7.7	8.0	0.71	0.38	0.70	0.71
P values:						
Technique (T)	<0.001	<0.001	<0.001	0.002	0.88	0.38
Feed (F)	<0.001	<0.001	0.006	0.005	<0.001	<0.001
T × F	<0.001	0.25	0.55	0.57	0.83	0.79
Repeatability[2]						
Closed bottles	5.9	6.3	2.38	2.22	1.91	1.93
Vented bottles	8.9	9.0	1.88	1.84	1.80	1.81

[1] Values of GP adjusted by adding the amount of dissolved gas computed from total gas pressure and CO_2 solubility, according to the Henry's law (Pell and Schofield, 1993).

[2] Repeatability, defined as the value below which the absolute difference between two single measures obtained with the same technique and under the same conditions (same incubation run, same feed) is expected with a 95% probability, computed as $2\sqrt{2\sigma^2_e}$ (IOS, 1994).

Results of GP and gas composition obtained from different researchers *in vitro* are often difficult to compare because of the influence of several confounding sources of variation. Among these, the major sources are represented by different operative conditions, such as the procedures of rumen fluid collection and treatment (Cornou et al., 2013), the type of buffer used (Patra and Yu, 2013), the ratios among feed sample size, fermentation fluid, and headspace volume (Ramin and

Huhtanen, 2012), and the type of GP equipment, such as syringes or bottles that can be closed or vented at fixed times or at fixed pressure (Gierus et al., 2008; Tagliapietra et al., 2010; Pirondini et al., 2012). The magnitude of the difference of unadjusted gas composition between closed and vented bottles found in this experiment indicates that GP equipment would affect the measured gas composition equally or more than that attributable to differences among diets or feeds. One major aspect that differentiates various GP systems concerns the different pressures generated within the fermentation units and the way by which such pressures are controlled. Despite the popularity of the various automated GP systems in use and the great effort of research done in this field, these aspects have been poorly investigated (Ramin and Huhtanen, 2012). Results of the current study show that closed bottles produced a notable underestimation of actual GP. The magnitude of this effect was small in the first phases of incubation (<6 h), when the headspace pressure was low, but it progressively increased at later incubation times as the headspace pressure increased (Figure 1).

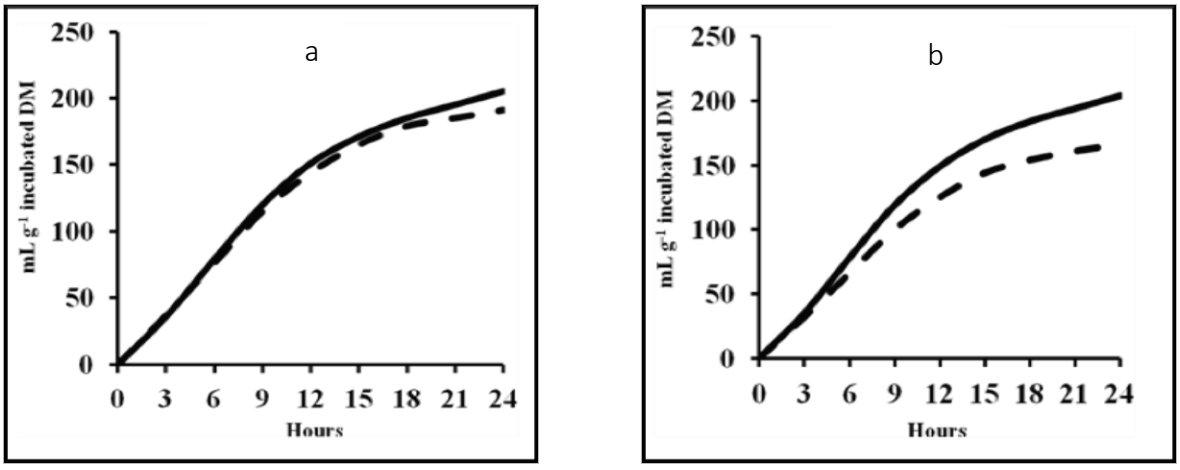

Figure 1. Gas production measurements (a) unadjusted, or (b) adjusted for dissolved CO_2 in closed (dotted lines) or vented bottles (solid lines) where, in 2 incubation runs, 5 feeds were incubated in 3 replications with buffered rumen fluid for 24 h.

Moreover, the magnitude of this effect mainly depends on the ratios among headspace volume, fermentation fluid, and sample size. In the current experiment, with 0.5 g of feed sample, the ratio between the volume of headspace and that of fermentation fluid was 4.3, a value greater than those frequently found in literature. This suggests that a greater underestimation of GP would be expected when smaller ratios between headspace and fermentation fluid are adopted, particularly when feeds are incubated for long times. In the vented bottles, the kinetics of GP were

not influenced by the adjustment for dissolved CO_2, as venting occurred when the 6.8 kPa threshold pressure was reached. On average, valve opening occurred 2 to 3 times for hays, 4 times for flaxseed, and 7 to 8 times for corn grain and sugar beet pulp. To overcome GP underestimation due to the dissolved CO_2, Pell and Schofield (1993) proposed that GP measures may be adjusted for dissolved CO_2, according to Henry's law, which considers the CO_2 solubility in saline solution, which may not be the same in buffered rumen fluid. In support of the results of Tagliapietra et al. (2010), our results confirm that this adjustment is not sufficient, particularly in closed systems, for a complete accounting of dissolved CO_2 in the buffered rumen fluid. A further adjustment is needed, either to compensate for more dissolved CO_2 than Henry's law allows or to account for differences caused by secondary interference of dissolved CO_2 on rumen fermentation. This implies that caution must be made in evaluating data of gas production and composition obtained from closed bottles, as an overestimation of CH_4 concentration and an underestimation of actual GP would be expected, even when an adjustment for dissolved CO_2, using Henry's law, is applied. In contrast, for bottles vented at low fixed pressures, the adjustment factor for dissolved CO_2 at the end of fermentation is small and results in minimal changes in the measures of GP and CH_4 concentration, suggesting that equipment operating at low pressure will provide a reliable measure of both total gas and CH_4 production. The main reason why closed bottles are preferred for measurements of CH_4 production is that all the CH_4 produced remains in the bottle headspace (often small) without the need for the connected bag. This saves space in the incubator equipment, allowing an increased number of replications, and avoids the risk of undesired gas loss. In the current experiment, irrespective of the GP adjustment, closed and vented bottles provided similar measures of CH_4 production, but only when these measures were expressed per unit of incubated DM. This was expected because, in contrast to CO_2, small or negligible amounts of CH_4 are dissolved in the fermentation fluid of closed bottles because of the very low CH_4 solubility in water (0.024 g/L at 20°C and 101.3 kPa of pressure). At the same time, the lack of difference in CH_4 production using the different gas collection techniques shows that gas losses from vented bottles connected to gas-tight bags used in this experiment are negligible or very low. The small or absent difference in CH_4 production between closed and vented bottles may also be due to the fact that care was taken to ensure that the internal pressure in the closed bottles would not reach 45 kPa after 24 h of fermentation. This value is lower than the threshold of 48 kPa above which microbial activity can be disturbed (Theodorou et al., 1994). This was done by considering the ratios between the volume of fermentation fluid, the

bottle headspace, the amount of feed incubated, and the expected GP at 24 h for each feed. The maximum headspace pressure found for corn samples after 24 h of incubation was, on average, 39 kPa. However, Patra and Yu (2013) demonstrated that headspace gas composition and bicarbonate concentration in the medium can affect CH_4 production and other characteristics of *in vitro* rumen fermentation. They proposed that a greater amount of dissolved CO_2 would stimulate CH_4 production. Reliable measurements of *in vitro* total GP and CH_4 would be particularly useful to explore effects of vitamins, additives, or bioactive molecules on rumen fermentation and microbial activity (Cattani et al., 2012; Hansen et al., 2012; Tagliapietra et al., 2013).

4.6 CONCLUSIONS

We conclude that closed systems permit reliable and comparable measurements of CH_4 production per unit of incubated substrate but not reliable measurements of GP, particularly in those cases where high gas pressures are generated from feed degradation. In these cases, GP could be indirectly computed from stoichiometric relations based on VFA production after *in vitro* fermentation, as described by Blümmel et al. (1997), but this requires time and additional analysis and costs, and uncertainty remains due to the possible influence of dissolved CO_2 on CH_4 production. Use of systems based on automated venting of gas at a low pressure threshold permits a reliable evaluation of both total GP and CH_4, with benefits in terms of less labor being required to control the fermentation process with respect to other semi-automated venting systems.

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5. THIRD CONTRIBUTION

Influence of main dietary chemical constituents on the in vitro gas and methane production in diets for dairy cows

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5.1 ABSTRACT

This study evaluated the influence of changes in main dietary constituents (starch:ADF ratio, protein and lipids contents), within ranges used in intensive farms of the North Italy, on *in vitro* degradability, gas (GP), and methane (CH_4) productions. A corn silage-diet (273, 361, 158, and 33 g/kg of starch, aNDF, CP, and lipids, respectively) was used as a reference diet. Other six diets had a low or high starch:ADF ratio (0.40 or 1.77, respectively), CP content (115 or 194 g/kg DM, respectively), or lipid content (26 or 61 g/kg DM, respectively). Four incubation runs were conducted (two lasting 24 h and two 48 h). The experimental design was: 4 incubations × 7 diets × 5 replications, plus 20 blanks (bottles without feed sample; 5 blanks/run), for a total of 160 bottles. In each run, diets (1.000 ± 0.0010 g) were incubated into bottles with 100 ml of buffer solution and 50 ml of rumen fluid. Bottles, equipped with a pressure detector, were vented at 6.8 kPa. At the end of each incubation, gas samples were collected from the bottle headspace and analyzed for CH_4. The proportion of CH_4 lost with venting was estimated. Fermentation fluids were analyzed for aNDF (NDFd) and true DM (TDMd) degradabilities, pH, ammonia N, and VFA. Data, averaged for run and diet, were analyzed using a model considering incubation time and diet as fixed factors and run within incubation time as a random blocking factor. An increasing starch:ADF ratio reduced significantly ($P < 0.01$ for both) acetate in the favor of butyrate and increased NDFd, TDMd, and GP per g DM and per g TDMd ($P < 0.001$ for all). The amount of CH_4 produced per g DM increased ($P < 0.001$), whereas that produced per g TDMd was unchanged. The increase of CP content increased ammonia N content ($P = 0.014$) and decreased propionate proportion in the favor of acetate ($P = 0.048$). No effects were observed for NDFd and TDMd, whereas GP decreased. The CH_4 production was reduced in low protein diet only when expressed as proportion of GP ($P < 0.001$). Lipid content had no effect on ammonia N and VFA. On the contrary, an increased lipid content reduced NDFd, TDMd, and GP per g DM. No influences were observed on CH_4 values. As expected, when incubation time was extended from 24 to 48 h, CH_4 produced per g DM and per g TDMd increased. Results of this study showed that the magnitude of effects exerted by the diets on *in vitro* fermentation was small. None of the diets reduced the CH_4 produced per g of degraded DM. Further research is needed to evaluate effectiveness of alternative mitigation strategies.

Keywords: Dietary manipulation, Dairy cows, *In vitro* techniques, Gas production, Methane production

5.2 INTRODUCTION

Mitigation of CH_4 production from rumen fermentation represents an important target for animal nutritionists, as this gas is responsible for global warming. Thus, the manipulation of dietary nutrient composition is often proposed as a strategy that farmers may exploit to reduce the proportion of energy lost by animals as eructated gases (CH_4) and to improve feed and energy efficiency (Cottle et al., 2011). There is evidence that the amount of CH_4 produced in the rumen is considerably influenced by the type and the content of dietary carbohydrates (Ellis et al., 2007) and lipids (Grainger and Beauchemin, 2011). However, in practice, notable reductions of CH_4 production compared to conventional high-roughages rations were more frequently observed for very concentrated diets (Johnson and Johnson, 1995) or when fat supplements (Grainger and Beauchemin, 2011) were used. In these cases the reduction in the gas emission was mainly a consequence of an overall impairment of rumen function with a reduction of fibre digestibility (Patra, 2012). Thus, these strategies do not always comply with the feeding standards used in the intensive dairy farms and they are usually not applied owing to the risks of negative health and economic consequences (Kumar et al., 2014). Compared to carbohydrates and lipids, minor effects on rumen gas production and methanogenesis are usually attributed to the crude protein (CP). In this regard, Bannink et al. (2005) observed that CH_4 production related to CP fermentation was lower compared to that due to carbohydrate fermentation. To date, changes in the dietary CP content have been mainly addressed to reduce feeding costs and N excretion (Yan et al., 2007; Schiavon et al., 2015). However, dietary strategies to reduce N excretion could also have an impact because CH_4 production may decline if starch or digestible nutrients escaping rumen fermentation replace CP (Dijkstra et al., 2011). Despite this, little information is currently available on the effects on CH_4 production due to changes in dietary CP content.

This study was aimed at evaluating *in vitro* the extent of alterations of true dry matter degradability (TDMD), total gas (GP) and CH_4 productions caused by changes in the proportions of the main feed ingredients and of the dietary constituents (structural and non-structural carbohydrates, CP and lipids), within ranges commonly used in intensive farms in North Italy.

5.3 MATERIALS AND METHODS

5.3.1 Chemical Composition of the Diets

Diets used in this experiment were defined after an analysis of a database containing information about ingredient and chemical composition of the rations used by 90 farms considered to be representative of the dairy farm system in North Italy (Dal Maso et al., 2009; Pirondini et al., 2012). The diets used in this study were formulated establishing a corn silage-diet, containing 273, 361, 158, and 33 g/kg of starch, aNDF, CP, and lipids, respectively, to be used as a reference (Table 1).

Table 1. Feed ingredients, chemical composition and gross energy content of seven diets

	Reference	Low starch:ADF	High starch:ADF	Low CP	High CP	Low Lipid	High Lipid
Ingredients							
Corn silage	351	-	430	375	281	351	351
Alfalfa hay	89	134	23	66	156	89	89
Ryegrass hay	47	231	-	43	52	47	56
Meadow hay	47	227	-	47	52	47	60
Corn grain	205	152	228	258	147	218	160
Barley grain	119	92	171	160	90	122	100
Soybean meal, (sol. extr., 44)	113	126	119	27	188	126	18
Whole soybean seeds	-	-	-	-	-	-	68
Extruded soybean seeds	-	-	-	-	-	-	68
Extruded flaxseed seeds	29	38	29	24	34	-	29
Chemical composition, g/kg DM							
Crude protein (CP)	158	161	152	115	194	161	158
Starch	273	100	273	332	176	265	233
aNDF	361	435	325	358	357	359	360
Hemicellulose	169	189	171	172	158	171	169
ADF	192	246	154	186	199	188	191
Cellulose	163	203	134	160	167	163	163
$Lignin_{(sa)}$	29	43	20	26	32	25	28
NFC[a]	395	302	443	446	357	402	367
Ether extract	33	38	34	34	34	26	61
Ash	53	64	46	47	58	52	54
Starch:ADF	1.42	0.40	1.77	1.78	0.88	1.41	1.21
Gross energy, MJ/kg DM[b]	16.8	17.3	16.9	16.8	17.3	16.5	16.7

[a] NFC = Not Fiber Carbohydrates computed as 100 - aNDF - CP - EE - Ash
[b] Measured by a bomb calorimeter method (ISO, 1998)

The other six diets were formulated to produce variations in the proportion of some feed ingredients, and hence in content of a given chemical constituent in favor or at the expense of starchy feeds, with respect to the reference diet. Two diets with a low (0.40) or a high (1.77) starch:ADF ratio were formulated by replacing roughages (corn silage, alfalfa hay, and ryegrass hay) with corn and barley grains in the form of meal. The diet with the lowest starch:ADF ratio did not contain corn silage, taking into consideration dairy farms that are not allowed to use this feed as they produce milk to be processed as Italian Protected Designation of Origin (PDO) Parmigiano-Reggiano cheese. Other two diets, with a low (115 g/kg DM) or a high CP content (194 g/kg DM), were formulated by replacing the soybean meal with cereal grains (corn and barley meals). It must to be considered that, in this way, the starch:ADF ratio of the low CP diet (1.78) was higher than that of the high CP diet (0.88). Two diets with different ether extract (EE) contents were also formulated. A low EE diet (26 g/kg DM) was achieved by excluding the extruded flaxseed (Linoies, Cortal Extrasoy, Cittadella, Italy), present in the reference diet, and increasing the content of corn, barley and soybean meal. The high EE diet (61 g/kg DM) was achieved by including whole soybean seeds, extruded soybean (Soyfull, Cortal Extrasoy, Cittadella, Italy) and extruded flaxseed. Diets were analyzed in triplicate for dry matter (DM: # 934.01; AOAC, 2003), N (# 976.05; AOAC, 2003), EE (# 920.29; AOAC, 2003), ash (# 942.05, AOAC, 2003) and aNDF content with amylase treatment (Van Soest et al., 1991). Neutral detergent fibre (aNDF), inclusive of residual ash, was determined (Mertens, 2002) with α-amylase using the Ankom220 Fibre Analyzer (Ankom Technology, NY, USA). Acid detergent fibre (ADF), inclusive of residual ash, and sulphuric acid lignin (lignin(sa)) were determined sequentially after aNDF determination (Robertson and Van Soest, 1981). Starch content was determined after hydrolysis to glucose (AOAC, 2003) by liquid chromatography (Bouchard et al., 1988). Gross energy content of diets (MJ/kg DM) was determined in duplicate by a bomb calorimeter method (ISO # 9831, 1998).

5.3.2 Incubation

The 7 diets were incubated in each of 4 repeated incubation runs, conducted in 4 successive wks. Two incubations were stopped at 24 h, whereas the other two were stopped after 48 h. In each of the four incubation runs we tested 7 diets × 5 replications, plus 5 blanks (bottles containing only the buffered rumen fluid; 5 blanks/run), for a total of 160 bottles incubated. A commercial GP apparatus (AnkomRF Gas Production System, Ankom Technology®, NY, USA) was used, consisting of

40 bottles equipped with pressure sensors (pressure range: from -69 to 3447 kPa; resolution: 0.27 kPa; accuracy: ± 0.1% of measured value) and wireless connected to a computer. Each bottle (317 ml) was filled with 1.000 ± 0.0010 g of diet, 100 ml of a buffer solution, and 50 ml of rumen fluid (headspace volume = 167 ml), keeping the headspace of bottles flushed with CO_2. The buffer solution was prepared according to Menke and Steingass (1988), heated in a water bath at 39 ± 0.4°C and purged continuously with CO_2 for 30 min, to maintain anaerobic conditions. Rumen fluid was collected by an esophageal probe, as described by Tagliapietra et al. (2012), 2 h before morning feeding from 3 dry Holstein-Friesian cows housed at the experimental farm of the University of Padova (Italy) and fed hay ad libitum and 2.5 kg/d of concentrates (0.5 kg of dry sugar beet pulp, 1 kg of corn grain, and 1 kg of sunflower meal). Rumen fluid was poured into thermal flasks preheated to 39 ± 0.5 °C, immediately transferred to the laboratory, strained through 3 layers of cheesecloth, to eliminate feed particles, and mixed with buffer solution in a 1 to 2 ratio (Menke and Steingass, 1988). Operations were conducted under anaerobic conditions, by flushing with CO_2, and required less than 30 min to be completed. Bottles were placed in a ventilated oven at 39 ± 0.4 °C and automatically vented at a fixed pressure (6.8 kPa), to avoid overpressure conditions and alterations of gas and CH_4 measures (Cattani et al., 2014b).

At the end of incubations (24 or 48 h), two aliquots (5 ml) of fermentation fluid were collected from each bottle and stored at -20°C with 1 ml of metaphosphoric acid (25%, w/v) to be later analyzed for ammonia N and volatile fatty acids (VFA). The content of ammonia N was measured using the FIAstarTM 5000 Analyzer (FOSS Analytical, Hilleroed, Denmark). The VFA profile was analyzed by GC with flame ionization detection (7820A GC system, Agilent Technologies, Milan, Italy) using a 30-m stainless steel column (J&W DB-FFAP, Agilent Technologies, Milan, Italy) and H2 as carrier gas (flow rate: 30 ml/min; isothermal oven temperature: 150°C). Fermentation fluids were filtered into weighed crucibles (30 ml, Robu Glasfilter-Geräte GMBH®, Hattert, Germany) and analyzed for residual aNDF using a Fibretech Analyzer (VELP® Scientifica, Milan, Italy).

At the end of each incubation (24 or 48 h), gas was collected with a 10-ml gas-tight syringe (Artsana S.p.A., Como, Italy) from the bottle headspace (HS). At each sampling, the syringe was flushed in order to collect a homogeneous sample, which was immediately transferred into a 9-ml vacuette (Greiner Bio-One GmbH, Kremsmunster, Austria). From each vacuette, an aliquot (10 µl) of gas was sampled with a gas-tight syringe (1701N, Hamilton, Bonaduz, Switzerland) and immediately

analyzed for CH_4 concentration by GC with flame ionization detection (7820A GC system, Agilent Technologies, Milan, Italy) using 15-m carbon layer column (GS-CarbonPLOT, Agilent Technologies, Milan, Italy) and H2 as carrier gas (flow rate: 1.6 ml/min; isothermal oven temperature: 40°C). An 11-point calibration curve was generated from eleven gas mixtures containing 2, 4, 8, 16, 24, 32, 60, 100, 140, 180, and 240 ml of CH_4/l (99.5% pure, SAPIO s.r.l., Monza, Italy), respectively, and known volumes of air. Mixtures were prepared using the same graduated gas-tight syringe (1701N, Hamilton). The calibration regression had $R^2 > 0.99$.

5.4 COMPUTATIONS AND STATISTICAL ANALYSIS

5.4.1 Computation

The aNDF degradability (NDFd) and the true DM degradability (TDMd) were calculated according to Goering and Van Soest (1970). Recently, Cattani et al. (2014b), using vented bottles connected to tight bags for gas collection, calculated CH_4 production (ml) as: [CH_4 concentration in HS] × [HS volume] + [CH_4 concentration in the gas bag × GP]. To evaluate the possibility of avoiding the use of bags, to save space and increase the number of replicates, data from a previous unpublished experiment (where different concentrates and forages were incubated for 6, 24, or 48 h with the same GP equipment under the same operative conditions of this experiment) were used. It was found that total CH_4 production is predictable, with acceptable precision and accuracy, as: -0.0064 × [CH_4 concentration in HS + (HS volume + GP)]^2 + 0.9835 × [CH_4 concentration in HS × (HS volume + GP)]. This equation had a residual standard deviation of only 0.1770 ml, and R^2 = 0.9993. Thus, the present experiment was conducted without the use of tight bags for gas collection. The CH_4 production was computed using the above described equation and it was expressed as ml/g DM incubated, ml/g TDMd, or ml/100 ml GP. *In vitro* GP and CH_4 were also predicted from VFA production, according to Blümmel et al. (1999).

5.4.1 Statistical Analysis

Data were averaged for run and diet and the 28 means were analyzed using PROC MIXED of SAS (SAS Institute, 2007). The statistical model included the incubation time (IT, 1 df) and the diet (D; 6 df) as fixed factors, the run within incubation time (2 df) as a random blocking factor and the

residual error term e (18 df). The Bonferroni adjustment was used for multiple comparison of the least square means of diets with different contents of a given chemical constituent.

5.5 RESULTS

Changes of the feed ingredients proportions and of dietary contents of chemical constituents had some influence on the various parameters of *in vitro* fermentation (Table 2). The final pH values, measured at the end of fermentation, were not influenced by the dietary changes. The ammonia N content increased with increasing dietary CP content ($P = 0.014$). No influence of the diets was observed on the VFA production, but the proportion of acetate or butyrate decreased ($P < 0.001$) or increased ($P < 0.001$), respectively, with an increase in the starch:ADF ratio, whereas the proportion of propionate decreased with increasing dietary CP ($P = 0.048$). Increasing proportions of CP also increased the proportion of other VFA ($P < 0.001$). Changes of dietary EE content had no consequence on the various rumen fluid parameters. The prolongation of the incubation time from 24 to 48 h increased only numerically the VFA production, but it did not influence pH and ammonia N values.

The NDFd, TDMd, and the GP expressed per unit of incubated DM or per unit of TDMd (Table 3) increased with an increase in the starch:ADF ratio ($P < 0.001$ for all). An increased starch:ADF ratio increased the CH_4 production per unit of incubated DM ($P < 0.001$), but the CH_4 yield per unit of TDMd did not increase. An increased dietary CP content had no influence on NDFd or TDMd, but GP was lowered and no influence was observed on the production of CH_4, except when this was expressed as a proportion of GP (ml CH_4/100 ml GP; $P < 0.001$). An increased inclusion of extruded oilseeds in the diet reduced both NDFd ($P = 0.003$) and TDMd ($P = 0.028$), and the measured GP expressed per g of incubated DM ($P = 0.017$), but no influences were observed on the CH_4 yield. A prolonged duration of the incubation, from 24 to 48 h, only numerically increased NDFd, TDMd, and GP, but CH_4 yield, per unit of incubated DM as well as per unit of TDMd, was increased. Predicted values of GP and CH_4 productions were not influenced either by dietary changes or by incubation time.

Table 2. Effect of diets and incubation time on pH, ammonia N concentration, volatile fatty acid production (VFA) and molar proportions of acetate, propionate, butyrate and other VFA.

	pH	Ammonia N, mg/L	VFA, mmol/l	Acetate, % VFA	Propionate, % VFA	Butyrate, % VFA	Other VFA, % VFA
Diet							
Reference	6.87	202	5.08	56.1	23.5	16.0	4.47
Low starch:ADF ratio	6.90	223	4.44	58.5^{A}	23.1	13.3^{B}	4.26^{B}
High starch:ADF ratio	6.88	192	5.37	55.8	23.7	16.2	4.29
Low CP	6.85	171	5.06	56.0	24.3	16.1	3.71
High CP	6.87	222	4.89	57.2	22.8	14.6	4.85^{A}
Low lipid	6.87	207	4.96	56.4	23.7	15.5	4.26
High lipid	6.90	197	4.99	56.7	23.5	15.4	4.36
SEM	0.043	28.5	0.231	0.25	0.64	0.91	0.158
P-value	0.46	0.008	0.25	<0.001	0.06	0.002	<0.001
P-value of contrasts							
Low *vs* High starch:ADF ratio	0.99	0.44	0.23	<0.001	0.99	0.004	-
Low *vs* High CP	0.99	0.014	0.99	0.049	0.048	0.99	<0.001
Low *vs* High lipid	0.99	0.99	0.99	0.99	0.99	0.99	-
Incubation time							
24 h	6.95	202	4.47	57.2	22.9	15.8	3.87
48 h	6.80	202	5.47	56.1	24.1	14.7	4.76
SEM	0.055	38.7	0.123	0.13	0.82	1.15	0.201
P-value	0.31	0.99	0.029	0.026	0.39	0.58	0.09
RMSE	0.032	17.5	0.462	0.49	0.60	0.87	0.147

Values with different superscripts within column are significantly ($P < 0.05$) higher (A) or lower (B) compared to the reference diet (containing 273, 361, 158, and 33 g/kg DM of starch, aNDF, CP, and lipids, respectively; starch:ADF ratio = 1.42); Low starch:ADF ratio = diet with a low starch:ADF ratio (0.40); High starch:ADF ratio = diet with a high starch:ADF ratio (1.77); Low CP = low protein diet (CP, 115 g/kg DM); High CP = high protein diet (CP, 194 g/kg DM); Low lipid = low lipid diet (EE, 26 g/kg DM); High lipid = high lipid diet (EE, 61 g/kg DM).

Table 3. Effects of diets and incubation time on in vitro degradability of aNDF (NDFd) and of true DM (TDMd), gas production (GP) and methane (CH_4) production, and predicted values of GP and CH_4 production.

	Degradability		Actual GP, ml per:		Actual CH_4 ml per			Predicted GP	Predicted CH_4
	NDFd, g/kg DM	TDMd, g/kg DM	g DM	g TDMd	g DM	g TDMd	100 ml GP	ml/g DM	ml/g DM
Diet									
Reference	567	840	274	325	34.2	40.8	12.5	244	38.8
Low starch:ADF ratio	480^{B}	767^{B}	228^{B}	300^{B}	30.8^{B}	39.9	13.5^{A}	220	35.9
High starch:ADF ratio	612^{A}	872^{A}	288^{A}	331	34.4	39.3	11.9	250	39.6
Low CP	545	835	277	333	33.4	39.9	12.0	242	38.0
High CP	542	831	255^{B}	310	34.0	41.0	13.3^{A}	234	37.8
Low lipid	583	845	265	315	33.1	39.0	12.3	251	37.2
High lipid	531	826	253^{B}	304^{B}	32.2^{B}	38.8	12.5	238	37.9
SEM	10.3	4.2	5.6	7.2	0.53	0.63	0.40	10.9	2.09
P-value	<0.001	<0.001	<0.001	<0.001	<0.001	0.025	<0.001	0.33	0.70
P-value of contrasts									
Low *vs* High starch:ADF ratio	<0.001	<0.001	<0.001	<0.001	<0.001	0.99	<0.001	0.99	0.99
Low *vs* High CP	0.99	0.99	<0.001	0.009	0.99	0.99	<0.001	0.99	0.99
Low *vs* High lipid	0.003	0.028	0.017	0.99	0.99	0.99	0.99	0.99	0.99
Incubation time									
24 h	472	801	250	314	29.6	37.0	11.9	214	34.9
48 h	631	860	276	320	36.7	42.6	13.2	260	41.5
SEM	10.5	3.5	7.4	8.8	0.59	0.66	0.54	7.6	1.69
P-value	0.009	0.007	0.14	0.69	0.014	0.027	0.22	0.06	0.12
RMSE	15.5	7.2	4.0	7.6	0.69	0.94	0.25	21.9	3.78

Values with different superscripts within column are significantly ($P < 0.05$) higher (A) or lower (B) compared to the reference diet (containing 273, 361, 158, and 33 g/kg DM of starch, aNDF, CP, and lipids, respectively; starch:ADF ratio = 1.42); Low starch:ADF ratio = diet with a low starch:ADF ratio (0.40); High starch:ADF ratio = diet with a high starch:ADF ratio (1.77); Low CP = low protein diet (CP, 115 g/kg DM); High CP = high protein diet (CP, 194 g/kg DM); Low lipid = low lipid diet (EE, 26 g/kg DM); High lipid = high lipid diet (EE, 61 g/kg DM).

5.6 DISCUSSION

5.6.1 General considerations

The diets used in this study were formulated using feed ingredients commonly used in the Po valley (North-East of Italy) and composed mainly by cereal grains, corn silage and modest amounts of various hays. In the scientific literature, *in vitro* evaluation of gas and CH_4 productions is commonly carried out using single feeds, mainly forages, whereas less information is available for complete diets (Getachew et al., 2005). *In vivo* measurement of gas and CH_4 production requires expensive equipment and it is labor and time consuming. *In vitro* techniques would permit a much more simple determination of the dietary characteristics which can influence the potential emission of gas and CH_4 from their fermentation in a simulated rumen environment (Cattani et al., 2014b). Studies of the relationships between *in vitro* and *in vivo* gas and CH_4 productions are lacking (Getachew et al., 2005). However, a recent study suggested that *in vitro* gas and CH_4 measurements can be indicative of the trend of *in vivo* CH_4 production originating from different combinations of feed ingredients (Hatew et al., 2015). This study was aimed at evaluating if changes in the diet composition might or might not have notable influence on gas and CH_4 productions. The average measured GP from the various diets after 24 h of fermentation was 250 ml/g DM, suggesting that a cow consuming 20 kg/d DM might produce about 5000 l/d of gas. The CH_4 production from fermentation of these diets ranged 30.8 to 34.4 ml/g DM, suggesting that, for a DM intake of 20 kg/d DM, a cow might produce 616 to 688 l/d of CH_4. Sauer et al. (1998) reported *in vivo* CH_4 production of lactating cows in the order of 622 l/d, corresponding to 38.9 ml/g DM intake. In the study of Holter and Young (1992), CH_4 production from dairy cows was 29.2 ml/g DM intake, whereas Moss (2002) reported for sheep an averaged CH_4 production of 31.0 ml/g DM intake. In a continuous culture fermenter, Eun et al. (2004) measured an averaged CH_4 production of 33.0 ml/g DM. The *in vitro* CH_4 production of the dairy rations tested by Getachew et al. (2005) varied from 30.1 to 35.9 ml/g DM, a range consistent with that found in this study. However, comparison with data from literature is difficult because huge variations in gas and CH_4 productions are commonly observed across experiments, even for diets with similar composition. This is the consequence of a combination of different biological, i.e. rumen fluid characteristics, and technical factors, i.e. fermentation procedures and equipment for collection, measurement and analysis (Maccarana et

al., 2015). In this experiment, as internal control of the consistency of the data, the observed GP and CH_4 were related to the GP and CH_4 values predicted from the stoichiometry of the VFA production. The correlation between measured and predicted values showed R^2 to be 0.78 and 0.74, respectively, for gas and CH_4 measures (data not shown), which are acceptable if the rather narrow range of variation in GP and CH_4 production caused by the relatively small dietary changes is considered. It was observed that the SEM of predicted GP and CH_4 production (21.9 and 3.78 ml/g DM, respectively) was about five times greater than the corresponding values of the measured GP and CH_4 production. In other words, the measure of the VFA production was less precise than the GP and CH_4 measures.

5.6.2 Effects on gas and CH_4 production due to changes in the starch:ADF ratio

Pirondini et al. (2012), in diets similar for ingredient composition to those used in this experiment, observed an increased CH_4 production (35.6 to 44.3 ml/g DM) with increasing starch:ADF ratio (0.60 to 2.23). In the present study, an increase from 0.40 to 1.77 of the starch:ADF ratio increased CH_4 production from 30.8 to 34.4 ml/g DM (+11.6%). This is consistent with an increased true degradability of the feed (+14%), but also of the aNDF fibrous fraction (+27%). This seems to be contradictory with current literature, as an increased starch:ADF commonly reduces the aNDF degradability (Pirondini et al., 2012). However, in this experiment such a result was achieved by an almost complete replacement of hays with corn silage and cereals. This is of interest in Italy as use of silages for the production of Parmigiano-Reggiano cheese is forbidden by specific feeding regulations. Results also evidenced that GP increased with an increase in the dietary starch:ADF ratio content even when expressed per unit of TDMd (+10%), but no influences were observed on the CH_4 production per unit of TDMd. A different trend emerged when CH_4 production was referred to total GP. In this case, an increased dietary starch:ADF ratio decreased the CH_4 proportion by about 12% (13.5 to 11.9 ml CH_4/100 ml GP, for the low and the high starch:ADF ratio, respectively). Results suggest that an increase of the dietary starch:ADF ratio, achieved from a complete replacement of hays with corn silage and cereals grains, might increase feed digestibility without changing GP and CH_4 produced per unit of digested material.

5.6.3 Effects on gas and CH_4 production due to changes in the CP content

In this experiment an increased proportion of CP, in replacement of starch, caused a reduction of GP. The negative influence of dietary CP on GP has been observed by others in the past. Such an effect was attributed to the buffer capacity of CP, that reduces the indirect CO_2 released from the buffered rumen fluid, and to the stoichiometry of protein fermentation, that differs from that of carbohydrates (Cone and van Gelder, 1999; Tagliapietra et al., 2011). In the present study, increasing the CP content decreased the production of propionate and butyrate, whereas other VFA (mainly iso-butyric and iso-valeric acids) were increased, being end-products of amino acid degradation. Changes in the dietary CP proportion had no effect on CH_4 production when expressed both per unit of incubated DM and per unit of TDMd. Thus, as CP depresses GP, an increased proportion of CP increases the CH_4 concentration on total GP.

5.6.4 Effects on gas and CH_4 production due to changes in the lipid content

Fat supplementation is often used to improve the fatty acid profile and nutritional value of milk and ripened cheese (Cattani et al., 2014a). However, the addition of fat to dairy cow diets can also be considered as a potential strategy to reduce CH_4 production (Hristov et al., 2013), although milk response to fat addition is not entirely predictable (Grainger and Beauchemin, 2011). The effect of dietary lipids on CH_4 production is dependent on the source, FA profile, level of inclusion, and diet composition (Knapp et al., 2014). The level of supplementation and the physical form of the lipid supplement affect its availability in the rumen, and these factors appear to be more important than the FA profile (Beauchemin et al., 2009). In this regard, Alstrup et al. (2015), using dairy cows housed in respiratory chambers, found that, compared to the control, the average reduction in CH_4 (l/kg DMI) per 10 g/kg of crude fat added was persistent throughout lactation. The same authors observed that the most effective lipid source in reducing methanogenesis was a commercial vegetal rumen protected fat fortified with hydroxy-methionine-analog-isobutyrate (-5.5% of CH_4), followed by vegetal rumen protected fat (-2.3%), and by whole cracked rapeseed (-0.8%). In the experiment of Beauchemin et al. (2009), only crushed canola seeds lowered CH_4 production per unit of digestible DM intake (-15%), whereas crushed flaxseeds and crushed sunflower seeds did not reduce CH_4 production compared to the control diet (a diet supplemented with a commercial source of calcium salts of long chain fatty acids). In this experiment changes in the dietary fat content of the

diets were achieved by changing the proportions of extruded flaxseeds, extruded soybean and whole soybean seeds. The threshold of 60 g fat/kg DM was considered to be the upper limit to avoid a possible impairment of feed digestibility (Jenkins, 1997; Kumar et al., 2014). Fat addition reduced feed degradability, particularly that of the aNDF fraction. No influences were found on total VFA production and on the proportion of acetate, propionate and butyrate, whereas GP and CH_4 productions decreased by 8 and 6%, respectively. However, differences were greatly reduced when GP and CH_4 were expressed per unit of TDMd suggesting that, under the commercial conditions evaluated in this study, small reductions of CH_4 might be achieved.

5.7 CONCLUSIONS

Changes of the ingredient and chemical composition of diets were analyzed to evaluate benefits in the amount of CH_4 produced, for the north eastern Italian dairy production chain. It was found that a replacement of hays with corn silage and cereals might increase GP and CH_4 per unit of DM intake, a decrease of the dietary CP content would reduce GP with no influences in the amount of CH_4 produced, and a moderate addition of cracked soybean seeds, and extruded flaxseed had few, or any, influence on the *in vitro* GP and CH_4 productions. In general, none of the various strategies tested in the present work was able to reduce the amount of CH_4 produced, especially if this production is expressed per unit of digestible DM intake. More research is needed to evaluate the effectiveness of strategies to reduce the CH_4 emissions, and relationships between *in vitro* and *in vivo* gas and CH_4 productions need to be developed.

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6. FOURTH CONTRIBUTION

Dose-response and inclusion effects of pure natural extracts and synthetic compounds on in vitro methane production

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6.1 ABSTRACT

This study explored effects of four pure plant extracts with antimicrobial properties (allyl-sulfyde, AS; cinnamaldehyde, CI; eugenol, EU; limonene, LI) and one ionophore compound (monensin, MO), on *in vitro* rumen fermentation and methane (CH_4) production of a commercial diet for dairy cows. Four consecutive incubations at 24 h were carried out using an automated gas production (GP) system, were the gas was automatically vented at fixed pressure. Bottles (317 ml) were filled with 1.0±0.010 g of diet and additive, and 150 ml of buffered rumen fluid, and incubated at 39±0.4°C. Two dosages of each additive were tested: 3 or 30 mg/g of diet for the pure plant extracts; 0.015 or 0.030 mg/g of diet for the ionophore compound. The experimental design was: 4 incubation runs×11 treatments×3replications plus twelve bottles (3 per each run) as blanks (bottles containing only the buffered rumen fluid), for a total of 144 bottles incubated. At the end of each incubation, gas (10 ml) was sampled from headspace of each bottle and analyzed for CH_4 by GC. Fermentation fluids were treated with neutral detergent solution to compute degradability of NDF (NDFd, %) and of true DM (TDMd, %). Data were submitted to ANOVA considering the treatment and incubation as sources of variation. High dosage of LI strongly depressed both NDFd (-68%) and TDMd (-14%) compared to the control; less marked reductions were noted for high dosage of EU (-15% and -4%, for NDFd and TDMd, respectively) and of MO (-16% and -3%, for NDFd and TDMd, respectively). No effects emerged for other additives on NDFd and TDMd, irrespective by the dosage. Compared to the control, high dosage of AS, CI, EU, LI, and MO significantly reduced *in vitro* GP (ml/g DM; -16%, -12%, -9%, -38%, -12%, respectively). *In vitro* CH_4 production was significantly reduced only by high dosage of AS, CI, LI, and MO (-32%, -12%, -43%, -18%, respectively, compared to the control). Only high dosage of AS and LI significantly reduce CH_4 proportion (-18%, -12% respectively, compared to the control). The most promising results were observed for CI, as the depression of gas emissions was not accompanied by a reduction of *in vitro* degradability.

Keywords: *In vitro* technique, Dose-response, Monensin, Pure natural extracts, Gas production, Methane production

6.2 INTRODUCTION

In the last years several *in vitro* studies have been performed to explore the capacity of plant compounds (i.e. essential oils) to improve efficiency of rumen fermentation, by increasing the

proportion of energy channeled towards the synthesis of VFA and microbial N (Hart et al., 2008; Klevenhusen et al., 2012). This interest has further raised after that the use of antibiotics as feed additives (i.e. ionophores) has been banned in the EU (Regulation 1831/2003/EC). Although results were sometimes encouraging, in other cases the dietary addition of these compounds was found to impair *in vitro* rumen fermentation, by reducing degradability and VFA production (Calsamiglia et al., 2007; Hart et al., 2008). In this regard, magnitude and kind of effects exerted by plant extracts on *in vitro* rumen parameters are influenced by the type of extract used, supplementation level, composition of the basal diet used for *in vitro* tests, pH conditions, and possible interactions among these factors (Klevenhusen et al., 2012). To date, the majority of *in vitro* studies was aimed at evaluating effects of plant extracts on rumen degradability, VFA production, and N metabolism, whereas effects on rumen CH_4 production are less documented (Hart et al., 2008). Moreover, most studies have been conducted using whole plant extracts or commercial mixtures (i.e. blends of essential oils). Two main shortcomings of using whole extracts and mixtures are the unambiguous definition of effects and the identification of compounds actually effective on rumen fermentation; in this regard, use of pure products is quite preferable (Martínez-Fernández et al., 2013). Thus, the present study was aimed at exploring effects of four pure products (allyl sulfide, cinnamaldehyde, eugenol, and limonene) on *in vitro* rumen fermentation of a commercial diet for dairy cows, with a particular focus on CH_4 production. The effect of monensin, a reference compound with a renowned effect on rumen fermentation, was also evaluated.

6.3 MATERIALS AND METHODS

Diet was analyzed in duplicate for proximate composition (AOAC, 2012). Starch was analyzed by HPLC (Bouchard et al., 1988). Neutral detergent fibre (NDF), inclusive of residual ash, was determined (Mertens, 2002) with α-amylase using the Ankom220 Fibre Analyzer (Ankom Technology, NY, USA). Acid detergent fibre (ADF), inclusive of residual ash, and sulphuric acid lignin (lignin(sa)) were determined sequentially after NDF determination (Robertson and Van Soest, 1981).

6.3.1 Incubation Procedures

A commercial GP apparatus (AnkomRF GP System, Ankom Technology®, NY, USA) consisting of 36 bottles equipped with pressure sensors (pressure range: from −69 to +3447 kPa; resolution: 0.27 kPa; accuracy: ± 0.1% of measured value) and wireless connected to a computer was used. Four

incubation runs were conducted in 4 successive wks. The experimental design was the following: 4 incubation runs×11 treatments×3replications plus twelve bottles (3 per each run) as blanks (bottles containing only the buffered rumen fluid), for a total of 144 bottles incubated (36 bottles per each run). The basal diets used as control group (CTR; bottles containing only the diet and the buffered rumen fluid), were formulated establishing a corn silage-diet, containing 273, 361, 158, and 33 g/kg of starch, aNDF, CP, and lipids, respectively (Table 1). The other 10 treatments were: five additives incubated in 2 different dosages: allyl sulfide (ALL; A35801, Sigma-Aldrich Chemical, Milan, Italy), cinnamaldehyde (CIN; W228613, Sigma-Aldrich Chemical, Milan, Italy), eugenol (EUG; E-51791, Sigma-Aldrich Chemical, Milan, Italy), limonene (LIM; 183164, Sigma-Aldrich Chemical, Milan, Italy), and monensin (MON; M5273, Sigma-Aldrich Chemical, Milan, Italy) that were added to the bottles containing the diet and the buffered rumen fluid.

Table 1. Ingredient and chemical composition (g/kg DM) of the control diet.

	Basal
Ingredients	
Corn silage	351
Alfalfa hay	89
Meadow hay	47
Ryegrass hay	47
Corn meal	205
Barley meal	119
Soybean meal, 48% CP	113
Extruded flaxseed	29
Chemical composition	
NDF	361
ADF	192
ADL	29
Crude protein	158
Ether extract	33
Starch	273
NSC	395
Ash	53

NSC = non-structural carbohydrates

Before to start incubations, two solutions were prepared for each pure product, containing 25 ml of 96% ethanol (v/v) plus 75 (low dosage) or 750 mg (high dosage) of additive. In the case of monensin, the two solutions contained 25 ml of 96% ethanol (v/v) plus 0.38 (low dosage) or 0.76 mg (high dosage) of additive. After that, the solutions were stored at 4°C until the incubation. The day of incubation, each bottle (317 ml) was filled with 1.000±0.0010 g of diet, 150 ml of fermentation

fluid (composed by 50 ml of rumen fluid and 100 ml of buffer solution), and 1 ml of the ethanol-additive solution, in order to achieve the final concentrations needed (3 or 30 mg of additive/g of diet, for the low and the high dosage of plant pure extracts; 0.015 or 0.030 mg of additive/g of diet, for the low and the high dosage of monensin). Such doses correspond to 20 or 200 mg/l of buffered rumen fluid, in the case of the pure compounds, and to 0.1 or 0.2 mg/l of buffered rumen fluid, in the case of monensin. To standardize fermentation conditions, 1 ml of ethanol was added also to the 3 blanks (bottles without feed sample and additive) incubated in each run. Such ethanol concentration (0.7% v/v) was assumed to not impair microbial growth and activity (Benchaar et al., 2007). The buffer solution was prepared according to Menke and Steingass (1988), heated in a water bath at 39°C and purged continuously with CO_2 for 30 min, to maintain anaerobic conditions. Rumen fluid was collected by an esophageal probe (Tagliapietra et al., 2012) 2 h before morning feeding from 3 dry Holstein-Friesian fed hay ad libitum and 2.5 kg/d of concentrates (0.5 kg of dry sugar beet pulp, 1 kg of corn grain, and 1 kg of sunflower meal). Rumen fluid was stored into thermal flasks preheated to 39 ± 0.5°C, transferred to the laboratory, filtered through 3 layers of cheesecloth, to eliminate residual feed particles, and mixed with buffer solution in a 1 to 2 ratio (Menke and Steingass, 1988). All operations required less than 30 min and were conducted under anaerobic conditions, by continuous flushing with CO_2. Bottles were placed in a ventilated oven at 39 ± 0.4°C and automatically vented at a fixed pressure (6.8 kPa), to prevent overpressure and alterations of gas and CH_4 measures (Cattani et al., 2014).

6.3.2 Sample collection and analytical procedures

At the end of each incubation, two aliquots (5 ml) of fermentation fluid were collected from each bottle and stored at −20°C with 1 ml of metaphosphoric acid (25%, w/v) until be analyzed for ammonia N (N-NH3) and volatile fatty acids (VFA). Moreover, pH of fermentation fluid of each bottle was measured using appropriate electrodes equipped with temperature gauge and connected to a pH meter (pH-Meter Crison Instruments - BASIC 20, Barcellona, Spain). The content of N-NH_3 was measured using the Method Cassette Ammonium of the FIAstarTM 5000 Analyzer (FOSS Analytical, Hilleroed, Denmark). The VFA concentration was determined by GC with flame ionization detection (7820A GC system, Agilent Technologies, Milan, Italy) using a 30-m stainless steel column (J&W DB-FFAP, Agilent Technologies, Milan, Italy) and H_2 as carrier gas (flow rate: 30 ml/min; isothermal oven temperature: 150°C). Fermentation fluids were filtered into weighed crucibles (30 mL, Robu

Glasfilter-Geräte GMBH®, Hattert, Germany) and analyzed for residual NDF using a Fibretech Analyzer (VELP® Scientifica, Milan, Italy). At the end of each incubation, gas was collected with a 10-ml gas-tight syringe (Artsana S.p.A., Como, Italy) from headspace of bottles. At each sampling, the syringe was flushed to allow the collection of a homogeneous sample, which was immediately injected into a 5-ml Vacuette (Greiner Bio-One GmbH, Kremsmunster, Austria). From each Vacuette, an aliquot (10 µl) of gas was sampled with a gas-tight syringe (1701N, Hamilton, Bonaduz, Switzerland) and immediately analyzed for CH_4 concentrations by GC with flame ionization detection (7820A GC system, Agilent Technologies, Milan, Italy) using a 30-m stainless steel column (GS-CarbonPLOT, Agilent Technologies, Milan, Italy) and H_2 as carrier gas (flow rate: 1.6 ml/min; isothermal oven temperature: 40°C). A six-point standard curve was obtained by mixing known volumes of CH_4 (99.5 % pure, SAPIO s.r.l., Monza, Italy) with known volumes of air using the same graduated gas tight syringe (1701N, Hamilton, Bonaduz, Switzerland). The 6 gas mixtures used for calibration contained 10, 15, 20, 25, 50, and 100 ml/l CH_4. The calibration regression showed an R^2 of 0.9999

6.4 COMPUTATIONS AND STATISTICAL ANALYSIS

6.4.1 Computations

Degradability of NDF (NDFd) and of true DM (TDMd) were calculated according to Goering and Van Soest (1970. The CH_4 production was computed using the equation suggested by Maccarana et al. (submitted article) and it was expressed as ml/g DM incubated, ml/g TDMd, or ml/100 ml GP.

6.4.2 Statistical analysis

Data were analyzed using PROC MIXED of SAS Institute (2007) with a model considering the treatment (CTR + 5 additives × 2 dosages; 10 df) as fixed effect and incubation run (3 df) as random effect. Least-squares means were separated using Fisher's test in SAS (2007). Orthogonal contrasts were run to test the effects of the additives and dosages with respect to CTR treatment. All parameters measured were graphically expressed as percentage variation of each additive and dosage compared to the CTR treatment.

6.5 RESULTS

Table 2. Effect of dietary treatment on final values of pH, in vitro gas production (GP), CH_4 production and proportion (CH_4/GP).

	pH	GP, mL/g DM	CH_4, mL/g DM	CH_4/GP
Treatment				
Control (CTR)	6.83	280	39.7	14.2
L-All	6.83	277	38.8	13.9
H-All	6.86	235	27.1	11.6
L-Cin	6.82	283	40.2	14.2
H-Cin	6.82	244	35.0	14.2
L-Eug	6.83	280	39.5	14.1
H-Eug	6.84	256	39.3	15.0
L-Lim	6.81	272	39.6	14.5
H-Lim	6.86	174	22.7	12.5
L-Mon	6.81	279	39.2	13.7
H-Mon	6.84	246	32.5	13.4
SEM	0.018	8.4	1.31	0.29
P value				
Treatment	0.003	<0.001	<0.001	<0.001
Contrasts				
Ctr vs. L-All	0.99	0.99	0.99	0.99
Ctr vs. H-All	0.33	<0.001	<0.001	<0.001
Ctr vs. L-Cin	0.99	0.99	0.99	0.99
Ctr vs. H-Cin	0.99	<0.001	0.004	0.99
Ctr vs. L-Eug	0.99	0.99	0.99	0.99
Ctr vs. H-Eug	0.98	0.009	0.99	0.99
Ctr vs. L-Lim	0.98	0.99	0.99	0.99
Ctr vs. H-Lim	0.43	<0.001	<0.001	0.001
Ctr vs. L-Mon	0.97	0.99	0.99	0.99
Ctr vs. H-Mon	0.99	<0.001	<0.001	0.99

L-All = low dosage of allyl sulfide; H-All = high dosage of allyl sulfide; L-Cin = low dosage of cinnamaldehyde; H-Cin = high dosage of cinnamaldehyde; L-Eug = low dosage of eugenol; H-Eug = high dosage of eugenol; L-Lim = low dosage of limonene; H-Lim = high dosage of limonene; L-Mon = low dosage of monensin; H-Mon = high dosage of monensin. For allyl sulfide, cinnamaldehyde, eugenol and limonene, low dosage = 3 mg/g diet (20 mg/L of fermentation fluid); high dosage = 30 mg/g diet (200 mg/L). For monensin, low dosage = 0.015 mg/g diet (0.1 mg/L); high dosage = 0.030 mg/g diet (0.2 mg/L).

6.5.1 Allyl sulphide

Effects of allyl sulfide on *in vitro* rumen fermentation are shown in Table 2 and in Figure 1. Compared to CTR, the low dosage did not affect any of *in vitro* parameters considered in this study. As regards to the main effects, the high dosage of allyl sulfide reduced *in vitro* GP and CH_4 production (both expressed as ml/g TDMd) by about -15 and -32%, respectively (P<0.001). The total VFA production (mg/l) was decreased by about -12% (P=0.009); acetate production (mg/l) was

markedly reduced (about -24%; P<0.001) at the favor of propionate (about +15%; P<0.001). No effects were observed on values of NDFd (g/kg NDF) and TDMd (g/kg DM). Similarly, ammonia N concentration (N-NH_3) of rumen fluids at the end of incubation was unchanged.

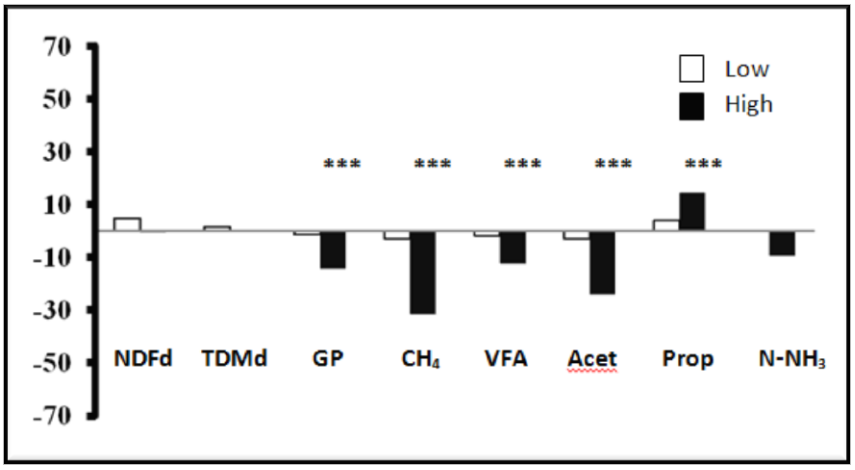

Figure 1. Percentage effect (increase or decrease of control values) of two dosages (low or high) of allyl sulfide on NDF (NDFd; g/kg NDF) and true dry matter degradability (TDMd; g/kg DM), GP (mL/g TDMd), CH_4 (mL/g TDMd) , VFA (mg/L), acetate (acet) and propionate (prop) proportions (both expressed as % on total VFA), and ammonia N (N-NH3) concentration of fermentation fluids (mg/L).

6.5.2 Cinnamaldehyde

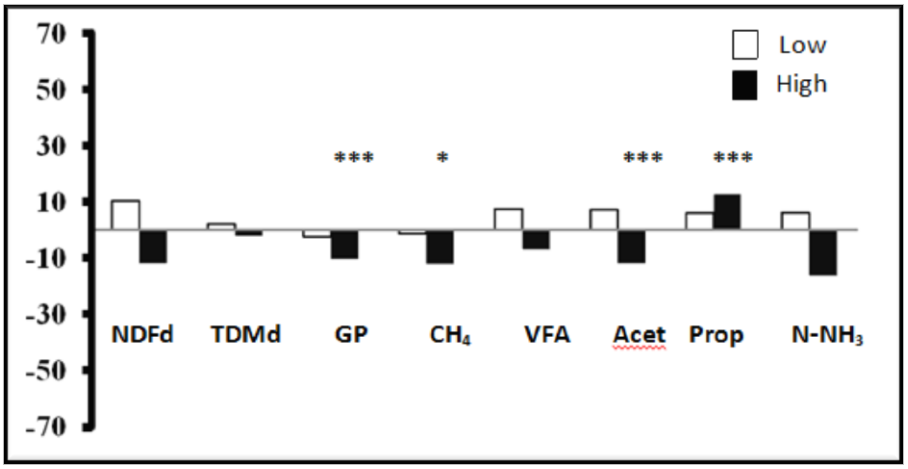

Figure 2. Percentage effect (increase or decrease of control values) of two dosages (low or high) of cinnamaldehyde on NDF (NDFd; g/kg NDF) and true dry matter degradability (TDMd; g/kg DM), GP (mL/g TDMd), CH_4 (mL/g TDMd) , VFA (mg/L), acetate (acet) and propionate (prop) proportions (both expressed as % on total VFA), and ammonia N (N-NH3) concentration of fermentation fluids (mg/L).

Effects of cinnamaldehyde on *in vitro* rumen fermentation are shown in Table 2 and in Figure 2. As observed for allyl sulfide, the low dosage of cinnamaldehyde did not exert any effect on *in vitro* fermentations compared to CTR. At the high dosage the additive caused a decrease of *in vitro* GP, as ml/g DM (P<0.001) and ml/g TDMd (about -10%; P<0.001), and of CH_4 production, as ml/g DM (P=0.004) and ml/g TDMd (about -12%; P=0.038). The high dosage of cinnamaldehyde reduced the acetate production (about -12%; P<0.001) and increased that of propionate (about +12%; P<0.001), without modifying the total VFA production (P=0.51). Similarly to allyl sulfide, cinnamaldehyde did not influence *in vitro* degradability and N-NH_3 concentration of rumen fluids.

6.5.3 Eugenol

Effects of eugenol on *in vitro* rumen fermentation are shown in Table 2 and in Figure 3. Compared to CTR, no effects were observed on the various *in vitro* parameters when the compound was added at the low dosage. At the high dosage, significant effects were only observed on a slight reduction of NDFd (about -18%; P=0.018), TDMd (about -3%; P=0.013), and GP as ml/g DM (about -9%; P=0.009). All other parameters were unchanged.

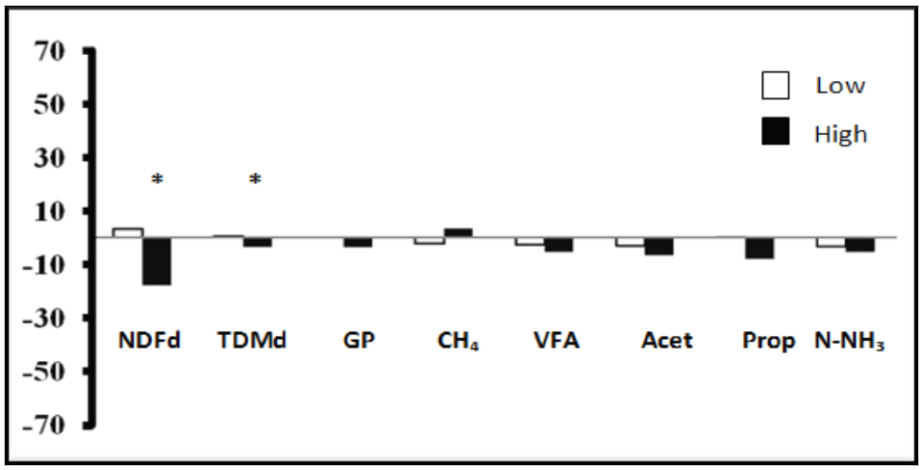

Figure 3. Percentage effect (increase or decrease of control values) of two dosages (low or high) of eugenol on NDF (NDFd; g/kg NDF) and true dry matter degradability (TDMd; g/kg DM), GP (mL/g TDMd), CH_4 (mL/g TDMd) , VFA (mg/L), acetate (acet) and propionate (prop) proportions (both expressed as % on total VFA), and ammonia N (N-NH_3) concentration of fermentation fluids (mg/L).

6.5.4 Limonene

Effects of limonene on *in vitro* rumen fermentation are shown in Table 2 and in Figure 4. Compared to CTR, the low dosage of limonene did not exert any significant effect on *in vitro* fermentations. When added at the high dosage, the most interesting effects of limonene were the depression of *in vitro* NDFd (about -66%; P<0.001), TDMd (about -14%; P<0.001), GP (about -25%; P<0.001) and CH_4 production as ml/g TDMd (about -34%; P<0.001). Compared to CTR, the high dosage of limonene reduced the total VFA, acetate, and propionate productions in the order of -22, -29, and -11%, respectively (P<0.001 for all). Also in the case of limonene, final concentration of N-NH_3 in rumen fluids was not modified.

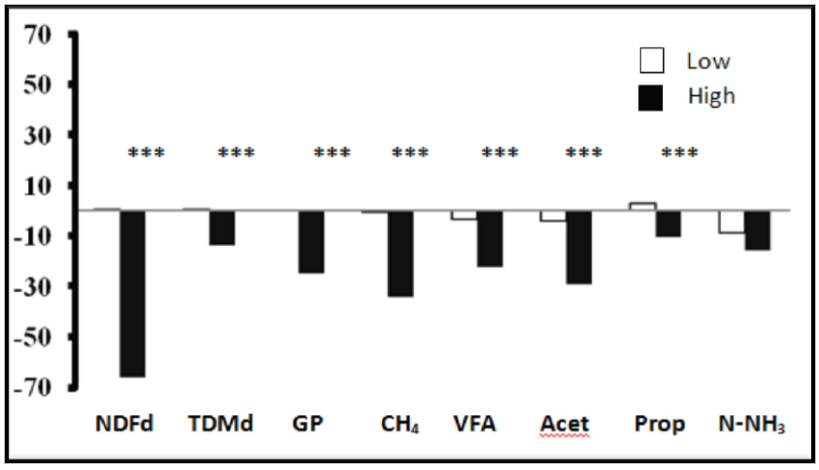

Figure 4. Percentage effect (increase or decrease of control values) of two dosages (low or high) of limonene on NDF (NDFd; g/kg NDF) and true dry matter degradability (TDMd; g/kg DM), GP (mL/g TDMd), CH_4 (mL/g TDMd) , VFA (mg/L), acetate (acet) and propionate (prop) proportions (both expressed as % on total VFA), and ammonia N (N-NH3) concentration of fermentation fluids (mg/L).

6.5.5 Monensin

Effects of monensin on *in vitro* rumen fermentation are shown in Table 2 and in Figure 5. As observed for other compounds, the low dosage of monensin did not affect *in vitro* fermentations compared with CTR. On the opposite, the high dosage reduced *in vitro* NDFd (about -17%; P=0.026) and TDMd (about -3%; P=0.028), and CH_4 production, expressed as ml/g TDMd (about -19%; P<0.001). The total VFA production was unchanged (P=0.17), as the lowering of acetate production (about -14%; P<0.001) was counterbalanced by a marked increase of propionate (+23%; P<0.001). Monensin did not influence N-NH_3 concentration of rumen fluids.

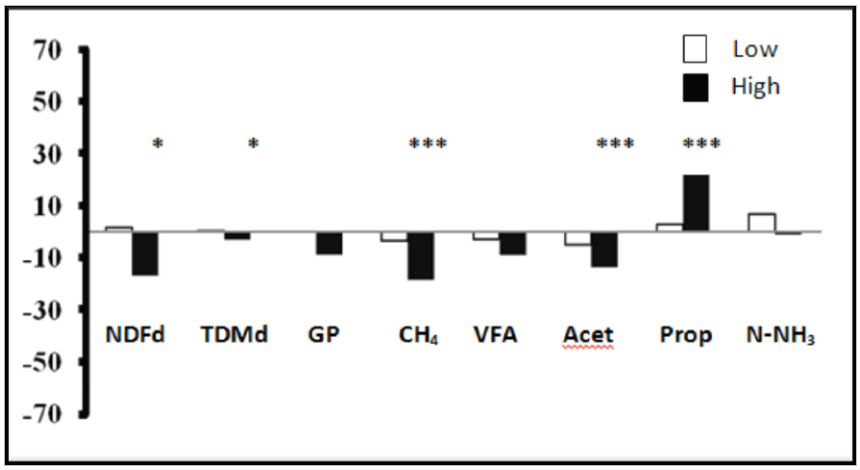

Figure 5. Percentage effect (increase or decrease of control values) of two dosages (low or high) of monensin on NDF (NDFd; g/kg NDF) and true dry matter degradability (TDMd; g/kg DM), GP (mL/g TDMd), CH_4 (mL/g TDMd) , VFA (mg/L), acetate (acet) and propionate (prop) proportions (both expressed as % on total VFA), and ammonia N ($N\text{-}NH_3$) concentration of fermentation fluids (mg/L).

6.6 DISCUSSION

6.6.1 Effects of plant extracts on in vitro fermentation

In this study commercial pure extracts of the four plant compounds had an effect only when tested at the high dosage (30 mg/g of diet; 200 mg/l of fermentation fluid), whereas no effects emerged at the low one (3 mg/g of diet; 20 mg/l). Other *in vitro* experiments found that cinnamaldehyde (Busquet et al., 2006), eugenol (Cardozo et al., 2005; Busquet et al., 2006), and limonene (Castillejos et al., 2006) did not influence DM degradability and CH_4 production when added at dosages similar to the low one used in this study. The same results emerged for some garlic compounds as allicin and allyl mercaptan (Busquet et al., 2005a,b); no data are available, to the best of our knowledge, about the effects of allyl sulfide. The short incubation time used in this study (24 h) could have further limited effects of the four compounds, when they were added at the low dosage. Castillejos et al. (2007) hypothesized that short-term incubations may not be long enough to evidence effects of plant extracts on rumen fermentation. On the other hand, use of long incubation times (i.e. 48 h) would led to microbial lysis into the batch culture systems, a process altering end-products of fermentation and, thus, results obtained *in vitro* (Cattani et al., 2013).

When added at the high dosage, the four compounds had an impact on rumen fermentation, but magnitude and kind of effects differed among the additives. The most pronounced effects were observed for limonene, which caused a marked depression of all *in vitro* parameters. Up to now, effects of limonene on rumen fermentation have been little documented. The majority of existing literature explored effects of mixtures containing limonene (Khiaosa-ard and Zebeli, 2013), whereas less information is available about effects of the pure compound. According to this study, Crane et al. (1957) found that limonene reduced strongly *in vitro* fibre degradability (from -40 to -70%) and *in vitro* GP (from –40 to -80%) and hypothesized that this compound may be toxic for fibrolytic bacteria. Dorman and Deans (2000) evidenced that limonene mainly impaired gram-negative bacteria; such a result corroborates the negative impact on fibre degradation observed in this study. Others (Castillejos et al., 2006) found that limonene had negative effects on *in vitro* fermentation, by decreasing the total concentration of VFA, at dosages greater than 50 mg/l of fermentation fluid. Magnitude and kind of effects exerted by limonene would suggest that this compound, when added at the high dosage, had a large negative impact on fermentation. As support of that, limonene was the only additive that reduced both acetate and propionate production, suggesting that fibrolytic and amilolytic bacteria were indiscriminately impaired. Further, the high dosage of such compound reduced N-NH_3 concentration of rumen fluids by about 16%, even if the effect did not reach the statistical significance. However, magnitude of such an effect would confirm that feed degradation by rumen microbes could have been reduced to some extent. Notwithstanding this large impact of rumen fermentations, any effect was observed when the limonene was added in proportion of 5 mg/l.

With respect to other additives, the high dosage of allyl sulfide and cinnamaldehyde showed intermediate effects on *in vitro* fermentation. Effects of garlic oil and extracts on N-NH_3 concentration of rumen fluid appear to be quite variable (Calsamiglia et al., 2007). In the present study allyl sulfide showed some small effects on N-NH_3 concentration, especially at high dosage (-11% compared to control) but, however, they were not significant. Such a result is in accord with findings of Busquet et al. (2005a). The latter authors hypothesized that major effects of garlic compounds might be addressed to the modification of carbohydrate metabolisms and that, subsequently, such effects could also influence N metabolism. According to such a hypothesis, in the present study allyl sulfide could have affected energy metabolism by reducing the total VFA

concentration without impairing *in vitro* digestibility. Busquet et al. (2005a) observed the same pattern for allyl mercaptan, a major compound of the garlic oil, and hypothesized that the effect of this extract on rumen microbes was not so strong to affect fermentation process. The reduction of acetate concentration at the favor of that of propionate is confirmed by Busquet et al. (2005a,b), which used similar dosages of other garlic compounds. This change in VFA proportions is consistent with the reduced CH_4 production. There is some evidence from literature that garlic and its compounds could influence *in vitro* methanogenesis in a dose-dependent manner. Kamel et al. (2009) found that the whole garlic oil caused a reduction of *in vitro* CH_4 production by 25 and 62%, respectively, at a dosage of 180 and 540 mg/l. Differently from the study of Kamel et al. (2009), this experiment evaluated a pure compound derived from the garlic oil. However, the magnitude of effect on *in vitro* methanogenesis (-32% in terms of ml CH_4/g TDMd) can be considered in line with the dosage used (200 mg/l). Overall effects would suggest that allyl sulfide may have potential benefits as a modifier of rumen fermentation, although the negative impact on total VFA concentration cannot be ignored.

In this regard, it is clear that additives with antimicrobial properties should reduce CH_4 production without impairing the overall fermentation (i.e. rumen degradability, VFA and microbial N production). The magnitude and kind of effects (positive or negative) exerted by plant extracts on *in vitro* fermentation seem to be mainly related to the dosage used (Calsamiglia et al., 2007). In some cases (i.e. thymol), the margin between the optimal and the toxic inclusion level is very narrow, thus the choose of the tested dosage can notably influence the results obtained *in vitro*. On the other hand, it is likewise clear that *in vitro* studies must to be performed using higher dosages compared to *in vivo* conditions, to increase the probability that additives actually interact with the rumen microbial population, that is less numerous *in vitro* than *in vivo* (Chow et al., 1994).

On the basis of such premises, the high dosage of cinnamaldehyde showed the most interesting and promising effects, as this compound reduced the proportion of CH_4 produced per g TDMd, without decreasing *in vitro* degradability and the total VFA production. Effects of cinnamaldehyde on *in vitro* rumen degradability have been scarcely explored. Busquet et al. (2005b) found that this compound, added at a dosage slightly greater (31.2 mg/l) than the low one used in this study, did not influence *in vitro* degradability and the total VFA concentration. Such results are in accord with the present study. In general, effects on VFA concentration were found to be minor

at lower dosages (i.e. 0.3, 3, and 30 mg/l), in accord with this experiment. In contrast, cinnamaldehyde reduced the total VFA concentration when added to high-concentrate diets (forage:concentrate equal to 10:90) at a dosage greater ≥ 300 mg/l (Busquet et al., 2005b, 2006; Cardozo et al., 2005); however, in this regard, results are sometimes controversial. In the present study cinnamaldehyde, when dosed at 200 mg/l, reduced acetate concentration (-12%) at the favor of propionate (+12%). Such an effect was also observed by Busquet et al. (2005b), which used similar dosages. Effects of cinnamaldehyde on rumen methanogenesis are very little documented. Macheboeuf et al. (2008) found that this compound, dosed at a level slightly greater (264 mg/l) compared to the high dosage used in this study, decreased *in vitro* CH_4 production by about 13%, without affecting the total VFA production. The extent of such reduction is similar to what observed in this experiment (about -12%). Effects of cinnamaldehyde on *in vitro* N-NH_3 concentration of rumen fluid and on the overall N metabolism are not clear, as influenced by the dosage used. Busquet et al. (2005a), using dosages similar to those tested in this study, did not observe any effect of cinnamaldehyde on N-NH_3 concentration. The overall effects of cinnamaldehyde would suggest that this compound had less marked effects on *in vitro* fermentation compared to limonene and allyl sulfide.

The overall effects of eugenol on *in vitro* rumen fermentation were of little magnitude. The compound was found to reduce *in vitro* NDFd in the order of 18%. However, considering the weight of the NDF fraction on the total DM incubated (37%), the overall impact on *in vitro* TDMd was nearly irrelevant (-3%), even though significant (P=0.013). Such effects were likely insufficient to determine appreciable variations of other parameters (i.e. VFA production and proportions, CH_4 production). Accordingly, Castillejos et al. (2006) did not evidence effects of eugenol on *in vitro* fermentations at a dosage of 50 mg/l. Other authors (Busquet et al., 2006; Lourenco et al., 2008), using dosages of eugenol similar to those tested in this study (from 30 to 300 mg/l of fermentation fluid), did not observe any effect on the total VFA production. However, in the study of Lourenco et al. (2008) a slight increase of acetate at the expense of propionate was found. Cardozo et al. (2004), using the same dosages of Busquet et al. (2006), found that effects of eugenol on the total VFA production was pH-dependent, with increments at pH 5.5 and decrements at pH 7.0. Effects of eugenol on rumen methanogenesis have been scarcely explored. Araujo et al. (2011) found that eugenol reduced *in vitro* CH_4 production by about 70%, but they used a dosage much greater (667 mg/l) than

those tested in the present experiment. Chaves et al. (2008) found that eugenol was able to reduce *in vitro* CH_4 production at dosages of 400 and 500 mg/l. In accord with findings of this study, some authors (Busquet et al., 2005c; Busquet et al., 2006) found that the addition of eugenol did not influence *in vitro* N-NH_3 concentration of rumen fluid. Lack of effects observed in this experiment could be attributed to the overall scarce effectiveness of eugenol. This would suggest that the two dosages used in this study (20 and 200 mg/l of fermentation fluid) were likely not sufficient, with regard to *in vitro* conditions, to make eugenol effective against CH_4 production and to influence the overall pattern of rumen fermentations.

6.6.2 Effects of monensin on in vitro fermentation

The impact of monensin on *in vivo* metabolism of dairy cows has been extensively studied (Duffield et al., 2008), whereas the number of *in vitro* experiments is limited. Literature provides evidence that effects of monensin are usually dose-dependent, both *in vitro* and *in vivo*. Castillejos et al. (2006) hypothesized that monensin could have a negative effect on *in vitro* rumen fermentation at dosages greater that 10 mg/l of fermentation fluid. However, others (Fellner et al., 1997) found that also a lower dosage of monensin (2 mg/l) can impair fermentation process.

In the present study, monensin showed to have an impact on fermentation at a dosage much lower (0.2 mg/l) compared to those cited above. Rumen fluid used in this study was collected from dry cows fed a high-forage diet. As the composition of rumen fluid used for *in vitro* tests is primarily influenced by the diet fed to donor animals (Rymer et al., 2005), it can be hypothesized that microbial population of rumen fluid used in this experiment was largely represented by fibrolytic bacteria. Some of these microorganisms (i.e. Cellulotytic ruminococci and Butyrivibrio fibrisolvens) are much sensitive to monensin (Schelling, 1984; Russell and Strobel, 1989), and this may partially explain the effectiveness of this additive at the very low dosage used.

The decrease of *in vitro* NDFd and acetate production, with the concurrent increment of propionate, seem to confirm that fibrolytic bacteria were negatively influenced by the high dosage of monensin. The present study also evidenced that monensin did not reduce the total VFA concentration, also when it was added at the high dosage. Such a result agrees with previous *in vitro* (Russell and Strobel, 1988; Busquet et al., 2005a; Castillejos et al., 2006) and *in vivo* studies (Yang and Russell, 1993). Reduced acetate production at the favor of propionate is a renown effect of

monensin (Schelling, 1984). Some *in vitro* studies (Chalupa et al., 1980; García-Lopez et al., 1996) support the fact that monensin is able to increase propionate concentration at the expense of that of acetate also when added at low dosage (0.5 mg/l of fermentation fluid). Even if this shift in VFA proportions is often associated with a decrease of CH_4 production in the rumen (Russell, 1998), effects of monensin on methanogenesis are still controversial. From a literature review, Beauchemin et al. (2008) concluded that monensin may affect CH_4 production in a dose-dependent manner. Russell and Strobel (1989) found that monensin can lead to notable reductions of CH_4 production (up to -30%), but such effects seemed to be related to an inhibition of rumen bacteria producing CH_4 precursors (i.e. formate and H_2) rather than of microorganisms directly involved in CH_4 production (*Archaea*). In this study, the high dosage of monensin reduced *in vitro* CH_4 production in the order of about 19%. Magnitude of this effect was lower compared to other *in vitro* studies (Chaves et al., 2008; Araujo et al., 2011), but such a result was likely due to the very low dosages used. To obtain more reliable and solid results, the two dosages of monensin tested in this study (0.015 and 0.030 mg/g of diet) were expressly chosen to be included within ranges recommended *in vivo* (185-660 mg/d; FDA, 2005).

6.7 CONCLUSIONS

Different modifications on fermentation parameters between pure extract of plant compounds tested in this study, allowed to select the best potential anti-methanogenic additive and its recommended dose. Specifically, cinnamaldehyde it was identified as an additive that, when used at the high dosage, reduced gas and methane production, without compromising the rumen degradability, the VFA production and microbial N production. Further investigations are required to confirm these positive potential effects *in vivo*, as well as its adequacy an its long-time effect as feed supplements for bovine.

6.8 ACKNOWLEDGMENTS

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7. FIFTH CONTRIBUTION

The effect of inhibiting methane production on bacterial population structure and function in a long term simulation of rumen fermentation

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7.1 ABSTRACT

Here we explore the effect of a potent direct inhibitor of methanogenisis (chloroform) with or without additives that act as H_2 sinks (nitrate) or decrease H_2 production (saponins, through inhibition of protozoa metabolism) on rumen function and the structure of the rumen bacterial population, in a long term rumen simulating fermenter. Both chloroform and nitrate decreased CH_4 production. Chloroform addition was associated with lower levels of volatile fatty acid production and the recovery of 2H in the fermenter, calculated from volatile fatty acid and CH_4 production, was lower in chloroform supplemented vessels in the absence but not the presence of nitrate, suggesting that 2H might have accumulated in chloroform supplemented vessels in the absence but not the presence of nitrate. The bacterial community determined by NGS sequencing was significantly altered by chloroform in control and saponin supplemented vessels but not when nitrate was present suggesting that direct CH_4 inhibition should be combined with the addition of alternative H_2 sinks in order to promote the adaptive process in the rumen and avoid deleterious effects on rumen fermentation.

Keywords: Rumen, Methane, Microbial community, Chloroform, Nitrate, Saponin

7.2 INTRODUCTION

Livestock are estimated to be responsible for 14.5% of the total greenhouse gas (GHG) emission from anthropogenic sources (Gereber et al., 2013), with CH_4 resulting from enteric fermentation the second largest source of anthropogenic GHG, representing 39% of the livestock sector emissions (Gereber et al., 2013). Methane is a normal product of ruminal fermentation, representing a pathway for the disposal of metabolic H_2 produced during microbial metabolism. During the oxidation of sugars via the Embden–Meyerhof–Parnas pathway, NAD+ is reduced to NADH which has to be reoxidised to NAD+ to allow fermentation to continue. Under the anaerobic conditions prevailing in the rumen, where NAD+ must be regenerated by electron transfer to acceptors other than oxygen, the major sink is the reduction of CO_2 to CH_4 (although other sinks include sulfate, nitrate and fumarate) (Morgavi et al., 2010). Because methanogens present in the mixed microbial ecosystem use H_2, it does not accumulate in the rumen. Indeed, even traces of H2 in the rumen are thought to inhibit hydrogenase activity and limit the oxidation of sugar when alternative pathways for the disposal of H_2 are absent (McAllister and Newbold, 2008). It has been

suggested that rumen function will be disrupted if rumen CH_4 production is inhibited without the provision of alternative H_2 sinks (Morgavi et al., 2010). Thus, while numerous studies have investigated the potential to decrease CH_4 from enteric fermentation in ruminants using dietary strategies or dietary additives, it has been suggested direct CH_4 inhibition should be combined with either an alternative H_2 sink or a decrease in H_2 production in order to promote the adaptive process in the rumen and avoid deleterious effects on rumen fermentation (McAllister and Newbold, 2008). Here we explore this concept by investigating the effect of a potent direct inhibitor of CH_4 production (chloroform) with or without additives that act as H_2 sinks (nitrate) or decrease H_2 production (saponins through inhibition of protozoa metabolism) on rumen function and the structure of the rumen bacterial population, in a long term rumen simulating fermenter.

7.3 MATERIALS AND METHODS

7.3.1 Apparatus and diets

The Rusitec rumen simulating fermenter was used (Czerkawski and Breckenridge, 1977) to investigate the effect of a control diet alone or supplemented with chloroform (80µL per kg DM: Chloroform anhydrous, ≥99%, containing 0.5-1.0% ethanol as stabilizer, Sigma-Aldrich, United States), nitrate (31.5 g/ kg diet DM: Bolifor® CNF, that contains 75% of nitrate DM, Yara International ASA, Norway) plus or minus chloroform, or saponin (50 g/ Kg DM Ivy fruit crude extract containing circa 35% saponins obtained by extraction of raw organic compounds from ivy fruit meal using ethanol) plus or minus chloroform in a 3 by 2 factorial arrangement. Urea was added to diets not containing nitrate to ensure equal levels of N addition (8 g/kg DM; Acros Organics, Belgium). The basal diet was a 70:30 forage-to-concentrate mix (Table 1) ground to pass through 2 mm^2 sieve size. Animal procedures were carried out according to the Home Office Scientific Procedures, Act 1986 (PLL 40/3653; PIL 40/9798). Rumen fluid was obtained from 4 barren rumen-cannulated Holsten-Frisian cows fed at maintenance level (diet composed of 67% perennial ryegrass hay and 33% concentrate, on a DM basis). Rumen contents were sampled before the morning feeding, filtered through a double layer of muslin and transferred to the laboratory anaerobically at 39ºC (circa 30 min). The trial consisted of a single incubation period using 24 vessels which were considered as experimental units.

Table 1. Ingredient and chemical composition of the basal diet used as a substrate

	Basal, g/Kg DM
Ingredients	
Grass hay	705
Barley	184
Molasses	102
Mineral premix	9
Chemical composition	
Dry matter (g/kg feed)	920
Crude protein	65
Crude fat	12
Crude ash	63
Starch	65
Sugar	129
NDF	486
ADF	228
Calcium	5.7
Phosphorus	2
Nitrate	<0.2

Thus, each dietary treatment had 4 replicates which were randomly allocated to the vessels and inoculated with rumen fluid from different cows. Vessels had an effective volume of 800 ml and were kept at 39ºC under permanent vertical agitation. On day 1 fermenters were inoculated with strained rumen fluid diluted 1:1 with artificial saliva (McDougall, 1948), then artificial saliva was continuously infused at a rate of 640 ml/d (dilution rate of 3.33%/h). Squeezed rumen contents (20g FM) were placed in nylon bags (110 × 60 mm, pore size 100 µm2) and incubated in each vessel for 1d to provide solid-associated bacteria, while experimental feed was supplied in a second bag. On subsequent days, the bag that had remained 2 days in each vessel was squeezed, under a CO_2 atmosphere. The liquid fraction of the washing was returned to the vessels and a new feed bag was inserted containing 20g DM.

7.3.2 Experimental procedure and sampling

The incubation trial lasted for 21 days, using the first 14 days for adaptation and the last 7 for sampling. Methane emissions and outflow of fermentation products was measured on days 15, 16, 17, 18 and 19. Fermentation gases were collected in gas-tight bags (TECOBAG 5L, PETP/AL/PE-12/12/75, Tesseraux container GmbH, Germany) to measure total gas and CH_4 production (ATI Unicam 610 Series, Gas Chromatograph, UK). Daily production ammonia and VFA were measured in the overflow flasks with 10ml of saturated $HgCl_2$ (diluted 1:5) added to stop the fermentation. The

pH was immediately recorded at the time of bag change in each vessel. At 2h post feeding on days 19 and 20, fluid from within vessels was sampled (20mL) by aspiration and stored at -80C for molecular analysis. To measure feed degradability at 48 and 24h all the feedbags retrieved at the last day of the experiment, were freeze dried and weighed for determination of DM degradability. CP degradation was determined from each feedbag residues. Crude protein content (N content × 6.25) was analysed by a combustion method using an elemental analyser (Vario Max Cube, Elementar Analysensysteme GmbH, Germany).

7.3.3 Sample analyses

Feed chemical composition was determined as described by Belanche et al. (2013). Measurements of pH were done using appropriate electrodes connected to a pH meter (S30 SevenEasy™ conductivity, Mettler-Toledo International Inc., Switzerland). VFA analysis, of overflow effluent preserved with 20% ortho-phosphoric acid (Isac et al., 1994), were made using GC (Varian CP3380 Gas chromatograph, Palo Alto, California, USA) with HP-FFAP column (J&W Scientific). Ammonia analysis, of overflow effluent preserved with 25% TCA, were made by a colorimetric method (Weatherburn, 1967).

From gas bags, total gas volume was measured by a dry test gas meter (model DC, shinagawa corporation, Japan). At the same time, gas was collected with syringe and immediately injected into a Vacutainer® (BD Vacutainer, New Jersey) (Cattani et al., 2014). From each Vacutainer®, an aliquot of 500 µL of gas was extracted with a gastight syringe (Hamilton SampleLock 1750SL) and immediately analyzed for CH_4 concentration by GC (Unicam 610 Gas Chomatograph, Cambridge, UK) fitted with a PORAPAK N packed metal column and flame ionization detector (FID). N_2 and H_2 were gas carriers. A 3 calibration point standard curve was generated using certified mix grade composed by CH_4, CO_2, N_2O, H_2, N_2. The 3 gas mixtures used for calibration contained 5, 10 and 15 ml of CH_4/l. To determine dry matter degradation at 48 and 24h all the feedbags retrieved at the last day of the experiment, were freeze dried and weighed.

True crude protein (N content × 6·25) was analyzed by a combustion method using an elemental analyzer (Vario Max Cube, Elementar Analysensysteme GmbH, Germany).

7.3.4 Protozoal counts

During the last 2 days of the trial, 0.5 ml of fermentation fluid sampled 2 h post feeding, preserved with 0.5 ml saline formalin solution (0.9% NaCl and 4% formalin), were stained with a drop of methylene blue to count protozoa using the protocol proposed by Dehority (1983).

7.3.5 Molecular procedures

Samples were stored at -80°C before freeze drying and physical disruption by beat beating as described by de la Fuente et al. (2014). DNA was extracted using the QIAamp® DNA Mini Stool Kit (Qiagen Ltd., West Sussex, England) as described previously (Skřivanová et al., 2010). Bacterial community composition was assessed through amplicon sequencing of the V1-V2 region of the bacterial 16S rRNA gene, using the IonTorrent PGM platform. Template DNA was used to amplify the V1-V2 region of the bacterial 16S rRNA gene. B-27F (sequence 5'-AGA GTT TGA TCM TGG CTC AG-3') and A1-R357 (sequence 5'-CTG CTG CCT YCC GTA-3') were used (Li et al., 2009) with barcoded tags and adaptors. For each sample, replicate PCR was performed in duplicate in a reaction solution consisting of reaction buffer (AccuBuffer 10X; 2mM MgCl2 final concentration), 0.2 mM dNTPs, 0.1 pmol/µL of both forward and reverse primer, 1 µL of 2.5 U/µl of FastStart high fidelity enzyme (ACCUZYME™ DNA Polymerase, London, UK) and 1 µL of template (approx. 75 ng/µL) in a total reaction volume of 25 µL. The conditions used were a hot start of 95°C for 10 min, 95°C for 2 min, followed by 22 cycles of 95°C for 30 s, 50°C for 30 s and 72°C for 30 s with a final extension at 72°C for 7 min. Verification of the fragment, pooling of the samples, library formation and purification using Agencout AMpure XP beads (Beckman Coulter Inc., USA) and the E-gel system (Invitrogen size select 2%, Life Technologies) were performed as described in de la Fuente et al. (2014). Purified libraries were assessed for quality and quantified on an Agilent 2100 Bioanalyzer with a High Sensitivity DNA chip (Agilent Technologies UK Ltd, Stockport, UK). The sample libraries were subsequently sequenced using the Ion Torrent PGM sequencer following the Ion PGM Template OT2 400 Kit (Life Technologies Ltd, Paisley, UK).

The emulsion PCR was carried out using the Ion PGM Template OT2 400 Kit (Life Technologies, Carlsbad, CA, United States) as described in the user Guide (Catalog number: 4479878, Revision 2.0) provided by the manufacturer. Sequencing of the amplicon libraries was carried out on the Ion Torrent Personal Genome Machine (PGM) system using the Ion PGM Sequencing 400 Kit v2 (Life

Technologies) following the corresponding protocol (Catalog number: 4482002, Revision 2.0). Raw sequence reads of all samples were deposited at the EBI Short Read Archive (SRA) from the European Nucleotide Archive (ENA). Sequences reads, binned by sample, were downloaded without any quality filtering employed. Sequences were trimmed at 250 bp, filtered on quality (expected error < 1.5) and chimeras were removed before clustering into OTUs at 97 % identity using USEARCH (Edgar, 2010). Samples were normalized using Daisychopper (www.genomics.ceh.ac.uk/GeneSwytch) and averaged across the two consecutive sampling days with OTUs present in only one day being discarded. OTUs were classified using RDP (Wang et al., 2007).

7.4 COMPUTATIONS AND STATISTICAL ANALYSIS

7.4.1 Calculations

Hydrogen balance was calculated as describe by Goel et al. (2009) with modification in that for the treatment with the supplementation of Bolifor®, as a H_2 sink, we calculated the H adapted and H recovery as:

H adapted = H accepted + 4 x ((0.02 x 20) / 62 x 1000);

H recovery (adapted), % = (H adapted / H released) x 100.

7.4.2 Statistical analyses

All data of last 5 days originating from overflow were analysed using a 2 x 3 factorial design in PROC MIXED of SAS 9.3 2 (SAS Institute Inc., Cary, NC, USA), according to the following model:

$$Y_{ijk} = \mu + D_i + CH_j + D_i * CH_j + Block_k + e_{ijk}$$

where D were diet and CH were presence or absence of chloroform.

RUSITEC unit and vessel nested to RUSITEC unit were included as the blocking factor. Kenward-Roger method to adjust denominator degrees of freedom was included as missing values were present (Kenward and Roger, 1997).

Dietary effects on NGS log-transformed data were analysed based on their Bray-Curtis distance metric within the function UPGMA. Data were then analysed by non-parametric

permutational multivariate analysis of variance using PRIMER-6 software (PRIMER-E Ltd., Plymouth, UK). Pairwise comparisons were also conducted to elucidate differences between treatments. The pseudo F-statistics and P-values were calculated after 999 random permutations of residuals under a reduced model using the Monte Carlo test. A canonical correspondence analysis (CCA) was also performed to investigate the relationships between the structure of the bacterial and methanogens communities and the fermentation pattern. The signification of each variable was calculated using 999 random permutations. Bacterial biodiversity indexes were calculated using normalized data to reduce over-inflation of true diversity in pyrosequencing data sets. Relative abundances data were tested for normality and homogeneity using the Shapiro-Wilk and the Bartlett's tests, respectively. When treatment or treatment interactions were significant a pairwise comparisons of means was performed with Tukey adjustment for multiple comparisons (Westfall, et al., 2011).

7.5 RESULTS

7.5.1 Methane production and fermentation parameters

Daily CH_4 production was decreased by 96, 66 and 22% by chloroform, nitrate and saponin respectively (Table 2), however neither nitrate nor saponin had any additional effect on CH_4 production in chloroform treated vessels. Total GP (on average 2.56 l/d) was not affected by diet or chloroform addition. Nitrate but not chloroform or saponin decreased dry matter degradation at 24 and 48 h (Table 2). However, none of the treatments had any effect on crude protein degradation (Table 2). On their own none of treatments had any influence on protozoal numbers, although nitrate in the presence of chloroform decreased protozoal numbers (Table 2). Saponin but not nitrate or chloroform caused a decrease in pH within the vessels (Table 3). Daily ammonia production was higher in nitrate supplemented vessels, but thus effect was reversed when chloroform was also added to the vessels (Table 3). Total volatile fatty acid output was lower in both nitrate supplemented vessels and those that received chloroform (Table 2). Chloroform but no other treatment decrease acetate production, whilst both chloroform and nitrate and saponin increased propionate production (Table 3). Conversely chloroform, nitrate and saponin all reduced the output of butyrate from the vessels (Table 3). Chloroform, nitrate and saponin all decreased the theoretical 2H release calculated from VFA production (Table 3). When corrected for direct reduction of nitrate, only chloroform decrease the acceptance of 2H in VFA and CH_4 (Table 3). As a result there was a

significantly lower recovery of 2H in VFA and CH_4 in the control and saponin supplemented vessels when chloroform was added, but not in the nitrate supplemented vessels.

7.5.2 Bacterial 16S rDNA sequencing

Bacterial 16S rDNA amplicon sequencing generated 6,7999,794 million raw sequences. Quality filtering resulted in 1, 231, 504 high quality sequences that were clustered in to 1771 unique OTUs with 8518 sequences per sample after normalization. The total OTUs recovered were 23% lower in nitrate supplemented vessels but there was no effect of treatments on either the Shannon or Simpsons indexes (Table 4). *Bacteroidetes* was the most abundant phyla (42% on average) followed by *Firmicutes* (32% on average) (Table 4). At phyla level, both nitrate and chloroform supplementation increased the relative abundance of *Bacteroidetes*, independently from each other (Table 4). The relative abundance of the candidate phylum *TM7* was decreased in both nitrate and saponin treatment compared to the control (Table 4). Chloroform supplementation did not affect the relative abundance of other phyla.

Permutational analysis of variance (Table 5) demonstrated a strong effect of chloroform, nitrate and saponin on the structure of the bacterial community. Pair-wise analysis showed that the structure of the bacterial community differed when chloroform was added on its own or in addition to saponin, but not when added to vessels already receiving nitrate (Table 5). This was confirmed in the Principal component analysis where there was clear clustering plus and minus chloroform in the control and saponin vessel, but no separation due to chloroform in the nitrate supplemented vessels (Figure 1).

At genus level, *Prevotella* was the most abundant genus (19% on average) and significantly increased in the nitrate supplemented vessels compared to the control (P=0.012 for the pairwise comparison, Table 6). Furthermore, an interaction between the general treatment effect and chloroform addition was observed (P=0.013), such that the nitrate and chloroform supplemented vessels showed a significant higher relative abundance compared to the control and control with chloroform supplemented vessels (Table 6). The relative abundance of the *Phocaeicola* genus was significantly increased in the nitrate treatment compared to the saponin treatment (P=0.034 for the pairwise comparison, Table 6). The genus of uncertain placement within the TM7 candidate phylum showed the same effects as the phylum with both nitrate and saponin decreasing its abundance

(Table 6). Chloroform supplementation significantly increased the relative abundance of the *Anaerophaga* genus (Table 6).

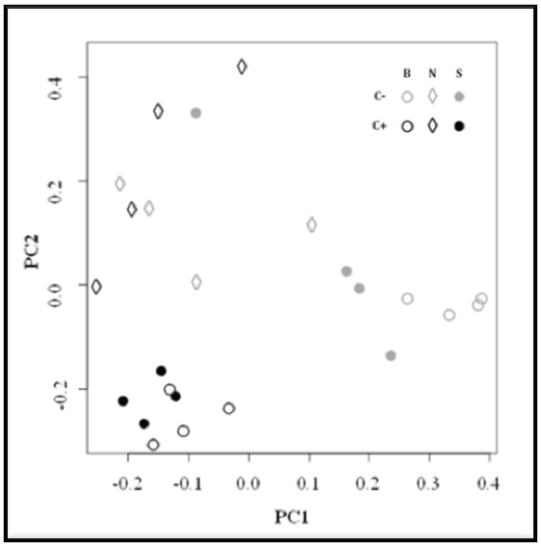

Figure 1. Principal component analysis plot of the first two principal components from the bacterial communities of the individual RUSITEC vessels supplemented with urea (○), nitrate (◊), saponin (●), nitrate with chloroform (○), nitrate with chloroform (◊) and saponin with chloroform (●). Symbols represent individual vessels after normalization and combining of data of day 19 and 20 at 2h post feeding

Table 2. Effects of saponin, nitrate or no addition plus or minus chloroform on rumen fermentation gases, rumen degradability, and protozoa number , obtained in the rumen simulation technique (RUSITEC)

	Treatments						SEM	P-value		
	BCH-	BCH+	NCH-	NCH+	SCH-	SCH+		D	CH	D*CH
CH_4 (ml/day)	384[a]	14[b]	129[b]	10[b]	284[a]	18[b]	44.7	0.002	<0.001	0.003
Gas production (l)	2.9	3.0	2.2	1.9	2.5	2.9	0.25	<0.001	NS	NS
Dry matter degradation (g/kg)										
24 h	632	632	598	551	632	663	28.6	<0.01	NS	NS
48 h	734	711	673	633	702	723	34.6	<0.01	NS	NS
True crude protein (g/kg)										
24 h	705	714	677	655	674	747	35.5	NS	NS	NS
48 h	729	729	680	724	740	757	30.4	NS	NS	NS
Total protozoa (log cells/ml)	4.16[a]	4.43[a]	4.34[a]	3.03[b]	4.01[a]	4.52[a]	0.410	NS	NS	0.011

[a,b,c] Mean values within a row with unlike superscript letters were significantly different considering D*C interaction (P<0·05).

BCH-, basal diets without chloroform (control treatment); BCH+, basal diets and chloroform. NCH-, nitrate diets without chloroform; NCH+, nitrate diets with chloroform. SCH-, saponins diet without chloroform; SCH+, saponins diet with chloroform

Table 3. Effects of saponin, nitrate or no addition plus or minus chloroform on rumen fermentation pattern and calculated hydrogen balance, obtained in the rumen simulation technique (RUSITEC)

	Treatments						SEM	P-value		
	BCH-	BCH+	NCH-	NCH+	SCH-	SCH+		D	CH	D*CH
pH	6.26	6.28	6.28	6.37	6.21	6.24	0.043	0.02	NS	NS
NH_3-N (mmol/day)	0.48[b]	0.64[ab]	1.15[a]	0.40[b]	0.29[b]	0.41[b]	0.188	0.017	NS	0.004
VFA production (mmol/d)										
Total	88.5	80.5	82.7	72.5	88.3	84.9	3.92	0.011	0.005	NS
Acetate	48.3	33.0	45.7	37.9	45.7	34.8	2.59	NS	<0.001	NS
Propionate	17.9	25.0	23.5	25.1	25.5	28.4	2.00	0.004	0.003	NS
Butyrate	18.0	13.7	9.4	4.7	13.0	12.1	2.21	<0.001	0.018	NS
Hydrogen balance (mmol/l)										
Released	191.4	159.6	161.0	127.0	174.4	163.1	11.12	0.002	<0.001	NS
Accepted adapted*	143.2	82.3	118.0	90.3	128.5	86.4	9.18	NS	<0.001	NS
Recovery adapted* (%)	75.1[a]	51.6[b]	73.4[a]	71.4[a]	73.6[a]	53.0[b]	2.57	<0.001	<0.001	<0.001

[a,b,c] Mean values within a row with unlike superscript letters were significantly different considering D*C interaction (P<0·05).

BCH-, basal diets without chloroform (control treatment); BCH+, basal diets and chloroform. NCH-, nitrate diets without chloroform; NCH+, nitrate diets with chloroform. SCH-, saponins diet without chloroform; SCH+, saponins diet with chloroform

* adapted for nitrate inclusion, assuming 1mol of nitrate accepts 4 moles of hydrogen gas when completely reduced to ammonia (based on analysed VFA production profile).

Table 4. Effects of saponin, nitrate or no addition plus or minus chloroform on relative abundance bacterial phylum present at more than 0·4% in the vessel of the rumen simulation technique (RUSITEC) system

	Treatments						SEM	P-value		
	BCH-	BCH+	NCH-	NCH+	SCH-	SCH+		D	CH	D*CH
Number of OTU	361	344	277	210	320	329	33.2	0.011	NS	NS
Shannon's index (H)	4.06	4.06	4.13	3.78	3.96	4.29	0.223	NS	NS	NS
Simpson's index (l-D)	0.937	0.934	0.963	0.950	0.941	0.964	0.0149	NS	NS	NS
Relative abundance (%)										
Bacteroidetes	32.4	40.1	42.2	60.2	39.4	39.3	4.46	0.009	NS	NS
Firmicutes	41.8	35.6	26.9	20.9	39.5	30.3	6.39	NS	NS	NS
Fibrobacteres	5.6	12.4	12.9	7.7	9.6	18.4	2.95	NS	NS	NS
Tenericutes	3.2	3.1	6.7	3.9	2.9	3.6	1.33	NS	NS	NS
Proteobacteria	2.8	3.2	5.5	3.0	2.1	3.5	1.14	NS	NS	NS
SR1	0.46	0.77	1.13	2.16	0.55	1.22	0.700	NS	NS	NS
Spirochaetes	0.09	0.15	1.08	0.25	0.88	0.67	0.410	NS	NS	NS
TM7	0.83	1.05	0.08	0.02	0.14	0.54	0.209	0.002	NS	NS

BCH-, basal diets without chloroform (control treatment); BCH+, basal diets and chloroform. NCH-, nitrate diets without chloroform; NCH+, nitrate diets with chloroform. SCH-, saponins diet without chloroform; SCH+, saponins diet with chloroform

Table 5 Effects of saponin, nitrate or no addition plus or minus chloroform on bacterial population structure in the rumen simulation technique (RUSITEC)

	Pseudo-F	P-value
Treatment effects		
Chloroform	3.71	0.046
Diet	3.49	0.002
Interaction	2.00	0.036
Pair-wise for diets		
BCH *vs* SCH	1.74	0.073
BCH *vs* NCH	1.99	0.036
SCH *vs* NCH	1.83	0.034
Pair-wise for Chloroform within diets		
BCH+ *vs* BCH-	2.11	0.03
SCH+ *vs* SCH-	1.73	0.071
NCH+ *vs* NCH-	1.15	0.273

BCH-, basal diets without chloroform (control treatment); BCH+, basal diets and chloroform. NCH-, nitrate diets without chloroform; NCH+, nitrate diets with chloroform. SCH-, saponins diet without chloroform; SCH+, saponins diet with chloroform

Table 6. Effects of saponin, nitrate or no addition plus or minus chloroform on relative abundance bacterial genera present at more than 0·4% in the vessel of the rumen simulation technique (RUSITEC) system

	Treatments						SEM	P-value		
	BCH-	BCH+	NCH-	NCH+	SCH-	SCH+		D	CH	D*CH
Relative abundance (%)	12·0[b]	11·8[b]	17·3[ab]	30·7[a]	27·0[ab]	15·5[ab]	3·72	0·012	NS	0·013
Prevotella	19·1	19·9	9·9	0·5	14·7	14·6	5·56	NS	NS	NS
Erysipelotrichaceae incertae sedis	5·6	12·4	12·9	7·7	9·6	18·4	2·95	NS	NS	NS
Fibrobacter	7·2	6·2	0·0	0·0	11·8	0·8	3·75	NS	NS	NS
Clostridium sensu stricto	3·1	3·1	6·7	3·9	2·9	3·6	1·33	NS	NS	NS
Anaeroplasma	1·3	2·3	2·2	3·6	1·6	4·3	1·28	NS	NS	NS
Streptococcus	0·0	0·0	3·2	6·7	0·0	0·0	3·05	NS	NS	NS
Lactobacillus	0·9	1·1	1·8	3·8	0·5	1·1	0·75	0·027	NS	NS
Phocaeicola	0·5	0·8	1·1	2·2	0·6	1·2	0·70	NS	NS	NS
SR1 genera incertae sedis	0·28	0·42	0·86	0·16	0·43	2·32	0·579	NS	NS	NS
Selenomonas	0·01	0·01	4·25	0·00	0·01	0·00	0·945	NS	NS	NS
Campylobacter	0·74	0·21	1·06	0·75	0·51	0·37	0·229	NS	NS	NS
Oribacterium	0·72	0·27	0·88	0·60	0·51	0·34	0·281	NS	NS	NS
Butyrivibrio	0·83	1·05	0·08	0·02	0·15	0·54	0·209	0·002	NS	NS
TM7 genera incertae sedis	0·03	1·06	0·17	0·60	0·00	0·09	0·244	NS	0·019	NS
Anaerophaga	45·1	36·1	34·7	35·7	26·7	31·8	6·49	NS	NS	NS
Unclassified	12·0[b]	11·8[b]	17·3[ab]	30·7[a]	27·0[ab]	15·5[ab]	3·72	0·012	NS	0·013

[a,b,c] Mean values within a row with unlike superscript letters were significantly different considering D*C interaction (P<0·05).

BCH-, basal diets without chloroform (control treatment); BCH+, basal diets and chloroform. NCH-, nitrate diets without chloroform; NCH+, nitrate diets with chloroform. SCH-, saponins diet without chloroform; SCH+, saponins diet with chloroform

7.6 DISCUSSION

The aim of this experiment was to investigating the effect of a potent direct inhibitor of methanogenesis (chloroform) with or without additives that act as H_2 sinks (nitrate) or decrease H_2 production (saponins, through inhibition of protozoa metabolism) on rumen function and the structure of the rumen bacterial population, in a long term rumen simulating fermenter.

The literature suggests that saponins mitigate methanogenesis mainly by reducing the numbers of protozoa, decreasing the supply of H to protozoal associated methanogens (Cieslak et al., 2013). Unfortunately, in this experiment protozoal numbers were low within the vessels and saponins had no effect on protozoal numbers, and thus the effect of saponins on CH_4 production and other aspect of rumen fermentation were small. Saponins are glycosylated triterpenes or steroids, where the saponin is the aglycone, while the glycone is a carbohydrate unit consisting of a monosaccharide or smaller oligosaccharide entity. The antiprotozoal effect of saponins is related to their interaction with the sterol moiety present in the membrane of protozoa (Patra and Saxena, 2009). The anti-protozoal action of saponins in the rumen is often transitory as when saponins are deglycosylated to sapogenins by rumen microbes they become inactive (Newbold et al., 1997). Possibly here deglycosylation, of the saponin may have limited the effects on rumen fermentation.

In contrast nitrate acts as an alternative H_2 sink in the rumen. The reduction of nitrate to nitrite, and the subsequent reduction of nitrite to ammonia, yields more energy ($\Delta G0 = -254$ kJ/mol of H_2 respectively) than the reduction of carbon dioxide to CH_4 ($\Delta G0 = -16.9$ kJ/mol of H_2; Ungerfeld and Kohn, 2006) and thus will be the principal route of H_2 disposal if sufficient nitrate is available. The reduction of nitrate to ammonia consumes 8 electrons and each mole of nitrate reduced could thus lower CH_4 production by 1 mole. Here CH_4 production was decreased by 66%. In the current experiment nitrate lead to a decrease in the number of bacterial OTUs recovered, but consistent with previous work (Veneman et al., 2015) had no effect species richness or alpha diversity. As with previous studies, Prevotella was the most abundant genus (Henderson et al., 2013, Kim et al., 2011) and was significantly increased by nitrate supplementation (Patra and Yu, 2013).

In agreement with previous studies, almost complete inhibition of CH_4 production was observed in vessels supplemented with chloroform (Bauchop, 1967). Furthermore, chloroform addition (without nitrate) decreased VFA production with a shift towards propionate and butyrate,

at the expense of acetate proportion. This shift has been described as a common feature of several anti-methanogenic compounds (Clapperton, 1974, Goel, et al., 2009, Knight, et al., 2011, Abecia, et al., 2012, Mitsumori, et al., 2012). When H_2 cannot be converted to CH_4, the pressure of dissolved H_2 increases, making fermentation pathways producing H_2 (including acetate production) less thermodynamically favourable (Janssen, 2010, Wang, et al., 2013). In agreement with previous work with bromochloromethane (BCM), chloroform changed the bacterial population structure in the vessels (Denman et al., 2015, Martinez-Fernandez et al., 2015), but at the doses used did not influence species richness or alpha diversity (Denman et al, 2015). Unlike previous studies with BCM (Denman et al, 2015, Martinez-Fernandez et al., 2015), chloroform did not decrease the occurrence of potentially cellulolytic bacteria with no change in the abundance of Firmicutes nor Fibrobacteres in chloroform supplemented vessels consistent, with the lack of effect on DM degradation at either 24 or 48h. Chloroform did result in a significant stimulation in the occurrence of the genera Anaerophaga, the exact role of this genera in the rumen is unclear but it has been associated with fatty acid metabolism and bio hydrogenation of fatty acids in the rumen (Petri et al., 2014), so it is perhaps not surprising the it might respond to the presence of greater free H_2 as the result of chloroform supplementation.

Perhaps, the most interesting results from the current study come from the combination of chloroform with other treatments. Few interactions of chloroform with saponin were observed, perhaps because saponin had a limited effect on CH_4 production, and thus potentially had a limited effect on rechannelling H_2 in the rumen. Indeed the change in the bacterial population structure due to chloroform, illustrated by the PCA plot in Figure 1, is similar in both control and saponin supplemented vessels. However, this is not so when chloroform and nitrate were combined, as chloroform did not significantly alter the bacterial population structure population structure in the presence of nitrate.

A number of recent papers have examined the effect of combining different strategies for decreasing CH_4 production in the rumen, with a general consensus that combinations are more effective than single additives (van Zijderveld et al., 2011,Guyader et al., 2015, Patra and Yu, 2015). However, we believe this study is the first to show that combining an alternative H_2 sink (nitrate) with a potent inhibitor of rumen methanogensis (chloroform) can prevent a shift in the rumen bacterial population structure. However this was not associate with a recovery in volatile fatty acid

production which was still decreased by chloroform in the presence of nitrate, despite the H_2 recovery in fermentation products and CH_4 being similar in control and chloroform plus nitrate supplemented vessels. Clearly, as noted by Ungerfeld (2013, 2015), there is a need to more fully understand the effect of different H_2 sinks on both CH_4 production and rumen function.

7.7 CONCLUSIONS

There is a need to develop strategies to decrease CH_4 emissions from ruminants, to improve the sustainability of ruminant food production systems. Ruminal metabolic processes are connected by common metabolic pathways and/or microbial species. Here we have shown that direct CH_4 inhibition should be combined with the addition of alternative H_2 sinks in order to promote the adaptive process in the rumen, but that further work is required to determine the consequences on rumen function.

7.8 ACKNOWLEDGMENTS

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The authors declare that there is no conflict of interest with any financial organization regarding the material discussed in the manuscript.

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8. MAIN CONCLUSIONS

Basing on the three years of research and on results obtained in the different experiments, the following main conclusions can be drawn:

i. *in vitro* techniques could represent an useful screening tool to evaluate effects of nutritional strategies to reduce CH_4 emissions in ruminants. However, these techniques are still plagued by a low degree of standardization, therefore a more standardized protocol on an international scale would be desirable;

ii. open system is the most promising technique inasmuch it showed greater repeatability of CH_4 proportion and production, compared to closed system;

iii. in order to select the best strategies to reduce CH_4 emission, it is important to consider the effect on the overall fermentation parameters;

iv. when the modifications of nutrient balance are not extreme, changes in chemical composition are ineffective on CH_4 production;

v. our results suggest that the most promising strategy is the use of natural pure compounds (i.e., cinnamaldehyde);

vi. further investigations are required to confirm *in vivo* positive potential effects of natural compounds, as well as the long-time effect, its palatability and its possible transfer of odours and taste to animal products, when they are used as feed supplements.

9. LIST OF PUBLICATIONS DURING THE PhD COURSE

1. L. Maccarana, M. Cattani, L. Bailoni. 2013. Rumen *in vitro* gas production of combinations between slowly and rapidly fermentable fibre sources. Agriculturae Conspectus Scientificus, 78(3): 229-233.

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7. M. Cattani, L. Maccarana, F. Tagliapietra, S. Schiavon, H.H. Hansen, L. Bailoni. 2014. *In vitro* incubation of dairy cow diets-2. Relations between measures and estimates of methane yield. Book of abstract n. 20 (2014). Copenhagen, Denmark, 25-29 August, p. 414, WAGENINGEN: Wageningen academic publishers.

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11. L. Maccarana, M. Cattani, R. Mantovani, F. Tagliapietra, S. Schiavon, L. Bailoni. 2015. Assessment of factors influencing *in vitro* gas and methane production by meta-analysis. Ital. J. Anim. Sci. vol. 14, suppl. 1, p 24

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10. RESEARCH GROUPS AND INTERNATIONAL COLLABORATIONS

The present doctoral thesis was developed under the supervision of Professor Lucia Bailoni.

The experimental activities were realised in two research centres:

i. for most of the time in the University of Padova:
   - at the Department of Comparative Biomedicine and Food Science (BCA)
   - at the Department of Agronomy, Food, Natural resources, Animals and Environment (DAFNAE), specifically in the chemical laboratory “La.Chi”
   - at the Experimental Farm "Lucio Toniolo";

ii. during the visiting period (from January to July 2014) at the Aberystwyth University, under the supervision of Professor Jamie Newbold:
   - at the Institute of Biological Environmental and Rural Sciences (IBERS).

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