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USE OF NATURAL ADDITIVES AS FEED SUPPLEMENTS TO REDUCE THE METHANE EMISSION FROM RUMINANTS

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THESIS FOR DEGREE OF DOCTOR OF PHILOSOPHY

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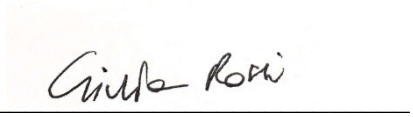
Declaration

I declare that this thesis has not previously been submitted as an exercise for a degree at the University of Padova, or any other university, and I further declare that the work embodied in it is my own.



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Giulia Rossi

Table of Contents

Abstract

Since the ban of antibiotics as growth promoting feed additives by the European Union in 2006, plant extracts and plant secondary metabolites have been considered as an alternative to manipulate the rumen. The term 'plant secondary metabolite' is used to describe a vast array of chemical compounds in plants that are not involved in the biochemical processes of plant growth and reproduction. These have been studied and used in medicines and preservation of foods for a substantial time.

The technology to assess methane emission *in vivo* is very slow, expensive, labour intensive and unsuitable for large-scale individual animal measurements. For this reason, a great deal of research has been conducted using *in vitro* simulation technique, which estimates the emission *in vivo*. However, the *in vitro* systems are often disapproved because of their short term duration (i.e. 24 hrs) and the lack of adaptation (they are conducted using rumen fluid which had not been exposed to the presence of the investigated compounds before). Based on this argument, this thesis aims to examine different aspects, regarding systems and procedures, used to investigate rumen fermentation and methane emission from ruminants, as follows:

1) Screening of different natural additives using a batch culture system;
2) Verify, through a batch culture system, the effect of the subministration *in vivo* of some natural additives in order to adapt the rumen fluid used as inoculum;
3) Asses the effect of one natural extract using a long-term continuous culture system;
4) Study the consequences on dairy products after the subministration of natural additives to lactating dairy cows.

The first contribution aimed to explored effects of four pure plant extracts (allyl-sulfyde, cinnamaldehyde, eugenol, and limonene) and one synthetic compound (monensin), on *in vitro* rumen fermentation and methane (CH_4) production of a commercial diet for dairy cows, using

a batch culture system, equipped with devices for automated gas venting at fixed pressure. Two dosages were tested: 3 or 30 mg/g of diet for the plant extracts; 0.015 or 0.030 mg/g of diet for Monensin. All the additives were ineffective at the low dosage. The high dosage of limonene caused a marked depression of neutral detergent fiber degradability (NDFd) (-68%) and true dry matter degradability (TDMd) (-14%) compared to the control; less marked reductions were noted for high dosage of eugenol (-15% and -4%, for NDFd and TDMd, respectively) and of monensin (-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 allyl sulfide, cinnamaldheyde, eugenol, limonene and monensin significantly reduced gas production (ml/g DM; -16%, -12%, -9%, -38%, -12%, respectively). *In vitro* CH_4 production was significantly reduced only by high dosage of allyl sulfide, cinnamaldheyde, limonene and monensin (-32%, -12%, -43%, -18%, respectively, compared to the control). Only high dosage of allyl sulfide, and limonene significantly reduce CH_4 proportion (-18%, -12% respectively, compared to the control). The most promising results were observed for the high dosage of cinnamaldehyde, that reduced *in vitro* CH_4 production without compromising degradability and VFA production.

Based on the results of the first trial, cinnamaldehyde, limonene and allyl-sulfide have been selected as the most effective in the reduction of methane emission, but also preserving the feed degradability and rumen functionality. The three compounds have been tested following a different procedure, indeed, the second trial aim was to evaluate the effect of rumen fluid which has not been adapted (NAF) and rumen fluid that has been adapted (AF) to the presence of the extract *in vivo*, before the *in vitro* tests. Therefore, four dry cows were fed diets with and without the pure compounds, according to a 4×4 Latin Square design, and the collected rumen fluids were used as *in vitro* inocula with or without the addition of the 3 compounds during the

incubation. Cinnamaldehyde, limonene and allyl-sulfide have been tested for *in vitro* gas production kinetics and end products of fermentation. In general, the results confirmed the first additive performed, however, regarding the adaptation *in vivo* of the rumen fluid, the use of AF did not affect any fermentation parameter compared to NAF. However, adding an *in vitro* diet with allyl-sulfide, the magnitude of the effects of this pure compound tended to be greater when incubated with AF compared to NAF. These results suggest that the administration of pure compounds to the cows can affect the rumen microbial activity and the response of *in vitro* experiments.

The third trial examined another category of plant secondary metabolites, flavonoids, which have recently gained interest because of their wide range of biological activities, but also because of their antimicrobial properties. Previously, it has been hypothesised that an extract of liquorice, rich in prenylated isoflavonoids and particularly glabridin, might potentially improve the efficiency of nitrogen utilisation and reduce methane production in the rumen. The experiment was carried out using a different *in vitro* system, the long-term rumen simulation technique (RuSimTec), which maintained the vitality and functionality of the rumen microbiome for long time and adapt the rumen fluid to the presence of the extract. The results obtained showed that when liquorice extract is added at 1 g/L, ammonia production decreased (-51%; $P<0.001$) without affecting the overall fermentation process. When added at 2 g/L, decreases in ammonia production (-77%; $P<0.001$), methane (-27%; $P=0.039$) and total VFA production (-15%; $P=0.003$) were observed. These effects in fermentation were probably related to decreases in protozoa numbers, a less diverse bacteria population as well as changes in the structure of both the bacterial and archaeal communities. The inclusion of an isoflavonoid-rich extract from liquorice in the diet may potentially improve the efficiency of the feed utilisation by ruminants.

Many studies have investigated the use of the plant secondary metabolites *in vitro*, but very limited research have examined the consequences may derived from feeding dairy cows with the additives. In particular, the focus of the fourth contribution is the use of garlic and garlic second metabolite as feed additives, recently proposed as modulators to reduce the enteric methane emissions of ruminants. Since there is no evidence available, this study aimed to investigate the influence of garlic and allyl sulfide on dry matter intake (DMI), productive performances, milk coagulation properties, cheese yield, milk and cheese sensory profiles, and rheological characteristics.

Four dairy cows were fed a total mixed ration either receiving 0 g/d (control) or supplemented with 100 or 400 g/d of garlic cloves or 2 g/d of Allyl sulfide in 4 consecutive experimental periods in a 4×4 Latin square design. Each experimental period consisted of 7 d of transition and 14 d of treatment. Milk samples were collected from each cow for chemical analysis and cheese-making. The organoleptic properties of the milk and 63 d ripened cheeses were assessed by a panel of 7 trained sensory evaluators. The experimental treatments had no effects on DMI, milk yield, feed efficiency (milk yield/DMI), milk coagulation properties, nutrient recovery and cheese yield. Garlic-like aroma, taste and flavour of milk and cheese were significantly influenced by the treatments, particularly the highest dose of garlic cloves, and we found close exponential relationships between milk and cheese for garlic-like aroma (R^2 = 0.87) and garlic-like flavour (R^2 =0.79). Allyl sulfide and 400 g/d of garlic cloves resulted in lower pH, shear force and shear work of ripened cheeses compared with the other treatments. Garlic cloves and Allyl sulfide had opposite effects on cheese colour indices. In conclusion, the addiction of 400 g/d of garlic to the feed of lactating dairy cows highly influences the sensory and rheological characteristics of cheese.

Riassunto

A causa del divieto di utilizzo di antibiotici come additivi alimentari promotori della crescita, da parte dell'Unione Europea nel 2006, gli estratti vegetali e i relativi metaboliti secondari sono stati considerati come una valida alternativa per la manipolazione dell'ambiente ruminale. Il termine "metabolita secondario" viene utilizzato per descrivere una vasta gamma di composti chimici vegetali che non sono coinvolti nei processi biochimici di crescita e riproduzione vegetali, ampiamente studiati ed utilizzati in medicina e nella conservazione degli alimenti.

La tecnologia disponibili per misurare l'emissione di metano in vivo è molto costosa, lenta, laboriosa e non applicabile su vasta scala. Per questo motivo sono state condotte numerose ricerche usando tecniche di simulazione *in vitro*, che consentono di stimare l'emissione *in vivo*. Tuttavia i sistemi *in vitro* sono spesso criticati a causa della breve durata (24 ore, per esempio) e della mancanza di adattamento dell'ambiente ruminale al composto in esame.

Sulla base di questi argomenti, i contributi della presente tesi hanno l'obiettivo di studiare le fermentazioni ruminali e l'emissione di metano aplicanto differente metodologicie sperimentali e differenti principi attivimetodologie per, come di seguito sintetizzato:

1) Screening di additivi naturali utilizzando un sistema batch culture;
2) Verificare, attraverso un sistema batch culture, l'effetto della somministrazione *in vivo* di alcuni additivi naturali allo scopo di adattare il liquido ruminale utilizzato come inoculo;
3) Misura l'effetto di un estratto naturale utilizzando un sistema continuo a lungo termine;
4) Studiare le conseguenze sui prodotti lattiero-caseari della somministrazione di additivi naturali a vacche da latte.

Nel primo contributo si è voluto esaminare gli effetti di quattro estratti vegetali puri (sulfuro di allile, cinnamaldeide, eugenolo e limonene) ed un composto sintetico (monensin), sulle

fermentazioni ruminali e la produzione di metano enterico di una dieta standard per vacche da latte. Il sistema utilizzato (batch culture) è dotato di valvole per il rilascio automatica del gas di fermentazione a pressione fissa ed è equipaggiato con un sistema wireless di trasmissione in continuo dei dati misurati. I dosaggi testati sono stati 2: 3 e 30 mg/g di dieta incubata, per ogni estratti vegetali, e 0.015 o 0.030 mg/g di dieta nel caso del Monensin. I risultati della prova hanno evidenziato una generale inefficacia di tutti gli estratti a basso dosaggio. Tuttavia l'elevato dosaggio del limonene ha causato una marcata riduzione della degradabilità della fibra NDF (NDFd) (-68%) e della degradabilità della sostanza secca (TDMd) (-14%) rispetto al controllo. anche l'alto dosaggio di eugenolo (-15% e -4%, per NDFd e TDMd rispettivamente) e del monensin (-16% e -3% rispettivamente per NDFd e TDMd) hanno ridotto la degradabilità della dieta ma tali effetti sono stati più contenuti. Non sono emersi effetti significativi nel caso degli altri additivi su NDFd e TDMd, indipendentemente dal dosaggio. Rispetto al controllo, l'elevato dosaggio di sulfuro di allile, cinnamaldeide, eugenolo, limonene e monensin ha significativamente ridotto anche la produzione di gas (ml/g DM, -16%, -12%, -9 %, -38%, -12% rispettivamente) mentre la produzione di CH_4 *in vitro* è stata significativamente ridotta solo da alti dosaggi di sulfuro di allile, cinnamaldeide, limonene e monensin (-32%, -12%, -43%, -18%, rispettivamente, confrontati con al controllo). Solo l'elevato dosaggio di sulfuro di allile e limonene riduce significativamente la percentuale di CH_4 nei gas di fermentazione (-18% e -12%, rispettivamente, rispetto al controllo). I risultati più promettenti sono stati osservati per l'alto dosaggio di cinnamaldehide, che ha ridotto la produzione di CH_4 *in vitro* senza compromettere la digeribilità della dieta e la produzione di acidi grassi volatili.

Sulla base dei risultati del primo contributo stati selezionati tre additivi considerati più efficaci nella riduzione dell'emissione di metano, ma preservando la degradabilità della dieta e la funzionalità dell'ambiente ruminale: cinnamaldeide, limonene e sulfuro di allile.

Nel secondo contributo gli additivi sono stati sottoposti a test *in vitro* (batch culture) in seguito ad una fase precedente di adattamento. L'obiettivo è stato di valutare l'effetto del liquido ruminante che non è stato adattato (NAF) e del liquido ruminale adattato (AF) alla presenza dell'estratto *in vivo*, prima di essere utilizzato come inocula microbico nel test *in vitro*. La prova ha previsto l'uso di quattro vacche in asciutta alimentate con dieta di riferimento per vacche da latte, addizionata dei composti puri, seguendo uno schema sperimentale a quadrato latino 4x4. I liquidi ruminali raccolti sono stati utilizzati come inoculo con o senza l'aggiunta dei 3 composti anche in sede di incubazione. Gli estratti sono stati testati per la cinetica di produzione di gas *in vitro* ed i prodotti di fermentazione. In generale, l'effetto dei diversi additivi sulle fermentazioni ruminali hanno confermato la prova di screeningprecedentemente realizzata. Per quanto riguarda l'adattamento *in vivo* del liquido ruminale, l'uso di AF non ha influenzato alcun parametro di fermentazione rispetto al NAF ma, aggiungendo il sulfuro di allile in sede di incubazione, la portata degli effetti di questo composto è stato maggiore quando incubato con AF rispetto a NAF. Questi risultati suggeriscono che la somministrazione di composti puri a vacche può influenzare l'attività della microflora ruminale e quindi la risposta di esperimenti condotti *in vitro*.

Nel terzo contributo si è voluto categoria di metaboliti secondari, i flavonoidi, che sono stati recentemente oggetto di interesse per la loro vasta gamma di attività biologiche, ma anche per le loro proprietà antimicrobiche. è stato ipotizzato che un estratto di liquirizia, ricco di isoflavonoidi prenilati ed in particolare glabridina, potrebbe potenzialmente migliorare l'efficienza dell'utilizzo dell'azoto e diminuire la produzione di metano ruminale.

L'esperimento è stato condotto utilizzando la tecnica di simulazione a lungo termine (RuSiTec), che consente di mantenere per lungo tempo la vitalità e la funzionalità del microbioma ruminale e adattare il liquido alla presenza dell'estratto, a differenza del sistema

batch culture. L'estratto è stato testato in due dosaggi: 1 g/L e 2 g/L. I risultati ottenuti hanno evidenziato che la dose di 1 g/L ha ridotto la produzione di ammoniaca (-51%, P <0,001) senza influenzare il processo di fermentazione complessivo. Quando invece sono stati aggiunti 2 g/L, non solo la produzione di ammoniaca è diminuita (-77%; P <0.001), ma anche la produzione di metano (-27%, P = 0.039) e la produzione totale di VFA (-15%; P = 0.003). Questi effetti in fermentazione sono probabilmente correlati ad una diminuzioni del numero di protozoi e ad una modificazione nella struttura della comunità battericha e degli arcaea con riduzione della biodiversità. In conclusione quindi, l'inclusione di un estratto di liquirizia, ricco di isoflavonoidi nella dieta, può potenzialmente migliorare l'efficienza dell'utilizzo della dieta da parte dei ruminanti, sulla base degli effetti verificati *in vitro*.

L'utilizzo di metaboliti secondari come additivi alimentari per vacche in produzione comporta necessariamente il possibile passaggio nel latte di sostanze che possono modificare la qualità dei prodotti lattiero-caseari. A questo proposito, la letteratura risulta molto limitata, e dal momento che non ci sono prove disponibili, l'obiettivo del quarto contributo è stato verificare come l'uso di aglio e sulfuro di allile, recentemente proposti come modulatori delle emissioni di metano enterico, influenzino i prodotti lattiero-caseari. L'effetto è stato verificato sull'ingestione di sostanza secca, le prestazioni produttive, le proprietà di coagulazione del latte, la resa casearia del formaggio, il profilo sensoriale di latte e formaggio e le caratteristiche reologiche di quest'ultimo. Quattro vacche in lattazione sono state alimentate con una dieta di riferimento senza la presenza di additivi (controllo) o integrata con 2 dosi crescenti di aglio, 100 o 400 g/d, o con 2 g/d di sulfuro di allile, in 4 periodi sperimentali consecutivi secondo uno schema a quadrato latino 4×4. Ogni periodo sperimentale era costituito da 7 d di transizione e 14 d di trattamento. Il latte è stato campionato per l'analisi chimica e la caseificazione. Le proprietà organolettiche del latte e dei formaggi, dopo stagionatura di 63

giorni, sono state valutate da un gruppo di 7 valutatori addestrati. I trattamenti sperimentali non hanno avuto effetti sull'ingestione alimentare, sulla produzione di latte, sull'efficienza alimentare, sulle proprietà coagulative del latte, sul recupero dei nutrienti e sulla resa casearia. L'aroma ed il sapore del latte e del formaggio sono stati particolarmente influenzati dai trattamenti sperimentali, in particolare dalla dose più alta di spicchi d'aglio. I risultati hanno evidenziato un'elevata correlazione tra latte e formaggio per quanto riguarda l'aroma di aglio (R^2 = 0,87) ed il flavor di aglio (R^2 = 0,79). Il sulfuro di allile e la dose più alta di spicchi d'aglio (400g/d) hanno provocato una riduzione del pH, dello sforzo di taglio ed il corrispondente lavoro di taglio dei formaggi stagionati. L'agli in spicchi ed il sulfuro di allile hanno mostrato effetti opposti sugli indici colorimetrici del formaggio stagionato. In conclusione, l'integrazione di 400 g/d di aglio in diete per vacche in lattazione influenza notevolmente le caratteristiche sensoriali e reologiche del formaggio.

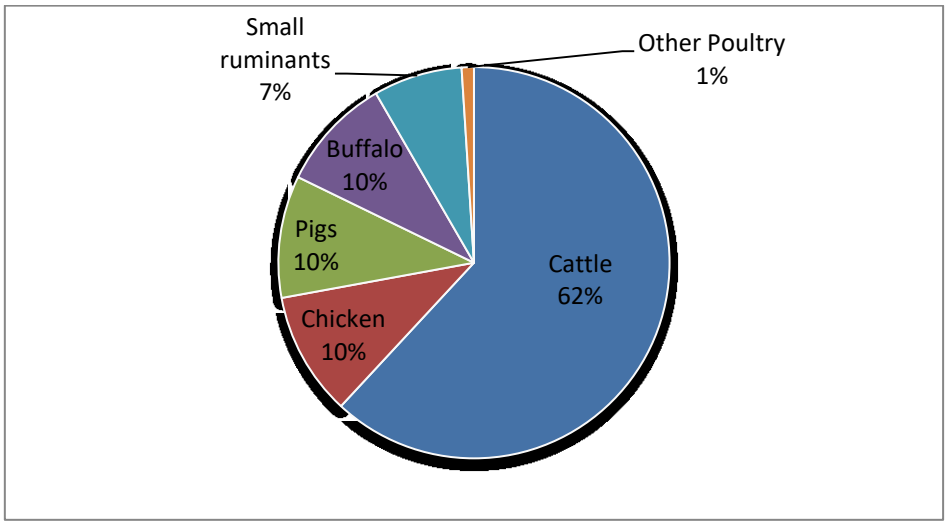
1 General Introduction

1.1 Greenhouse gas emission: livestock production chain

Carbon dioxide (CO_2), methane (CH_4), nitrous oxide (N_2O) and halocarbons are greenhouse gases (GHG) that enhance the effect of solar and thermal radiation on surface and atmospheric temperatures. They are often expressed on a CO_2-equivalent (CO_2e) basis. The impact agriculture has globally is likely to increase over the next several decades because of increases in population growth and income-dependent dietary shifting towards more meat-based diets (Springmann et al., 2016).

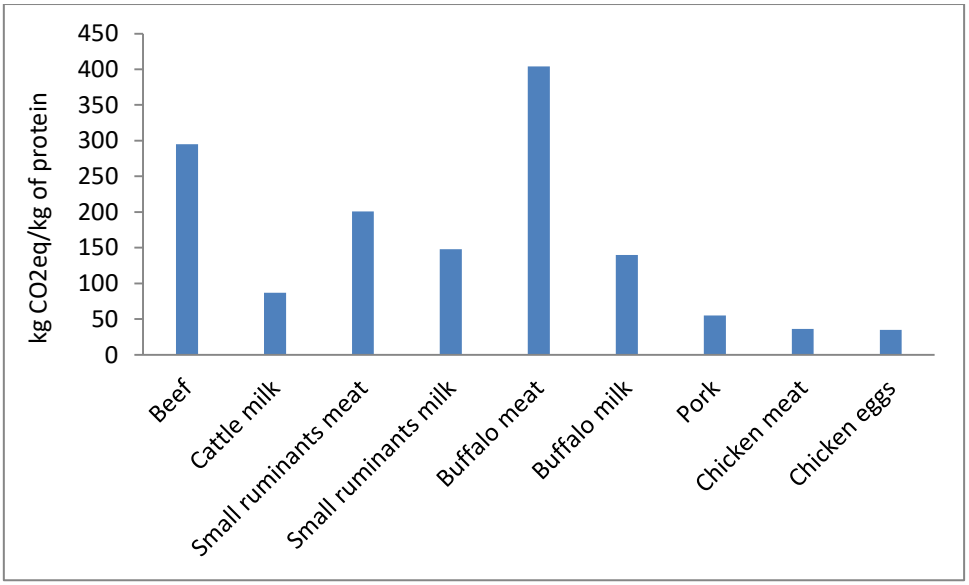
The emissions can be expressed in different ways and this issue can lead to confusing communication. Methane emission from livestock supply chains can be expressed as per unit protein basis, allowing comparisons between species and products to be made. Also, it can be related to the feed intake by the livestock. Even if the proportion of GHG emission from livestock is confusing, it is certain that the livestock sector is a significant contributor to global human-induced GHG emissions (Knapp, 2014). Livestock chains emitted an estimated total of 8.1 gigatonnes CO_2-eq in 2010, with methane (CH_4) accounting for about 50 percent of the total, while N_2O) and CO_2 represent almost equal shares with 24 and 26 percent, respectively. By species, cattle are the main contributor, with about 62% of sector's emissions, with pigs, poultry, buffaloes and small ruminants ranged from 7 to 11% of sector's emissions (FAO, 2017), as shown in figure 1.

Figure 1:% Global estimates of emissions by specie. FAO (2017).



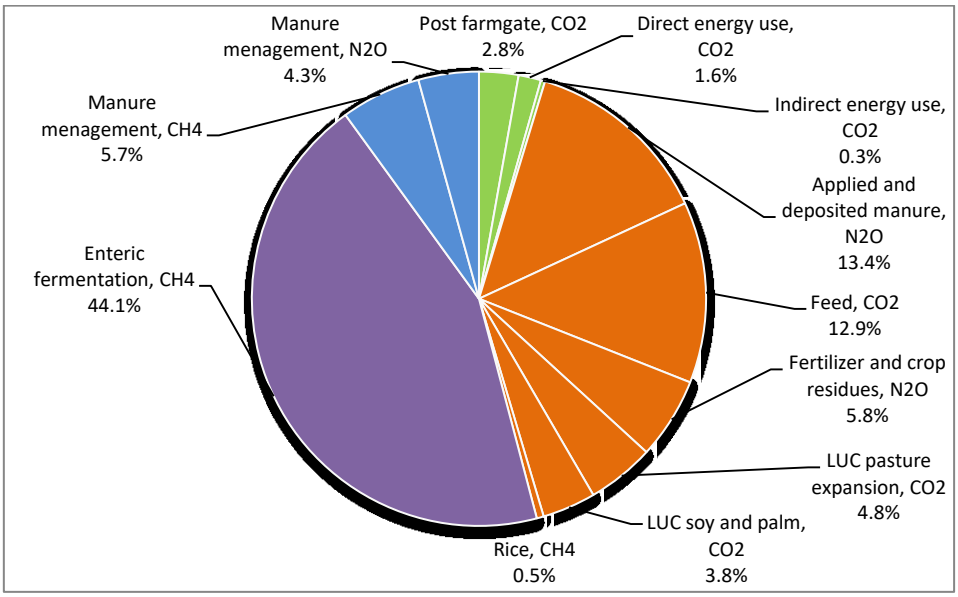
However, FAO (2017) stated that, in terms of a per protein basis (Figure 2), buffalo meat is the commodity with highest emission intensity, followed by beef meat. Dairy cattle, chicken meat and eggs and pork have lower emission intensities.

Figure 2:Global emission intensities by commodity, expressed as kg of CO2-equivalent per kg of protein. FAO (2017).



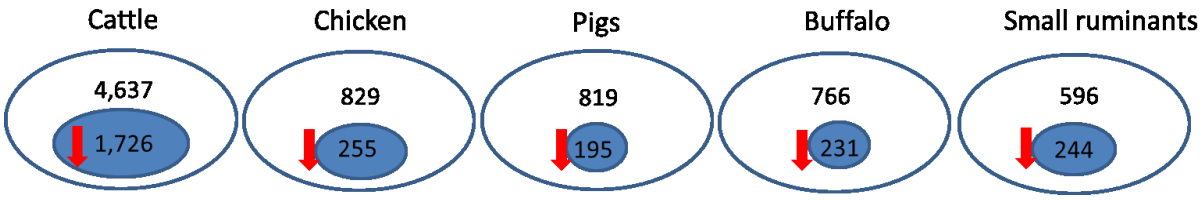
Global emission by source indicates that enteric fermentation accounts for almost 44% of total sector's emissions (Figure 3). Feed production is the second largest source of emissions, with 41% of total emissions, after enteric emissions with 44.1%.

Figure 3:Global emission by source. FAO (2017).



Since emission intensities vary greatly among agro-ecological conditions, farming practices and supply chains management, it is interesting to ascertain where opportunities for mitigation can be found (Figure 4). FAO (2017) stated that a lowering in emissions from the livestock sector can be achieved by reducing production and consumption, by lowering emission intensity in production, or by a combination of the two.

Figure 4:Mitigation potential of the global livestock sector, expressed as Millitons of CO2-eq. FAO (2017).

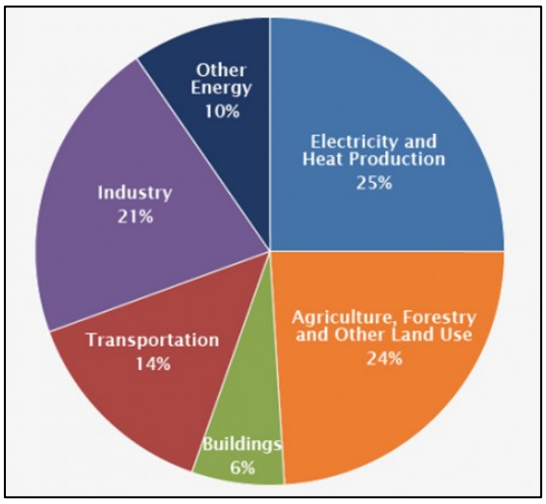


1.2 Methane as impacting factor of livestock sector

The 100-year global warming potential of methane is 28 times more, which means that it traps 28 times more heat per mass unit, than carbon dioxide and 32 times the effect when accounting for aerosol interactions (IPCC, 2014). Global methane levels have risen from 722 parts per billion (ppb) in pre-industrial times to 1800 ppb by 2011. An increase by a factor of 2.5 and the highest value in

at least 800,000 years. Many are the causes of the rapid raising of surface temperature of the earth, but all of them are because of human activities. Most come from the combustion of fossil fuels in cars, factories and electricity production. The gas responsible for the most warming is carbon dioxide, but other contributors, especially methane, released from landfills and agriculture, have an important impact on global warming. In 2011, Herrero et al., considering the positive trend of CH_4 emission since before the Industrial Revolution increased, especially in the decade of 1990s and 2000, concluded that it is necessary to impose short-term reduction of CH_4 emissions. Later, in 2014, the IPCC reported that "it is extremely likely that human influence to have been the dominant cause of the observed warming since the mid-20th century". The methane produced by agriculture, forest and other land use represent the 24 %, of the global emission (Figure 5).

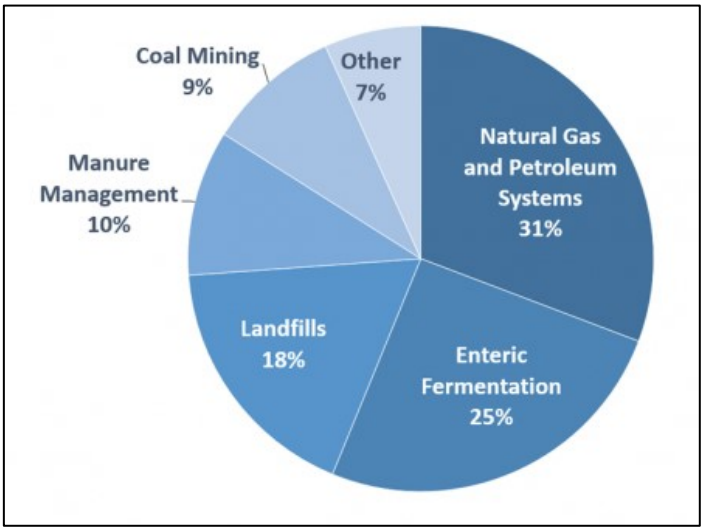
Figure 5: Global CH_4 emission by economic sector, IPCC (2014).



Moreover, the U.S. Environmental Protection Agency (EPA) in 2014, estimated that enteric fermentation associated with the domestic livestock sector is the main sources of CH_4 emission, followed by natural gas system and other sources (Figure 6). Therefore, the impact of enteric methane emission is controversial. FAO (2006) stated that 18% of total anthropogenic greenhouse gases are directly or indirectly related to world's livestock. Herrero et al. (2011) reported that, depending on different assessments, GHG emissions by livestock range from 8 to 51%. Conversely,

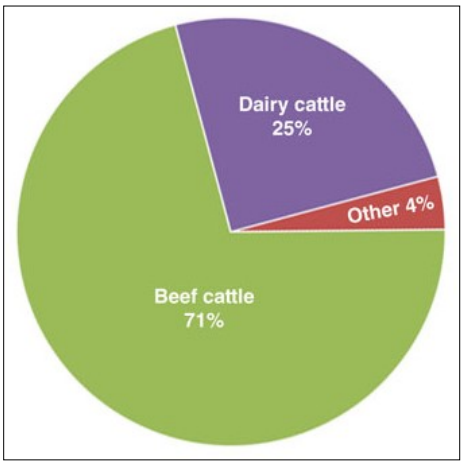
the majority of scientists agreed with the estimation of EPA (2015) where it has been reported that about 25% of GHG (Figure 6) arise from enteric fermentation in livestock.

Figure 6: Global CH_4 emission by source (1990-2015), EPA (2015).



The EPA (2014) estimated that enteric fermentation was responsible for 141 MMT of CO_2e emissions in 2012, and based on livestock type, beef and dairy cattle were responsible for the overwhelming majority of CH_4 emissions (Figure 7) with 71% of the methane emission, followed by dairy sector with 25%. All other classes of livestock contributed by 4 percent of the global CH_4 emissions.

Figure 7: Global enteric methane emissions by livestock type. EPA (2014).



1.3 Methanogenesis in ruminants

Methane production by enteric fermentation is a part of the normal digestive process in animals, especially ruminants, such as cattle, sheep and goats. Although methane production can also occur in the lower gastrointestinal tract, as in non-ruminants, 89% of methane emitted from ruminants is produced in the rumen and exhaled into the atmosphere through the mouth and nose (Murray et al., 1976). This results in a loss of ingested feed-derived energy of approximately 2–12% (Patra, 2016). Methane losses from ruminants can vary greatly. It can be based on feed quality, but also on geographical location, feed intake, feed composition, and the processing of the feed. Estimations has been made by Johnson and Ward (1996), where diet-derived energy losses from methane for dairy cattle, range-cattle, and feedlot cattle vary from 5.5–9.0%, 6.0–7.5%, and 3.5–6.5%, respectively.

The rumen is a large anaerobic fermentation chamber in which nutritional components (carbohydrates, proteins and, in lesser extent, lipids) are degraded by the rumen microbial community (bacterial, protozoal and fungal species) and transformed mainly into volatile fatty acids (acetate, propionate and butyrate) (Mitsumori and Sun, 2008), but also formate, ethanol, lactate, succinate. In addition, and branched chain volatile fatty acids are formed, even if in a lower amount, with ammonia, carbon dioxide and H_2 gas as end products (Hook et al., 2010). Methane arises from microbial activities in the rumen of the gastrointestinal tract and can be released via three routes (Ricci et al., 2014):

1) CH_4 from rumen and lower gut absorbed into the blood and exhaled from the lungs via expiration;
2) CH_4 emitted directly from the rumen by eructation;
3) CH_4 emitted from the hindgut in the flatus.

Collectively, expiration and eructation have been encompassed by the term ‘exhaled’ gas, as the majority of eructated gas from the rumen is inhaled into the lungs before being exhaled (Hoerneckie

et al., 1965; Berends et al., 2014). The 87% of total CH_4 production in ruminants is mainly involved the rumen, whereas 13% is produced in the lower digestive tract (Murray et al., 1976). Rectal emissions ranged from 2 to 11% of the total CH_4 emissions in sheep and dairy cows (Muñoz et al., 2012); while up to 89% is excreted through the breath (Murray et al., 1976). Methane emissions are closely related to the amount of rumen fermented organic matter (OM) or the amount of digestible OM. When the digestibility of energy increases by 10%, energy losses as methane increase by 0.47 points in a roughage diet and by 0.74 points in a mixed diet (Blaxter and Clapperton, 1965). Carbon dioxide and hydrogen are the precursors of methane (Moss et al., 2000), which is the major way of H_2 elimination through the following reaction:

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O,$$

acetogenesis is another way to remove hydrogen, represented by the following reaction:

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O,$$

but also by enhancing the propionate production, while decreasing acetate and butyrate (producers of H_2):

$$C_6H_{12}O_6 + 2H_2 \rightarrow 2C_3H_6O_2 + 2H_2O.$$

CH_4 is produced in anaerobic conditions by the Archaea microbes as a physiological end-product of microbial fermentation of carbohydrates, which are able to metabolise the hydrogen formed by other fermentative microbes. Many studies have been performed with the aim to understand this community (Henderson et al., 2015, Seedorf et al., 2015). However, the mechanism of CH_4 production in ruminants is still unclear (Danielsson et al., 2017a). About 40 species of Archaea were identified and Methanobrevibacter, Methanobacterium and Methanosphaera are the main genera (Wedlock et al., 2013). Methanobrevibacter seems to be the dominant genus of the archaeal domain (Leahy et al., 2013; Henderson et al., 2015). Danielsson et al. (2012, 2014) found out that certain groups of Methanobrevibacter species (M. smithii, M. gottschalkii, M. millerae, and M. thaueri), were not only correlated to individuals with higher CH_4 production but also to feed additive. This indicating that specific substrates favour certain Methanobrevibacter species

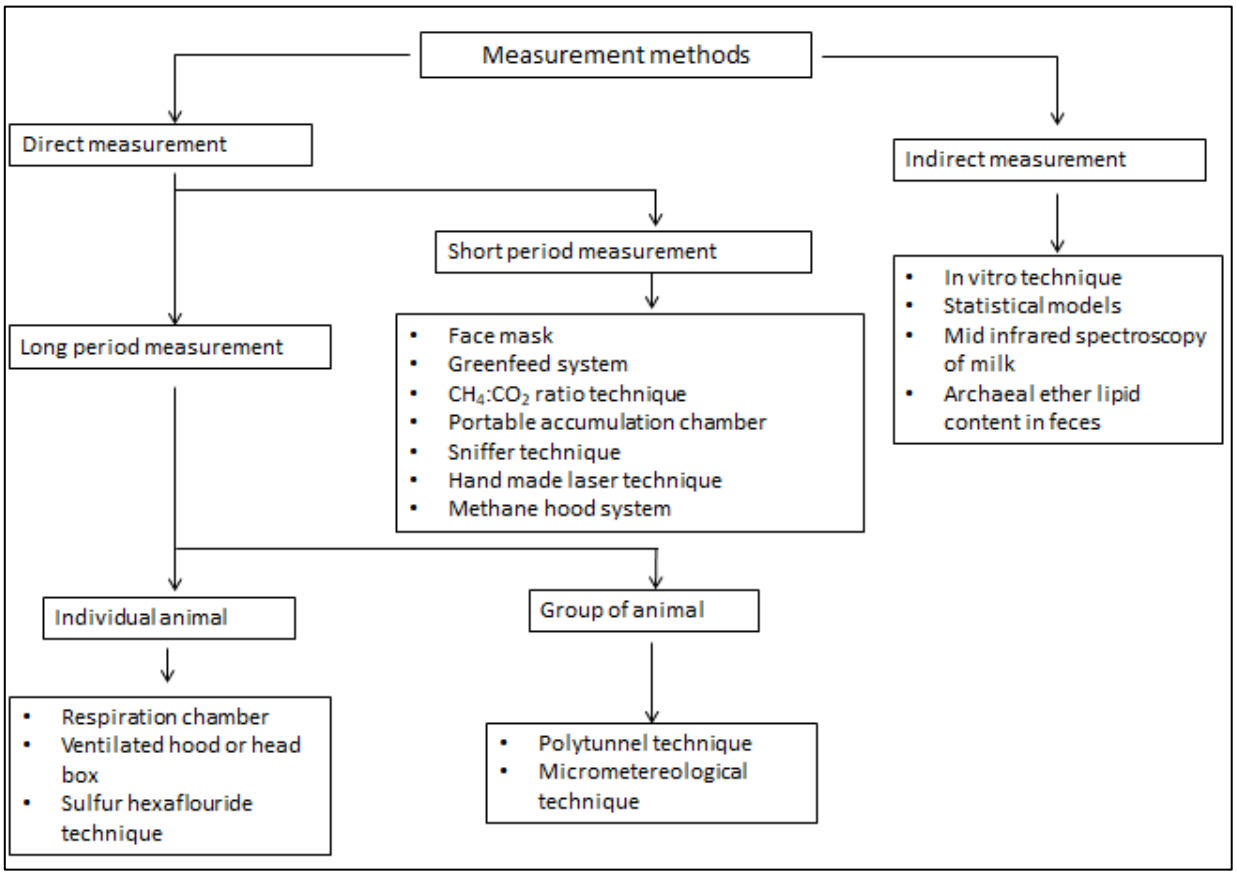
(Danielsson et al., 2012 and 2014). Thanks to new molecular techniques, such as next-generation sequencing, knowledge of rumen microbiology has increased in recent years. However, the correlation to level of CH_4 emissions is still not clear. Moreover, it is unclear whether cows producing comparatively lower amounts of CH_4 have less efficient feed degradation, resulting in lower milk and meat production (Danielsson et al., 2017a).

1.4 In vivo methane measurement: crucial point

Measurements of individual methane emission were applied to assess the energy loss as a component of energy balance and for the estimation of heat production based on respiratory exchange (Reynolds, 2000). Many of the available measurement techniques, so far, are either slow, expensive, labour intensive and/or are unsuitable for large-scale individual animal measurements (Negussie et al., 2017). However, recently, the need for high throughput measurement of enteric methane emission has led to development of a variety of approaches, which differ in their application, cost, accuracy and precision (Hammond et al., 2016). Many are techniques currently used to directly measure enteric CH_4 emission from ruminants, for instance, respiration chambers, sulphur hexafluoride tracer, GreenFeed, carbon dioxide as tracer, estimation based on eructated air and handheld laser detector (Figure 8).

Despite the technology available, accurate and repeatable measurements of CH_4 emission from large numbers of animals are needed for investigating possible mitigation options, screening animals for breeding programmes, assessment of alternative management strategies, and decreasing uncertainties associated with national GHG inventories (Pickering et al., 2015). The criteria of non-invasive and non-intrusive techniques is the most important for appropriate and acceptable CH_4 measurement, measuring the animals in their environment, but also, rapid, cost effective, ideally automated and with the minimum error.

Figure 8: Schematic presentation of different CH_4 measurement techniques (Patra, 2016).



In addition, the analysis of the complex matrix needs an understanding of physics associated with airflow, air mixing, background gas concentrations and ambient conditions, as well as an appreciation of animal behaviour and an understanding of how applicable the data is to the environment under evaluation.

CH_4 emission can be accurately measured using respiration chambers. These are typically designed for measurements of one animal at a time, normally over the course of successive 24 h periods for 3–7 daily measurements. This is to account for between-day variation. Their limitations have been well documented, particularly, the rate of throughput (numbers of measurements over time), construction and operation cost, animal behaviour changes, constraints by their 'artificial' environment, limit their use for larger scale experiments (Reynolds, 2000; Hegarty, 2013). In addition, animals need to be acclimatised before readings can be recorded and the lack of activity within the chambers inevitably lowers energy expenditure compared with loose housing or grazing

environments (Hammond et al., 2016). Reynolds (2000), supported that, even with all the correct disposition, DMI and therefore CH_4 emission may decrease during chamber housing, depending on the level of production, diet composition and feeding level. However, Hellwing et al. (2012) found no effects on DMI were observed in studies using transparent chambers. The rate of CH_4 emission is variable according to the source (low when derived from the absorbed in blood) whereas concentration is high when you include eructated gas from the rumen. CH_4 varies considerably throughout the day in relation to feeding pattern and rate of fermentation. In this regard, breath analysis techniques only consider CH_4 concentrations during eructation events (Garnsworthy et al., 2012). Also monitoring head position is crucial, it is also important to measure both absorbed and eructated CH_4 emitted through expiration, otherwise emission may be underestimated if only eructation emission is measured, for instance with the laser tracer.

The sulphur hexafluoride (SF6) tracer technique (Johnson et al., 1994) generates values for CH_4 flux that are correlated with chamber measurements, but the between-cow variability is greater than with chamber measurements (Pinares-Patiño et al., 2011). The method is also relatively labour intensive and, therefore, not suitable for comparing large number of animals (Huhtanen et al., 2015).

Regarding the sniffer methods, a sampling inlet is placed in the feed trough of an Automatic Milking System, and gas concentrations of exhaled air are continuously sampled (Huhtanen et al., 2015). Garnsworthy et al. (2012) developed an on-farm method, following this principle, based on an index of CH_4 emission that is calculated during each milking as the product of peak frequency and mean peak area of CH_4 concentration. Studies using the sniffer method have reported emissions with relatively high between-animal variability (Garnsworthy et al., 2012; Lassen et al., 2012; Bell et al., 2014) compared with data from studies using respiration chambers (Blaxter and Clapperton, 1965; Yan et al., 2010) or the flux method (Zimmerman et al., 2013).

A recently patented gas-flux quantification system, the GreenFeed, uses a similar principle for measuring gas emissions as for respiration chambers (flux method) where an active airflow is

induced to capture emitted air. This system integrates measurements of air flow, gas concentrations, and detection of muzzle position to allow direct measurement of CH_4 and CO_2 fluxes to be measured during each animal visit to the feed trough. The sensors identify the animal and its head position, allowing the measurement of air flow, methane and carbon dioxide within the hood, in exhaust air (Hammond et al., 2015). Even though this technique is applicable on larger number of animals and on farm conditions, the animal head position is critical for successful measurements, as the animal is free to move in and out of the hood (Hammond et al., 2015). The risk of interrupted data is relevant. There is also a risk of bias if data are rejected on the basis of number of eructations, using the GreenFeed or sniffer methods (Hammond et al., 2016).

1.5 Prediction of in vivo methane emission: in vitro technology

A rapid and less expensive alternative to bypass the *in vivo* measurments, which are known to be expensive, laborious, time consuming and involve a large number of animal, is the use of *in vitro* fermentation techniques. The alternative *in vitro* techniques have important advantages:

- they do not involve the direct use of animals;
- they are less laborious and more suitable for a large-scale evaluation of ruminant feeds;
- they are less time-consuming and less expensive.

The potential of diets and modifiers of rumen fermentation have been tested across this technology to rapidly obtain results on their suitability for further avaluation. As reported in a recent review by Yáñez-Ruiz (2016) Czerkawski and Breckenridge in the 1970's developed the first system that involved recording the direct displacement of a piston by gases produced during the fermentation of feeds by rumen fluid in a glass syringe, later improved by Menke in 1979. The syring system was also modified by Blümmel and Ørskov in 1993 allowing the gas production and the kinetics of fermentation recording at different time-point.

The *in vitro* fermentation systems are divided in two main categories:

Continuous culture system, as described by Czerkawski and Breckenridge (1977), is design for maintaining a normal microbial population of the rumen under strictly controlled conditions over long periods of time. The apparatus (Figure 9) is simple to construct and operate. The complete apparatus consisted of vessels (fermentation unite, figure 10) and allow the control of temperature of the water bath and the movement of the feedbag.

Figure 9: RUmen SImulation TEChnique apparatus.

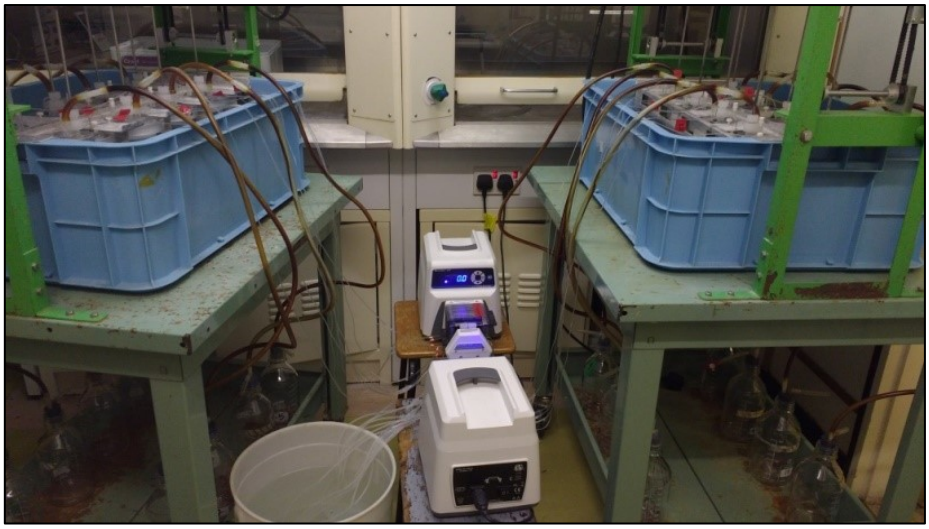
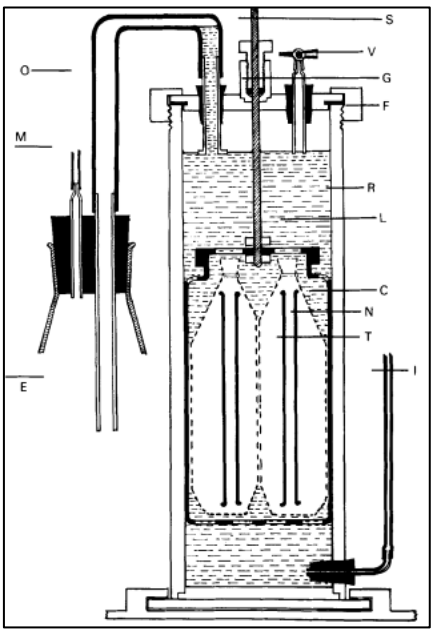


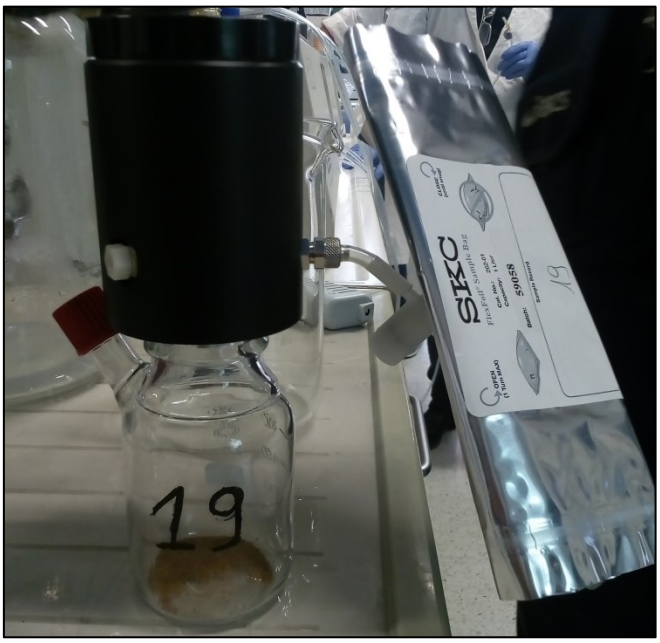
Figure 10: RUmen SImulation TEChnique's vessel (Czerkawski and Breckenridge, 1977).



Batch culture system, as described by van Nevel and Demeyer (1981), are commonly used for evaluating the effects of diets and additives on enteric CH_4 production. Recently, Ramin and Huhtanen (2012) developed an *in vitro* method for prediction of CH_4 production in the rumen of cows using the kinetic parameters from an automated *in vitro* gas production (GP) system in a 2-compartment rumen model. Danielsson (2017b) confirmed that this approach takes rumen dynamics (digestion kinetics) into account and may have advantages compared with single time point batch culture systems. Pressure data is recorded and then converted in terms of volume of total gas produced. Moreover, gas can be collected and analyzed for composition. The commercial apparatus Ankom^{RF} Gas Production System (Ankom Technology, NY, USA; Figure 11) consisting of a set of fermentetion units (Figure 12) equipped with pressure sensors and wireless connected to a computer. During the incubation, the headspace pressure of each bottle is recorded, and the electromechanical valve in each unite, allowing the control of the release of gas (Tagliapietra et al., 2011).

Figure 11: Gas Production system (Ankom^{RF}).

Figure 12: Fermentation unite of the Gas Production system (Ankom^{RF}).



As suggested by Calsamiglia (2007) the criticism of the batch system, compared to continuous system, is the difficulty to adapt the rumen microbiome, due to the very short duration of the experiments (i.e., 24 h) and also to the use of rumen fluid which had not been exposed to the presence of the investigated compounds before. The incubation of a non-adapted rumen fluid for short times might generate misleading effects causing the discarding of compounds that may have needed a longer time to be effective on rumen fermentation, and the acceptance of a compound that is effective in the short term but not in the long term. Recently innovative approaches, for DNA sequencing and fermentation products estimation on fermentation residues, i.e. the Ion Torrent Personal Genome Machine and the Fourier Transform Infrared (FTIR) spectroscopy, have been combined to the *in vitro* system allowing the analysis, at very very low cost, improving accuracy in quantification and estimation.

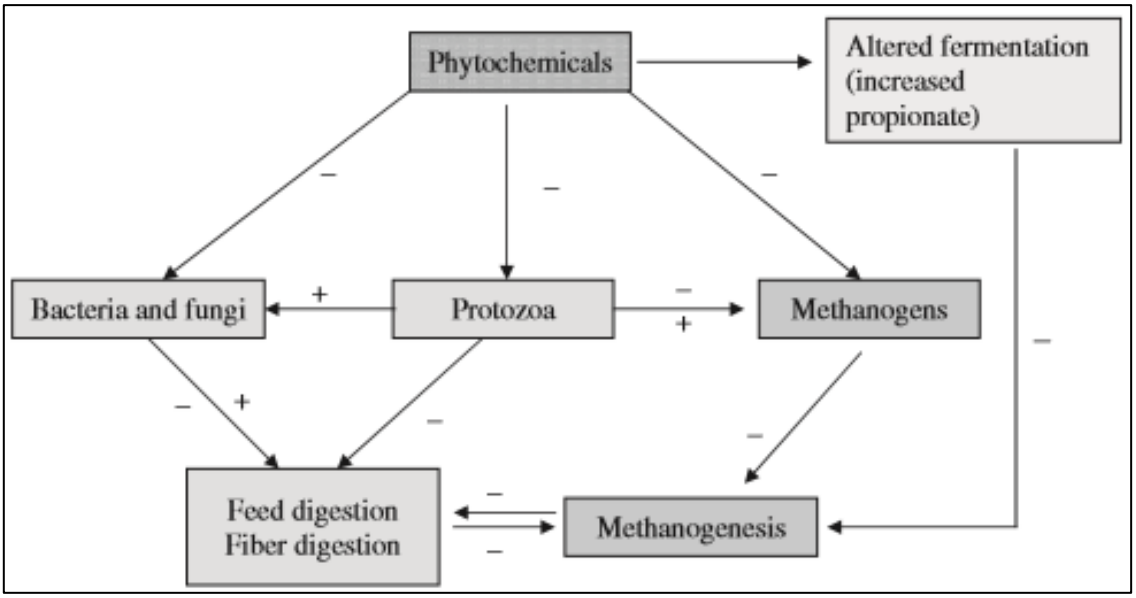
1.6 Use of natural compounds as rumen methane modulators

Since the ban of antibiotics as growth promoting feed additives by the European Union in 2006, plant extracts and plant secondary metabolites (PSM) have been considered as an alternative to manipulate the rumen (Hart et al., 2008). The term 'plant secondary metabolite' is used to describe a vast array of chemical compounds in plants that are not involved in the biochemical processes of plant growth and reproduction. These have been studied and used in medicines and preservation of foods (Patra and Saxena, 2010). The mode of action (Figure 13) depends on the specific additive, but can include (Knapp et al., 2014):

1) direct inhibition of methanogens or methanogenesis;
2) suppression of ciliate protozoa;
3) providing or stimulating a competitive pathway for H_2 disposal.

These natural plant bio-chemicals can generally be classified into five major groups: tannins, saponins, flavonoids, essential oils and organosulphur compounds. They have been shown to selectively regulate the rumen microbial populations (Patra and Saxena, 2009a) resulting in an improvement of rumen fermentation and nitrogen metabolism. This may leads to a decrease in methane production (Kamra et al., 2006; Rochfort et al., 2008). Recently, a number of reviews have discussed the potential of plant bioactives as modifiers of rumen microbial fermentation and ruminant production (Calsamiglia et al., 2007; Hart et al., 2008; Spanghero et al., 2008; Patra and Saxena, 2009b; Soliva, 2011) and results of these studies are very promising.

Figure 13: Schematic presentation of mode of action of phytochemicals on methanogenesis (Patra and Saxena, 2010).



Tannins, as well as saponins, have been extensively studied and show the most mitigating potential (Hristov et al., 2014). The tannin compounds are widely distributed in many species of plants, seeds, bark, wood, leaves, and fruit skins. They play a role in protection from predation, pesticides, and might help in regulating plant growth in plants. Tannins are usually considered anti-nutritional although they can have considerable potential to reduce intestinal parasites (Niezenet al., 1995). On the other hand, high levels of tannins, as reported by some authors (Waghorn, 2008; Patra, 2010), reduced digestibility of diets. Thus they will inevitably be anti-nutritional when dietary crude protein concentrations are limiting production because they reduce absorption of amino acids (Waghorn, 2008). Beauchemin (2007) reported that tannins showed a mitigation potential by up to 20%. Therefore, it is important that benefits of reduced CH_4 emission do not negatively affect the digestion and production, as observed by Grainger et al. (2009). In the same study, CH_4 emissions were reduced by up to 30%, but milk production of the cows was also reduced by about 10%. In 2012, Jayanegara stated a relatively close relationship between dietary tannin concentration and CH_4 production per unit of digestible OM. Also a decrease in feed intake and nutrient digestibility was observed, particularly crude protein. In a recent review, Goel and

Makkar (in 2012), concluded that the risk of impaired rumen function and animal productivity with tannins is greater than with saponins. For decreasing CH_4 production, the concentration range for tannins is narrower than for saponins.

The second group of SPM includes saponins. Their mode of action have been investigated and seems to decreased the number of protozoa and consequently methanogens species associated with them (Patra and Saxena, 2010). The effectiveness of saponins-extracts in reducing CH_4 emission has been demonstrated both *in vitro* (Pen et al., 2008; Holtshausen et al., 2009) and *in vivo* studies (Holtshausen et al., 2009; Wang et al., 2012).

However, some studies have indicated that saponins may decrease the activities of methane producing genes or rate of methane production in each methanogenic cell (Guo et al., 2008). Additionally, Guo stated that methane production may also be affected by saponins as a result of reduced rate of methanogenesis via diminished activity of methane producing gene without changing the total methanogen population. On the other hand, saponins may decrease methanogenesis indirectly via inhibition of protozoa (Patra and Saxena, 2010).

Flavonoids are a large class of polyphenolic compounds and the most numerous. The flavonoids generally act against microorganisms through inhibition of cytoplasmic membrane function, inhibition of bacterial cell wall synthesis, or inhibition of nucleic acid synthesis (Cushnie and Lamb, 2005). Also, it has been suggested that the flavonoids, directly or through new derivatives produced upon biotransformation or degradation, affect the rumen microbial activity (Oskoueian et al., 2013). Flavonoids are contained in plants such as liquorice, parsley, onions, blueberries and other berries, black and green tea, bananas, all citrus fruits, Ginkgo biloba, and food, such as red wine, sea-buckthorns, buckwheat and dark chocolate (with a cocoa content of 70% or greater). Moreover, plant extracts rich in flavonoids have gained importance in improving animal production. Tedesco et al. (2004) reported the increase in milk yield and lactation performance in dairy cows upon 25 d administration of sylimarin (10 g/d) which mainly consist of flavonolignans. Currently, various flavonoids-rich feed additives, which suppress the methane production, are available in the market.

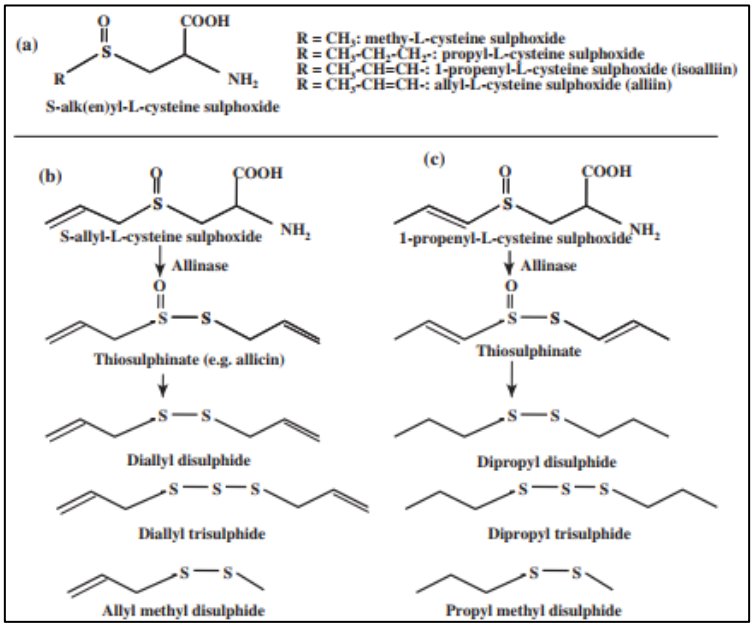
Furthermore, the information on the effect of flavonoids in the pure form on rumen microbial activity is still lacking (Gohlke et al., 2013).

Essential oils are a group of secondary plant metabolites obtained from volatile fractions of plants by steam distillation process (Gershenzon and Croteau, 1991), which are used as antimicrobial agents and preservatives. Essential oils have diverse chemical composition, natural and biological properties. It has been suggested that they increase the phylogenetic distribution of methanogenic archaea, which may have resulted from changes in associated protozoal species (Ohene-Adjei et al., 2008). Many essential oils, such as chinnamaldehyde, juniper berry, limonene, eucalyptus, thymol, peppermint, and many others, have shown methane suppressing effects (Patra and Saxena, 2010). However, effects of essential oils on protozoal population vary. Some studies reported a lack of effect on protozoal numbers (McIntosh et al., 2003; Benchaar et al., 2007; Newbold et al., 2004), while others have found a stimulatory effect of essential oils on protozoa (Patra and Saxena, 2009b). There are also studies that indicate an anti-protozoal effect (Ando et al., 2003; Cardozo et al., 2006; Fandiño et al., 2008) where concentrations of total, entodiniomorph and holotrich protozoa have been reduced. Although the mechanism of action is poorly understood. It may be related to the lipophilic nature of compounds such as Anethol, which facilitates permeation of essential oil across the protozoal membrane (Cardozo et al., 2006).

The last big group is represented by the organosulphur compounds. They occur mainly in two plant families: (1) Alliaceae family e.g., Allium sativum (garlic), Allium cepa (onion) and Allium porrum (leek) containing alliin–alliinase system and (2) Cruciferae (Brassicacae) family e.g. Brassica juncea, Wasabia japonica (wasabi), Armoracia rusticana (horseradish) and Brassica oleracea (cauliflower) containing glucosinolate–myrosinase (Mithen, 2006). The primary sulphur-containing constituents in Alliums pp. are S-alk(en)yl-L-cysteine sulphoxides, ranging from 0.53 to 1.3% of fresh garlic (largest contributor), and cglutamyl-S-alk(en)yl-L-cysteins sulphoxides (Ross and Milner, 2007). This can be seen in figure 14. By the action of allinase, these compounds are converted into thiosulphinates, which are then spontaneously and enzymatically converted into a

large array of volatile compounds e.g. diallyl disulphide, diallyl trisulphide, allyl methyl disulphide, dipropyl disulphide (Mithen, 2006) mainly after the cloves' fraction.

Figure 14: Major organosulfur compounds found in Alliacae family: (a) organosulfur compounds in intact plants, (b) compounds. Produced from allyl cystein sulphoxide (in garlic) and (c) 1-propenyl cystein sulphoxide (in onion) by aliinase (Patra and Saxena, 2010).



A number of studies have been conducted on garlic oils or its components for modification of rumen fermentation and inhibition of methane production (Busquet et al., 2005; Kamel et al., 2008; Chaves et al., 2008; Kongmun et al., 2010; Patra et al., 2006). The knowledge of the mode of action on the microbiota is still incomplete, but it has been suggested by Ohene-Adjei et al. (2008) that organosulphur compounds may change the associated protozoal species. Patra et al. (2010) did not not support the hypothesis of protozoal associated methanogenesis reduction, but because the garlic extracts did not appreciably affect degradability of feeds, he believed that garlic oil may specifically inhibit methanogenic archaea. Moreover, it has been suggested that the organosulphur compounds found in garlic oil may directly inhibit the rumen methanogenic archaea through an inhibition of the enzyme 3- hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase (Busquet et al., 2005). In the same study, valuating the effects of garlic oil and four of its main components (diallyl sulphide,

diallyl disulphide, allyl mercaptan, and allicin) in batch culture, he observed that garlic oil and diallyl disulphide (300 mg/l of ruminal fluid) reduced methane production by 74 and 69%, respectively. Furthermore, methanol and ethanol extracts prepared from fresh garlic caused an inhibition of methanogenesis *in vitro*, but not water extract of garlic (Patra et al., 2010). However, there has been a concern that allicin may be unstable in the rumen, resulting in loss of activity with time. In this regard, Kamel et al. (2008), observed that allicin and diallyl disulphide were effective at 6 hrs, but had no effect at 12 or 24 hrs. However, no adaptation was founded by Hart et al. (2006) to allicin even up to 17 days. Several authors also reported the effect on the rumen fermentation: no effect on degradability of organic matter (Patra et al., 2010); no effect on VFA concentration (McAllister and Newbold, 2008); while Busquet et al. (2005) observed a reduction of volatile fatty acids concentrations at high doses. In particular, garlic oil and its components increased the proportions of propionate and butyrate. Patra et al. (2010) observed that garlic extract from cloves increased total volatile fatty acid and butyrate production and decreased acetate to propionate ratio.

A few experiments investigated the effect *in vivo* of garlic, or its sulphur constituents, on digestion, ruminal fermentation, milk production and quality in dairy cows (van Zijderveld et al., 2011; Oh et al., 2013; Blanch et al., 2016). In a trial with sheep, the supplementation with garlic (10 g/kg of dry matter intake) decreased methane formation expressed relative to organic matter digested (Patra et al., 2008a). Therefore, the response of organosulphur compounds on intake by ruminants is limited. Garlic oil or garlic cloves appear to have no effect on intake (Nolte and Provenza, 1992; Bampidis et al., 2005; Yang et al., 2007; Patra et al., 2008a). Thus, garlic oil seems to have great potential for mitigation of methanogenesis, without affecting nutrient utilisation . Even, garlic fed to sheep, led to an increase in digestibility (Patra et al., 2008a; Kongmun et al. (2010) that might have been due to increased number of fibre degrading microbial population (Patra et al., 2008b).

Therefore, it appears that organosulphur compounds have great potential to improve rumen fermentation with suppression of methanogenesis. They are potentially more specifically effective against methanogens, which usually changes rumen fermentation characteristic to chemical anti-

methanogenic compounds i.e. increased propionate and decreased acetate concentrations with no apparent change in total volatile fatty acids (Patra et al., 2010).

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2 Aim and Objectives

The general objective of the present thesis was to study different natural additives to reduce enteric CH_4 emission in ruminants using in vitro techniques. A screening of four pure plant extract and one synthetic have been evaluated using a batch culture system (first contribute) on rumen fermentation and methane emission. Based on the results obtained in the first experiment, the three more promising compound were interesting to verify the influence of the rumen fluid adaptation before been used as inocula (second contribute). The third contribution was carried using a long term technology, a continuous culture system (RuSiTec), to test the effect of liquiorice extract on fermentations, methane emission and rumen microbiome at at IBERS (Aberysthwyth, Wales). After these *in vitro* studies, the interest was focused on the effect, of natural additives, on the dairy product. Specifically, how feeding the dairy cows with them can modify the organoleptic profile of dairy products, in particular garlic and sulfur compounds (fourth contribute).

3 List of the Contributions

This Ph.D. Project is composed by the following four Contributions (the manuscripts are named "Contribution" preceded by ordinal number in the text).

1st Contribution Cattani Mirko, Maccarana Laura, Rossi Giulia, Tagliapietra Franco, Schiavon Stefano, Bailoni Lucia. 2016. **Dose-response and inclusion effects of pure plant extracts and synthetic compounds on in vitro methane production.** Animal Feed Science and Technology, 218, 100-109.

2nd Contribution Rossi Giulia, Cattani Mirko, Maccarana Laura, Vieira Vanesa, Tagliapietra Franco, Schiavon Stefano, Bailoni Lucia. **Effect of rumen fluid collected from cows exposed or not to pure compounds on the *in vitro* gas production kinetics, fermentations and methane production.** Submitted to International Dairy Journal.

3rd Contribution Ramos Morales Eva, Rossi Giulia, Jones Eleonor, Braganca Radek, Newbold C. Jamie. **Effect of an isoflavonid-rich liquorice extract on rumen fermentation, methanogenesis and microbiome in the Rumen Simulation Technique.** Submitted to FEMS microbiology ecology.

4th Contribution Rossi Giulia, Schiavon Stefano, Lomolino Giovanna, Cipolat-Gotet Claudio, Simonetto Alberto, Bittante Giovanni, Tagliapietra Franco. **Garlic (Allium sativum L.) fed to dairy cows to reduce enteric methane emissions do not modify the cheese-making properties of milk but affects color, texture, and flavor of ripened cheese.** Submitted to Journal of Dairy Science, under minor revision.

Chapter 4

1st Contribution

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

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ABSTRACT

This study explored effects of four pure plant extracts (allyl-sulfyde, cinnamaldehyde, eugenol, and limonene) and one synthetic compound (monensin), on *in vitro* rumen fermentation and methane (CH_4) production of a commercial diet for dairy cows. Four incubations at 24 h were conducted using a gas production (GP) system equipped with devices for automated gas venting at fixed pressure. Bottles (317 ml) were filled with 1.0±0.010 g of diet, additive (except in the control group), 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 plant extracts; 0.015 or 0.030 mg/g of diet for the ionophore compound. The resulting experimental design was: 4 incubations × 11 treatments (5 additives × 2 dosages, plus the control group) × 3 replications. Twelve bottles (3 per each run), containing only the buffered rumen fluid, were included as blanks. 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 effects of treatment and incubation as fixed and random factor, respectively. All the additives were ineffective at the low dosage. The high dosage of limonene caused a marked depression of NDFd (-68%) and TDMd (-14%) compared to the control; less marked reductions were noted for high dosage of eugenol (-15% and -4%, for NDFd and TDMd, respectively) and of monensin (-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 AL, CI, EU, LI, and MO significantly reduced in vitro GP (ml/g DM; -16%, -12%, -9%, -38%, -12%, respectively). In vitro CH4 production was significantly reduced only by high dosage of AL, CI, LI, and MO (-32%, -12%, -43%, -18%, respectively, compared to the control). Only high dosage of AL and LI significantly reduce CH4 proportion (-18%, -12% respectively, compared to the control). The most promising results were observed for the high dosage of cinnamaldehyde, that reduced *in vitro* CH_4 production without compromising degradability and VFA production.

Keywords: Dose-response, Inclusion effects, Pure natural extracts, Gas production, Methane production

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). 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 complex 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. To this regard, use of pure products could allow to obtain more accurate results (Martínez-Fernández et al., 2013).

Thus, this 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.

MATERIALS AND METHODS

The commercial diet used for *in vitro* tests 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 with α-amylase using the Ankom^{220} 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).

Incubation Procedures

A commercial GP apparatus (Ankom^{RF} 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 11 treatments were: a control group (CTR) where the bottles containing the diet and the buffered rumen fluid were incubated; 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. 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, for the pure compounds, and to 0.1 or 0.2 mg/l of buffered rumen fluid, for 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).

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-NH_3) 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 FIAstar^{TM} 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 hydrogen 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 hydrogen 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.

Computations and Statistical Analysis

Degradability of NDF (NDFd) and of true DM (TDMd) were calculated according to Goering and Van Soest (1970). Recently, Cattani et al. (2014), using vented bottles connected to tight bags for gas collection, calculated CH_4 production (ml) as follows: [CH_4 concentration in the bottle headspace] × [headspace volume] + [CH_4 concentration in the gas bag × total GP volume]. 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 and the same operative conditions of those of the current experiment) were used. It was found that total methane production can be

predicted, with acceptable precision and accuracy, as: total CH_4 production = - 0.0064 × [CH_4 concentration in the headspace × (headspace volume + total GP volume)]^2 + 0.9835 × [CH_4 concentration in the headspace × (headspace volume + total GP volume)]. The equation had a residual standard deviation of only 0.1770 ml, and a R^2 = 0.9993. Therefore, data of total CH_4 production (ml) computed with this equation were expressed as ml/g DM incubated, as ml/g TDMd, and as ml/100 ml GP.

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). All parameters measured were graphically expressed as percentage variation of each additive and dosage compared to the CTR treatment.

RESULTS

The proximate composition of the commercial diet used for *in vitro* tests is given in Table 1. The diet contained 361, 158, and 33 g/kg of NDF, CP, and lipids, respectively (Table 1).

Allyl sulfide

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 ($N\text{-}NH_3$) of rumen fluids at the end of incubation was unchanged.

Cinnamaldehyde

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.

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.

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.

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.

DISCUSSION

Effects of plant extracts on in vitro fermentation

From a recent meta-analysis (Klevenhusen et al., 2012) it emerged that studies exploring effects of bioactive compounds on *in vitro* rumen fermentation have tested a wide range of dosages, from very low (0.03 mg/g DM) to very high (500 mg/g DM). Despite these divergences, it is 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).

In this study the four pure extracts did not affect *in vitro* fermentation when added at the low dosage (3 mg/g of diet; 20 mg/l of fermentation fluid). Such results agree with other *in vitro* studies where similar dosages of cinnamaldehyde (Busquet et al., 2006), eugenol (Cardozo et al., 2005; Busquet et al., 2006), and limonene (Castillejos et al., 2006) were found to not influence DM degradability and CH_4 production. The same results emerged for 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. In our case, it could be hypothesized that the ineffectiveness of the low dosage could be partially related to the short incubation time used (24 h). To this purpose, Castillejos et al. (2007) affirmed that short-term incubations may not be long enough to evidence

effects of plant extracts on rumen fermentation. On the other hand, use of longer incubation times (i.e., 48 h) would led to microbial lysis into the batch culture systems, with a consequent alteration of *in vitro* results (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.

With respect to other additives, the high dosage of allyl sulfide showed intermediate effects on *in vitro* fermentation. Interestingly, the pure extract from garlic reduced the total VFA concentration without impairing *in vitro* degradability. Busquet et al. (2005a) observed the same pattern for allyl mercaptan, a major compound of garlic oil, and hypothesized that main effects of garlic compounds

might be addressed to the modification of carbohydrate metabolism but without a negative impact on the overall fermentation process. As regards to this study, the reduced VFA production caused by allyl-sulfide could suggest a decreased energy metabolism. Together with the total production, the high dosage of allyl-sulfide showed to influence also the proportion among the main VFA. The reduction of acetate concentration at the favor of that of propionate is confirmed by Busquet et al. (2005a,b), which used similar dosages. 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, 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). 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 the 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). Overall effects of allyl-sulfide would suggest that this compound 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 obvious 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 strictly related to the dosage used (Calsamiglia et al., 2007). In some cases (i.e. thymol), the margin between the optimal and the toxic level of inclusion is very narrow, thus choose of the dosage could notably influence kind of *in vitro* effects.

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* $N\text{-}NH_3$ 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.

Effects of monensin on in vitro fermentation

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).

CONCLUSIONS

Results of the present study showed that the pure compounds influenced *in vitro* fermentation and CH_4 production in a dose-dependent manner. The high dosage of limonene and allyl-sulfide showed to reduce notably *in vitro* CH_4 production, but such positive effect was accompanied by an overall impairment of rumen fermentation, especially in the case of limonene. Most promising results were observed for cinnamaldehyde that, when used at the high dosage, reduced gas and CH_4 production, without compromising the rumen degradability and the VFA production. The high dosage of monensin determined the expected effects on rumen fermentation.

This study allowed to better elucidate effects of pure extracts on *in vitro* rumen fermentation. However, such results should be investigated and confirmed using dosages and long-term feeding that are proper of *in vivo* conditions.

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Table 1. Ingredient and chemical composition (g/kg DM) of the diet used for *in vitro* tests.

	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

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).

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 ($N\text{-}NH_3$) concentration of fermentation fluids (mg/L).

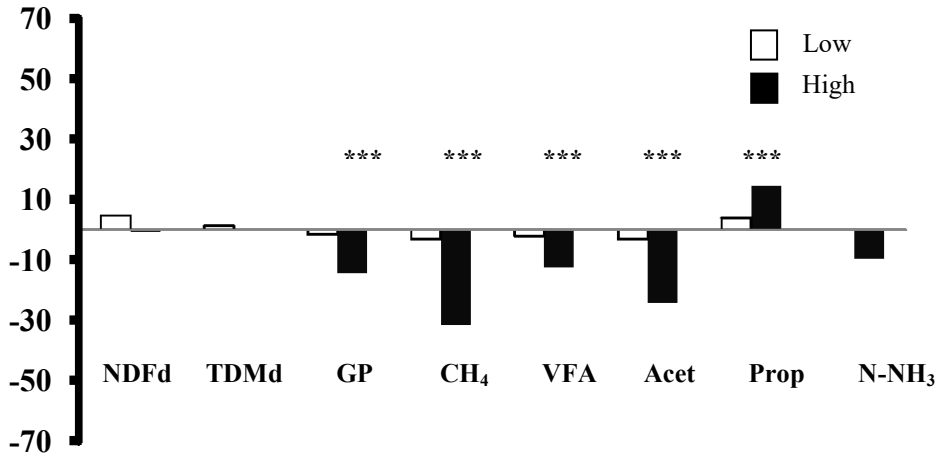


Figure 2. Percentage effect (increase or decrease of control values) of two dosages (low or high) of cinnamaldehyde on NDF (NDFd; g/kg NDF) and true dry matter degradability (TDMd; g/kg DM), GP (mL/g TDMd), CH_4 (mL/g TDMd) , VFA (mg/L), acetate (acet) and propionate (prop) proportions (both expressed as % on total VFA), and ammonia N ($N\text{-}NH_3$) concentration of fermentation fluids (mg/L).

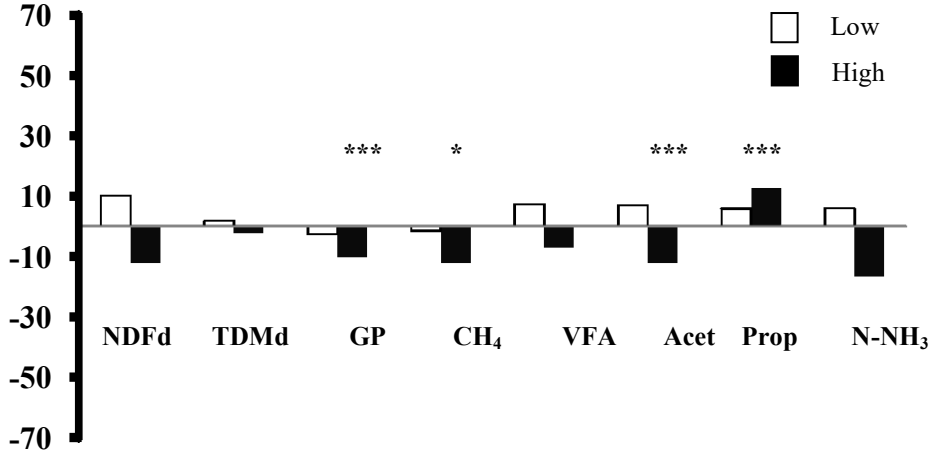


Figure 3. Percentage effect (increase or decrease of control values) of two dosages (low or high) of eugenol on NDF (NDFd; g/kg NDF) and true dry matter degradability (TDMd; g/kg DM), GP (mL/g TDMd), CH_4 (mL/g TDMd) , VFA (mg/L), acetate (acet) and propionate (prop) proportions (both expressed as %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

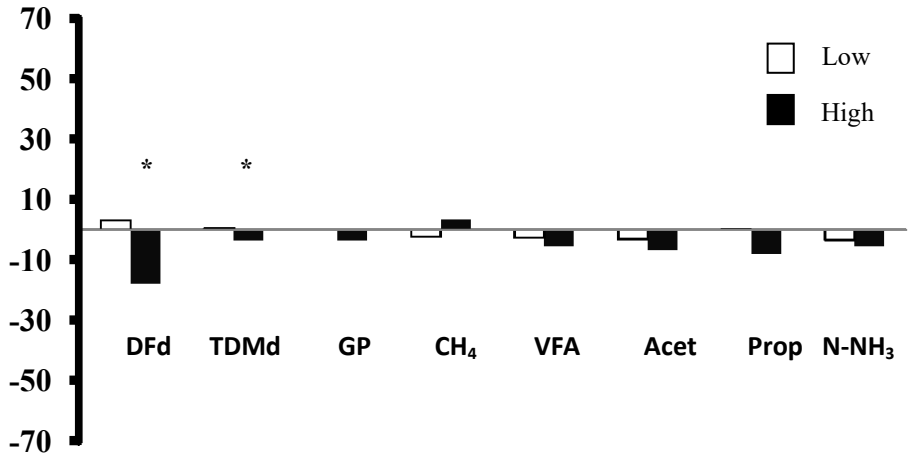


Figure 4. Percentage effect (increase or decrease of control values) of two dosages (low or high) of limonene on NDF (NDFd; g/kg NDF) and true dry matter degradability (TDMd; g/kg DM), GP (mL/g TDMd), CH_4 (mL/g TDMd) , VFA (mg/L), acetate (acet) and propionate (prop) proportions (both expressed as % on total VFA), and ammonia N ($N\text{-}NH_3$) concentration of fermentation fluids (mg/L).

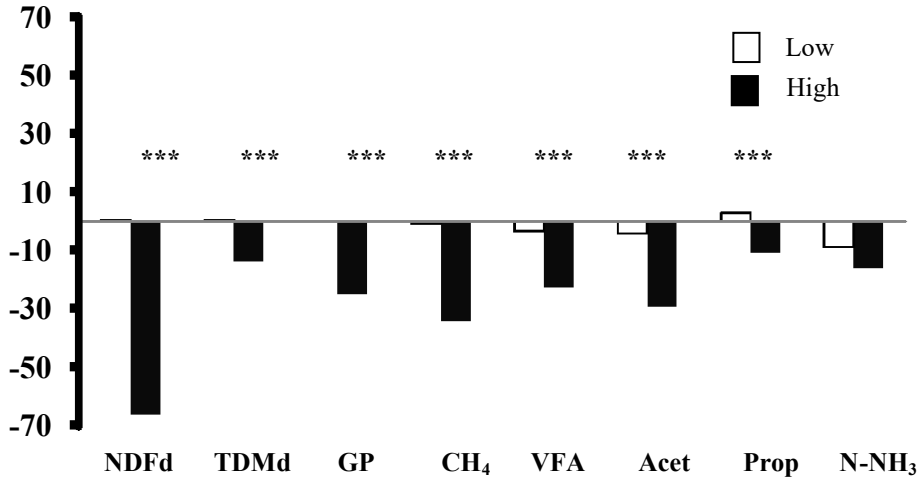
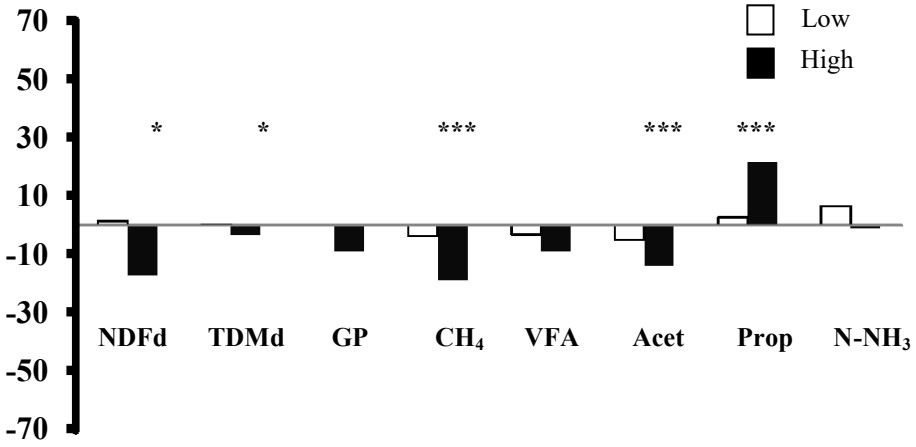


Figure 5. Percentage effect (increase or decrease of control values) of two dosages (low or high) of monensin on NDF (NDFd; g/kg NDF) and true dry matter degradability (TDMd; g/kg DM), GP (mL/g TDMd), CH_4 (mL/g TDMd) , VFA (mg/L), acetate (acet) and propionate (prop) proportions (both expressed as %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



Chapter 5

2nd Contribution

Effect of rumen fluid collected from cows exposed or not to pure compounds on the *in vitro* gas production kinetics, fermentations and methane production

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INTERPRETIVE SUMMARY

By Rossi *et al.*

The effect of rumen fluid, used as inoculum for *in vitro* short-term experiments, has been investigated. We adapted or not the rumen fluid of dry cows to three pure compounds and the collected rumen fluids were used to evaluate *in vitro* the activity of the pure compounds. This experiment highlight that pure compounds, actives in the short term on *in vitro* fermentations, maintain their activity when incubated with rumen fluid adapted to the some compounds for a long period. However, the administration of a pure compound to the cows can affect the rumen microbial activity and the response of *in vitro* experiments.

ABSTRACT

This study evaluates the effect of rumen fluid which has not been adapted (NAF) and rumen fluid which has been adapted (AF) to three pure compounds (cinnamaldehyde, limonene, allyl-sulfide) on *in vitro* gas production kinetics and end products of fermentation. According to a 4x4 Latin Square design, four dry cows were fed diets with and without 3 pure compounds and the collected rumen fluids (NAF and AF, respectively) were used as *in vitro* inocula with or without the addition of the 3 compounds. In general, the use of AF did not affect any fermentation parameter compared to NAF but, adding *in vitro* diet with allyl-sulfide, the magnitude of the effects of this pure compound tended to be greater when incubated with AF compared to NAF. These results suggest that the administration of pure compounds to the cows can affect the rumen microbial activity and the response of *in vitro* experiments.

Key words: rate of gas production, methane, compound, rumen fermentation, inoculum

INTRODUCTION

In recent years *in vitro* gas production (**GP**) technique has been largely employed to evaluate the effects of various compounds (i.e., plant compounds with antimicrobial properties) on rumen fermentation (Klevenhusen, Muro-Reyes, Khiaosa-ard, Metzler-Zebeli & Zebeli, 2012). In most

cases, experiments were of short duration (i.e., 24 h) and were conducted using rumen fluid which had not been exposed to the presence of the investigated compounds before. The incubation of a non-adapted rumen fluid for short times might generate misleading effects on the rumen microbial population and the tested compounds, giving the possibility of two types of error: i) the discarding of compounds that may have needed a longer time to be effective on rumen fermentation; ii) the acceptance of a compound that is effective in the short term but not in the long term (Calsamiglia, Busquet, Cardozo, Castillejos & Ferret, 2007). Another aspect concerns the use of short-term batch culture system as a tool to evaluate the fermentative properties of rumen fluid of cows receiving specific compounds. Commonly, the *in vitro* system is used to evaluate the fermentative properties of feed or additives incubated in a buffered rumen fluid with a standard diet. A different approach could be to feed the animals with the additives and to evaluate *in vitro* the fermentative properties of rumen fluid collected from these animals (adapted rumen fluid, **AF**). This approach could combine the advantage of *in vivo* trials (longer adaptation time and direct evaluation of effects *in vivo*) with those of *in vitro* techniques (low cost, standardization of conditions, evaluation of traits hardly measurable *in vivo,* such as the kinetics of fermentation, methane production, etc.).

Therefore, the aim of the present research is to study whether the rumen fluid adaptation to the presence of pure compounds might influence both the kinetics of feed fermentation *in vitro* and the response of the same compounds incubated *in vitro*.

MATERIAL AND METHODS

All experimental procedures involving animals were approved by the Ethical Committee for the Care and Use of Experimental Animals of the University of Padua. Four Holstein-Friesian cows were housed in single pens at the experimental farm of the University of Padova (Legnaro, Italy).

Animal, diets and experimental design

One week before the beginning of the experiment, cows were randomly assigned to 4 experimental groups in a 4 × 4 Latin Square design. Each cow received the TMR without any

supplementation, or supplemented with 1 g d^{-1} of three pure compounds with known effects on rumen fermentation (Calsamiglia, Busquet, Cardozo, Castillejos & Ferret, 2007): cinnamaldehyde (SIGMA-ALDRICH Corporation, Milan, Italy; W228613; Purity ≥95%; **CIN**), limonene (SIGMA-ALDRICH Corporation, Milan, Italy; 183164; Purity 97%; **LIM**), and allyl-sulfide (SIGMA-ALDRICH Corporation, Milan, Italy; A35801; Purity 97%; **ALL**). The TMR was based on wheat silage and corn silage, 355 and 257 g kg^{-1} DM respectively (Crude protein, 154 g kg^{-1} DM; Lipids, 33 g kg^{-1} DM; NDF, 378 g kg^{-1} DM; Starch, 238 g kg^{-1} DM). The amount of each pure compound supplemented (1 g d^{-1}) was chosen according to the dosage commonly used in dairy cow feeding (Benchaar, Petit, Berthiaume, Whyte & Chouinard, 2006) and was chosen in an attempt to evaluate a reduction of methane production while avoiding a depression of microbial growth and activity. The amount of TMR fed to each cow was computed according to nutrient recommendations for cows (NRC, 2001), and resulted 14.1, 16.3, 16.9, and 14.6 kg DM, respectively, for the four cows. A portion of the TMR (about 10 kg of feed), supplemented or not with the compound, was distributed in the morning at 8 a.m. The remaining part of the TMR, without supplementation for any of the cows, was fed in the early afternoon at 1 p.m., after the complete consumption of the first aliquot, to be sure that the three supplemented cows had consumed the whole dose of compound. In order to have a homogenous distribution throughout the TMR, the three compounds were diluted in 150 mL of water and homogeneously sprayed over the TMR in the manger of each cow. Each experimental period, during which the cows received the TMR with the supplementation lasted 14 d; followed by a transition period of 7 d during which the four cows received the TMR without supplementation.

Rumen fluid collection and incubation procedures

On the first day of each transition period, rumen fluids to be used for *in vitro* tests were collected from each cow, before morning feeding, using an esophageal probe (Tagliapietra et al., 2012). The four rumen fluids were separately stored into 4 thermal flasks preheated to 39 ± 0.5°C, transferred to the laboratory, and filtered through 3 layers of cheesecloth, to eliminate residual feed particles.

Before starting incubation, a solution was prepared for each compound, containing 25 mL of 96% ethanol (v/v) plus 750 mg of CIN, LIM, or ALL. *In vitro* tests (4 incubations in total) were conducted on the same days as the rumen fluid collection. A commercial, fully automated wireless GP apparatus (Ankom^{RF} GP System, Ankom Technology®, NY, USA) was used, consisting of 44 bottles equipped with pressure sensors (pressure range: from −69 to +3447 kPa; resolution: 0.27 kPa; accuracy: ±0.1% of measured value) and a wireless connection to a computer (Tagliapietra, Cattani, Bailoni & Schiavon, 2010). In each incubation, ten treatments were analysed: the non-adapted fluid (**NAF**), collected from the non-supplemented cow was incubated alone or added *in vitro* with 30 mg of CIN (NAF+CIN), LIM (NAF+LIM), or ALL (NAF+ALL); the adapted fluids (**AF**), collected from the cows supplemented with CIN, LIM, or ALL, were incubated alone (AF_{CIN}, AF_{LIM}, and AF_{ALL}) or added *in vitro* with CIN (AF_{CIN}+CIN), LIM (AF_{LIM}+LIM), or ALL (AF_{ALL}+ALL). Each of the 10 treatments was incubated in 4 replications. Four bottles were included as blanks, containing only the buffer solution and rumen fluid collected from each cow (1 blank/rumen fluid), giving a total of 44 bottles tested in each incubation. Each bottle (317 mL) was filled with 1.000±0.0010 g of diet (the same TMR fed to the cows, ground to 1 mm), 50 mL of rumen fluid, 100 mL of buffer solution (Menke and Steingass, 1988), and 1 mL of the ethanol-compound solution, to achieve the concentration of 30 mg g^{-1} DM of diet. To standardize fermentation conditions, 1 mL of ethanol was also added to the blank bottles. Such a concentration of ethanol (0.7% v/v) was assumed not to influence microbial growth and activity (Chaves, Fraser, Beauchemin, & McAllister, 2007). Bottles were placed in a ventilated oven at 39±0.4°C and automatically vented at a fixed pressure (6.8 kPa). Vented gas was collected into airtight plastic bags connected to each bottle. During the incubation the total gas production was recorded (**GP24**), and the gas production rates from 0-3 hours (**GPR 0-3h**) and 3-6 hours (**GPR 3-6h**), were computed. At the end of the incubation, gas samples were collected from bottle headspaces and from the airtight plastic bags and analysed for methane (**CH4**) concentration (Cattani et al., 2014). Fermentation fluids were filtered into weighed crucibles (30 mL, Robu Glasfilter-Geräte GMBH®,

Hattert, Germany) and analysed for residual NDF using a fibre analyser (FIWE 6; VELP® Scientifica, Milan, Italy). Degradability of NDF (**NDFd**) and of true DM (**TDMd**) were computed according to Goering and Van Soest (1970). The TMR was analysed in three replicates for proximate composition (AOAC, 2012). Neutral detergent fibre (NDF), inclusive of residual ash, was determined with α-amylase using the Ankom^{220} Fibre Analyser (Ankom Technology, Macedon, NY, USA).

Statistical analysis

All data (mean of 4 replications) were analysed by PROC MIXED of SAS Institute (2007) using a model that included the treatment (n=10) as a fixed factor, the period (n=4) and the interaction period×cow (n=16) as random factors. The Bonferroni adjustment was used for multiple comparison of the least square means of the various treatments. For each compound, contrasts were run to evaluate the effect on *in vitro* fermentations: i) of the addition of a pure compound to the non-adapted rumen fluid ("NAF vs NAF+pure compound"); ii) of two types of rumen fluid (non-adapted vs adapted rumen fluid, "NAF vs AF"); iii) of the addition of a pure extract to the adapted rumen fluid ("AF vs AF+pure compound").

RESULTS

The addition *in vitro* of cinnamaldehyde and also the use of adapted or non-adapted rumen fluid did not produce any effect (P>0.05) on rumen fermentation parameters (Table 1). Notwithstanding the total GP after 24 h of incubation (**GP24**) was not affected by the treatments, a reduction of the GP rate during the first 3 h of incubation was observed when CIN was added *in vitro* both to NAF (NAF vs. NAF+CIN; P = 0.003) and to AF (AF_{CIN} vs. AF_{CIN}+CIN; P = 0.002 and, the peak of GP moved from 3 h to 9 h after the beginning of the incubation when the additive was added *in vitro* to both the tested rumen fluid (NAF and AF) (Fig 1.a).

When limonene was added *in vitro* to NAF, values of NDFd, GP24, and **CH4** production were reduced, respectively, by 31% (P < 0.001), 20% (P < 0.001), and 31% (P < 0.001). Similar results

were obtained when limonene was added to AF_{LIM}. Also the proportions of the main VFAs and the proportion of methane on total GP24 (**CH4** mL/100mL GP24) were changed ($P < 0.01$) and, as shown in fig 1.b, the kinetics of GP exhibited a similar behaviour to CIN but even stronger with a clear differentiation of NAF+LIM and AF_{LIM}+LIM from NAF and AF, respectively ($P < 0.001$).

The addition of allyl-sulfide to the non-adapted fluid caused a shift from acetate (-28%) to propionate (+12%) and butyrate (+16%), and a decrease of **CH4** production (-34%; $P < 0.001$) and **CH4** proportion (-28%; $P < 0.001$), whereas it had a weak effect on NDFd (-6%; $P = 0.09$) and on GP24 (-8%; $P = 0.04$). The addition of the allyl-sulfide to the adapted fluid showed the same but stronger effect on all the above mentioned parameters (i.e. on GP24: -15%; $P < 0.001$) and also changed the rate of gas production (Fig 1.c) during the first 3 h of incubation (-20%; $P < 0.001$).

Generally, for all the additives, the use of AF instead of NAF as inoculum of in vitro fermentation (NAF vs AF) did not change the fermentative parameters.

DISCUSSION

The dual-flow continuous fermenter is used to simulate the rumen environment by incubating the rumen fluid with the tested compounds for a given period of time (i.e., 10 d), in order to evaluate the evolution over time the effects the compounds have on fermentation and to highlight any ability of rumen microorganisms to adapt to the presence of the compounds (Cardozo, Calsamiglia, Ferret & Kamel. 2004; Busquet, Calsamiglia, Ferret, Cardozo, & Kamel, 2005; Castillejos, Calsamiglia, Ferret & Losa, 2007). Differently from the above mentioned studies, the logic of the experimental design used in this trial was to test the effect of pure compounds, feeding the cows with the compound and using the adapted or non-adapted rumen fluid as inoculum of *in vitro* incubations. The hypothesis of the experiment was that rumen fluid activity, when used as inoculum for *in vitro* incubations, can be affected by the administration to the cows of the additive. Therefore, the effects of the compound were tested in two ways: using the conventional procedure of *in vitro* incubation (NAF vs NAF+pure compound), and using the rumen fluid adapted or non-adapted to the extracts

(NAF vs AF) as inoculum of incubation. Moreover, the effects of the additive *in vitro* using the adapted fluid were tested (AF vs AF+pure compound). Only the study of Mlambo et al. (2007) used this methodology to compare the effects of adapted or non-adapted fluid on fermentations but they did not used a Latin square design model that evaluates the effect of the rumen fluid donor on *in vitro* parameters.

Compound effect (NAF vs NAF+compound)

Results of this study showed that three tested compounds developed differentiated effects on rumen fermentations when incubated with the conventional non-adapted fluid, in agreement with other experiments (Crane et al., 1957; Kamel et al., 2009; Cattani et al., 2016). These studies observed that similar doses of limonene and garlic compounds were found to reduce *in vitro* GP, and **CH4** production and, as observed in previous research (Cattani et al., 2016), allyl-sulfide appears as an interesting additive to manipulate the rumen fermentation, changing the VFA profile, decreasing the acetate/propionate ratio and decreasing the methane production without evident inhibitory effects on fibre degradability. Such depression of rumen fermentation could be due to the incubation time used (24 h), that was probably too short to allow an adaptation of rumen population which, as a consequence, was sensitive to *in vitro* addition of limonene and allyl-sulfide. This hypothesis seems to be supported by some authors (Cardozo, Calsamiglia, Ferret & Kamel. 2004; Molero, Ibars, Calsamiglia, Ferret, & Losa, 2004; Castillejos et al., 2007), who observed that rumen bacteria are able to build up a tolerance mechanism *in vitro* as well, but this process requires more than 24 h of incubation. In the present study, *in vitro* addition of cinnamaldehyde to the non-adapted fluid produce only weak effects on fermentation, reducing the rate of gas production but without effects on the overall gas and methane production and feed degradation. Recently, Cattani et al. (2016) observed the same effects on *in vitro* degradability and GP but, in that case, a reduction of **CH4** and a change in VFA production were also found. Results provided by the current literature highlight that effects of cinnamaldehyde on rumen fermentation are quite controversial. However, major effects seem to be related to the use of doses greater than those used *in vitro* (200 mg/L of

fermentation fluid) in the present study (Calsamiglia, Busquet, Cardozo, Castillejos & Ferret, 2007).

Rumen fluid type (NAF vs AF)

Regardless of the tested compounds fed to the cows, the fermentations were not affected by the use, as *in vitro* inoculum, of adapted or non-adapted rumen fluid. The lack of effect of rumen fluid type on *in vitro* fermentation could be due to the low dosage of additive fed to the cows (1g d^{-1}). In any case, this amount was in line with the dosage commonly used in dairy cow diets (Benchaar, Petit, Berthiaume, Whyte & Chouinard, 2006) and was chosen in an attempt to evaluate a reduction of methane production while avoiding a depression of microbial growth and activity. Moreover, it cannot be excluded that the microbial population could have developed a mechanism of tolerance to the compounds during the period of *in vivo* administration, thus microorganisms may be able to alleviate biological activity of the same compounds. Development of a tolerance mechanism could be plausible as the feeding period of compounds to the cows (14 d) was probably sufficient to allow an adaptation of rumen bacteria, considering that they usually require a time of about 10 d to get accustomed to new components introduced in the ration (Warner, 1962).

Compound effect on adapted rumen fluid (AF vs AF+pure* compound*)

For cinnamaldehyde and limonene, the pattern of fermentation traits were comparable to those previously described when the compounds were added to the NAF. In this case the two rumen fluids, adapted and non-adapted, not only gave comparable fermentative patterns but also showed similar activity when the compound was added *in vitro* and the administration *in vivo* of the compound seems to have no effect on fermentations *in vitro,* at least under present conditions. Differently, the magnitude of the *in vitro* effects of allyl-sulfide tended to be greater when incubated with adapted compared to non-adapted rumen fluid. For instance, the use of this additive with NAF reduces the rate of gas production at the beginning of the fermentation by 7% while with AF the reduction was as much as 15%. Similarly, the use of allyl-sulfide on NAF did not change the peak of gas production, while the use of this additive on AF postponed the peak from 3 h to 6 h of incubation. Therefore, the effect of allyl-sulfide on fermentations was amplified when it was

incubated with rumen fluid of cows receiving the same additive (Fig 2). Under present experiment conditions, these observations support the hypothesis that the use of rumen fluid adapted or non-adapted to a pure compound can influence the *in vitro* fermentation. In a previous experiment, Mlambo et al. 2007 did not observe an adaptation of rumen fluid to the presence *in vitro* of the same compound. In fact, these Authors explored the effect on *in vitro* fermentation of rumen fluid collected from donors fed or not with a pure compound combined with the use of the same compound *in vitro*, and these effects appeared to show an additive pattern.

CONCLUSIONS

This experiment highlights that pure compounds, active in the short term on *in vitro* fermentations, did not change the rumen fluid *in vitro* activity when fed or not to cows for a long period. It is clear that effectiveness of a given compound is real when it has a long-term effect on rumen fermentation. On this basis, *in vitro* experiments should be conducted using rumen fluids that were adapted for a given period to the presence of tested compounds, to highlight possible mechanisms of defence built up by microbial population against the same compounds.

The prospective of using the *in vitro* batch culture technique to test the effects of specific compounds on rumen fermentation using the rumen fluid collected from adapted and non-adapted cows requires further investigation.

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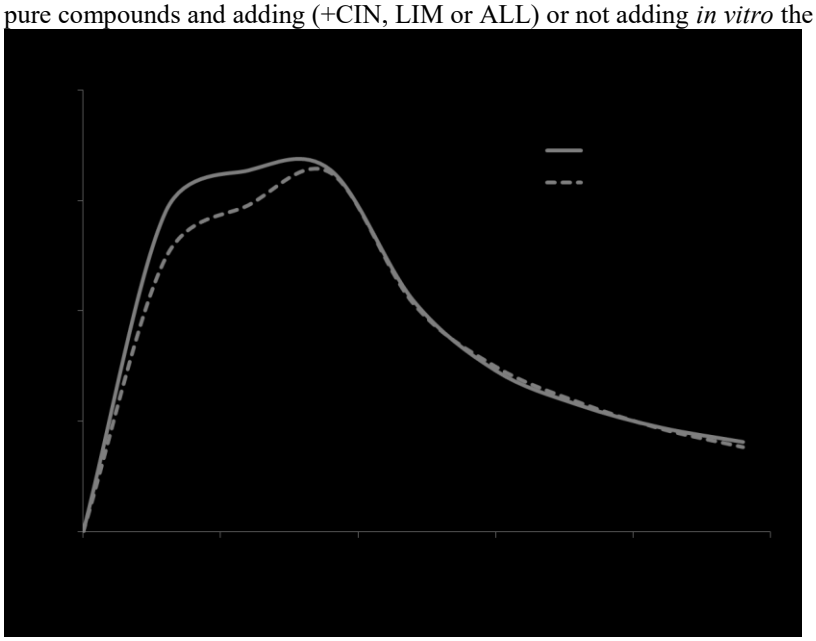
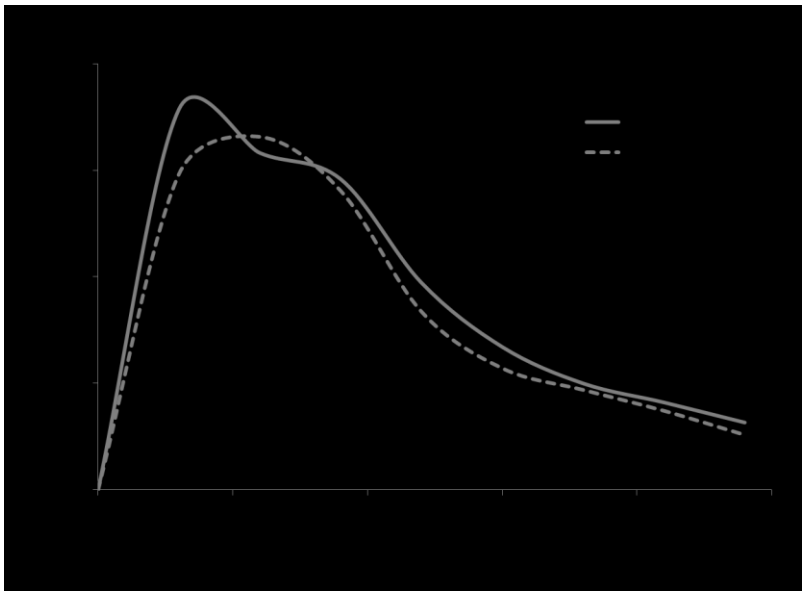
Table 1 *In vitro* degradability of NDF (NDFd) and true DM (TDMd), gas production rate from 0-3 hours (GPR 0-3h) and 3-6 hours (GPR 3-6h), total gas production after 24 h (GP 24h), VFAs, CH4 production and concentration at 24 h of incubation as affected by the type of rumen fluid (non-adapted rumen fluid NAF; adapted rumen fluid, AF) and by the addition *in vivo* and/or *in vitro* of cinnamaldehyde (CIN), limonene (LIM), and allyl-sulfide(ALL).

Rumen fluid	NDFd	TDMd	GPR 0-3h	GPR 0-6h	GP 24h	Acetate	Propionate	Butyrate	CH4	
	g kg^{-1} NDF	g kg DM	mL h^{-1} g^{-1} DM	mL h^{-1} g^{-1} DM	mL g^{-1} DM	% VFA	% VFA	% VFA	mL g^{-1} DM	mL 100 mL^{-1} gas
Not adapted fluid (NAF)	476	787	18.4	17.3	287	57.2	19.1	16.3	50.4	15.4
Cinnamaldehyde (CIN)										
NAF+CIN[1]	452	776	14.5	16.4	274	56.9	18.8	16.7	47.3	15.0
AF_{CIN}[2]	461	778	16.0	15.9	259	57.9	19.8	15.2	43.4	14.5
AF_{CIN}+CIN[3]	435	768	12.4	14.7	260	57.1	20.4	15.3	41.0	13.7
Limonene (LIM)										
NAF+LIM[4]	327	725	11.7	14.6	230	54.7	21.2	17.3	34.8	12.7
AF_{LIM}[5]	465	781	16.7	16.7	266	58.3	20.1	14.7	43.2	14.1
AF_{LIM}+LIM[6]	313	718	9.5	12.6	196	55.6	21.1	16.7	30.1	11.8
Allyl-sulfide(ALL)										
NAF+ALL[7]	446	775	17.7	15.9	265	51.1	21.5	19.0	33.4	11.1
AF_{ALL}[8]	466	781	18.3	14.5	280	57.5	20.1	15.0	52.0	16.4
AF_{ALL}+ALL[9]	464	780	14.6	16.5	243	51.9	22.4	17.8	28.3	10.2
SEM	2.1	0.8	2.57	0.80	14.3	0.72	0.98	0.67	4.37	1.03
P value										
Treatment	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001
Contrasts										
Cinnamaldehyde										
NAF vs. NAF+CIN	0.15	0.15	0.003	0.08	0.09	0.62	0.74	0.33	0.40	0.70
NAF vs. AF_{CIN}	0.59	0.59	0.51	0.20	0.17	0.51	0.60	0.27	0.25	0.52
AF_{CIN} vs AF_{CIN}+CIN	0.15	0.15	0.002	0.05	0.94	0.23	0.48	0.85	0.55	0.46
Limonene										
NAF vs. NAF+LIM	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.004	0.05	<0.001	0.01
NAF vs. AF_{LIM}	0.70	0.70	0.64	0.60	0.30	0.28	0.48	0.09	0.24	0.39
AF_{LIM} vs AF_{LIM}+LIM	<0.001	<0.001	<0.001	<0.001	<0.001	<0.001	0.19	<0.001	<0.001	0.02
Allyl-sulfide										
NAF vs. NAF+ALL	0.09	0.09	0.55	0.01	0.04	<0.001	0.001	<0.001	<0.001	<0.001
NAF vs. AF_{ALL}	0.73	0.73	0.99	0.84	0.74	0.72	0.46	0.19	0.79	0.48
AF_{ALL} vs AF_{ALL}+ALL	0.89	0.89	0.001	0.09	<0.001	<0.001	0.004	<0.001	<0.001	<0.001

[1] non-adapted fluid added *in vitro* with 30 mg of cinnamaldehyde; [2] fluid adapted *in vivo* to cinnamaldehyde; [3] fluid adapted *in vivo* to cinnamaldehyde and added *in vitro* with 30 mg of cinnamaldehyde; [4] non-dapted fluid added *in vitro* with 30 mg of limonene; [5] fluid adapted *in vivo* to limonene; [6] fluid adapted *in vivo* to limonene and added *in vitro* with 30 mg of limonene; [7] non-adapted fluid added *in vitro* with 30 mg of allyl-sulfide; [8] fluid adapted *in vivo* to allyl-sulfide; [9] fluid adapted *in vivo* to allyl-sulfide and added *in vitro* with 30 mg of allyl-sulfide;

Fig 1 Effect of the pure compounds (A. cinnamaldehyde, CIN; B. limonene, LIM; C. allyl-sulfide, ALL) on *in vitro* kinetics of gas production rate (mL h^{-1} g^{-1} DM): each graph shown, the kinetics obtained incubating the basal diet with rumen fluid as inoculum of fermentation collected from cows not fed (non-adapted, NAF) or fed (adapted, AF) with the pure compounds and adding (+CIN, LIM or ALL) or not adding *in vitro* the pure compound.





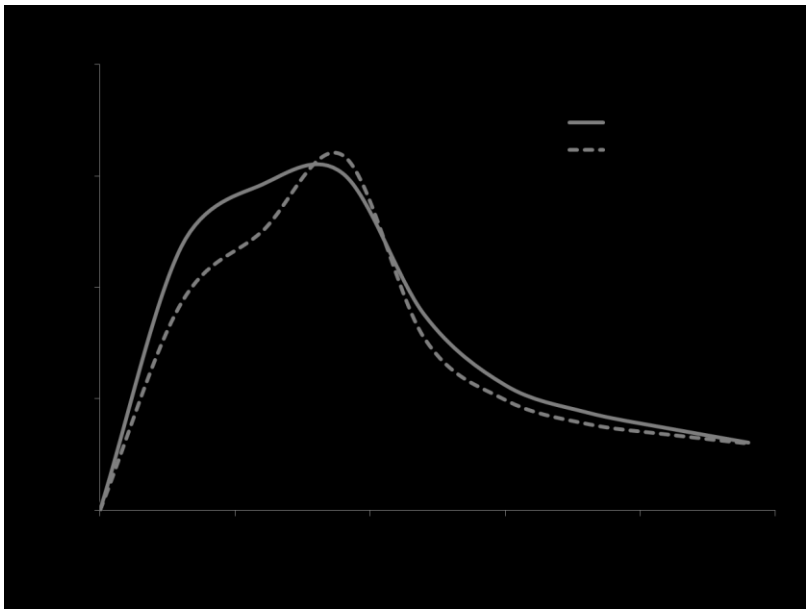
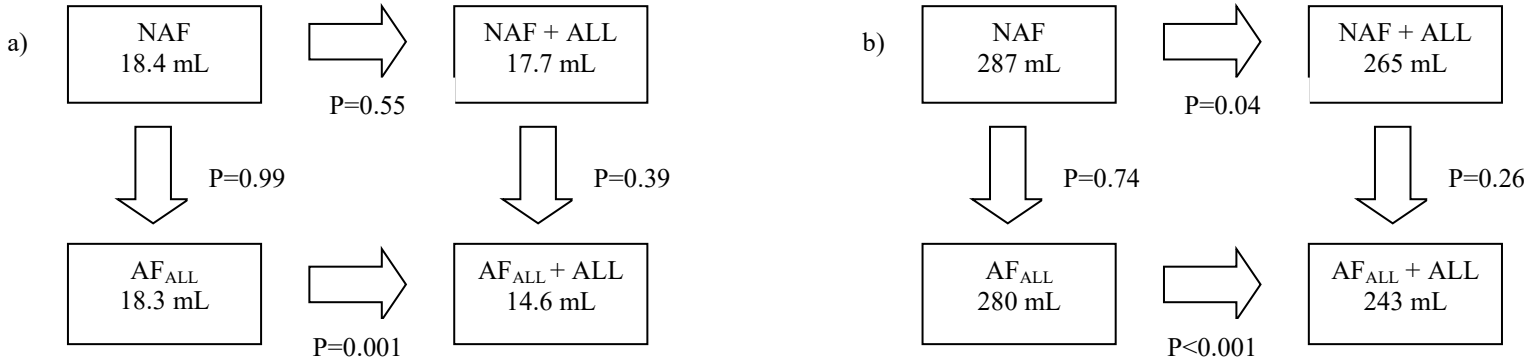


Fig 2. Effect of rumen fluid used as inoculum of fermentation collected from cow fed without (not adapted rumen fluid, NAF) and with allyl-sulfide (adapted rumen fluid, AF_{ALL}) and incubated adding (+ ALL) or not adding also *in vitro* the allyl-sulfide on (a) GP at 3 h (mL h^{-1} g^{-1} DM) and on (b) cumulated GP at 24 h (mL g^{-1} DM).



Chapter 6

3rd Contribution

Effect of an isoflavonid-rich liquorice extract on rumen fermentation, methanogenesis and the microbiome in the Rumen Simulation Technique

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Under minor revision to FEMS microbiology ecology

ABSTRACT

Due to the antimicrobial activity of flavonoids it has been suggested that they may provide a possible alternative to antibiotics to stimulate productivity and reduce the environmental load of ruminant agriculture. We hypothesised that an extract of liquorice, rich in prenylated isoflavonoids and particularly glabridin, might potentially improve the efficiency of nitrogen utilization and reduce methane production in the rumen. When added to a long-term rumen simulating fermentor (RUSITEC) liquorice extract at 1 g L^{-1} decreased ammonia production (-51%; $P<0.001$) without affecting the overall fermentation process. When added at 2 g L^{-1}, decreases in not only ammonia production (-77%; $P<0.001$) but also methane (-27%; $P=0.039$) and total VFA production (-15%; $P=0.003$) were observed. These effects in fermentation were probably related to a decrease in protozoa numbers, a less diverse bacteria population as well as changes in the structure of both the bacterial and archaeal communities. The inclusion of an isoflavonoid-rich extract from liquorice in the diet may potentially improve the efficiency of the feed utilization by ruminants.

Keywords: glabridin, isoflavonoids, liquorice, methane, rumen fermentation

INTRODUCTION

Since the ban of antibiotics as growth promoting feed additives by the European Union in 2006, plant extracts and plant secondary metabolites have been considered as alternatives to manipulate rumen fermentation to boost productivity and decrease the environmental burden of livestock production (Hart et al., 2008). Among plant secondary metabolites, flavonoids have recently gained interest because of their wide range of biological activities, particularly antimicrobial properties (Oskoueian et al. 2013).

Flavonoids are polyphenolic compounds consisting of a fifteen-carbon skeleton in which two benzene rings are linked via a heterocyclic pyran ring (Kumar et al., 2013). According to substitution pattern variations, flavonoids can be classified into different

subclasses, providing an extremely diverse range of derivatives (Wang et al., 2017). Depending on their chemical structure, flavonoids can then have different antimicrobial effects (Wang et al., 2017) which ultimately determine the extent in which rumen fermentation can be altered (Oskoueian et al., 2013).

The effect of flavonoids on rumen fermentation has not been extensively evaluated (Patra et al., 2017). In addition, and taking into account their great variability in structure (over 9,000 different compounds identified; Wang et al., 2017), only a small number of flavonoid-rich plant extracts or pure compounds have been tested so far. Some flavonoids, or the derivatives produced by microbial degradation in the rumen, have been reported to affect rumen microbial activity causing, amongst other effects, a decrease in methane production (Oskoueian et al., 2013; Kim et al., 2015; Ma et al., 2017). Furthermore, flavonoids have been shown to be effective in attenuating the effects of excessive grain feeding on rumen pH (Balcells et al., 2012; Nardi et al., 2014). However, to our knowledge, a detailed characterization of the changes in rumen microbial communities associated with the effects of flavonoids on rumen fermentation has not yet been published. In this study, we tested an extract of liquorice, rich in prenylated isoflavonoids and particularly glabridin (Asl and Hosseinzadeh, 2008), for its effect in *in vitro* batch culture and its long-term effect on rumen fermentation and methanogenesis whilst also characterising its effect on bacterial and methanogen communities.

MATERIAL AND METHODS

Liquorice extract

Liquorice extract was obtained from the dried roots of liquorice (*Glycyrrhiza glabra*; 40 g) after extraction with 95% ethanol (2 L for 2 h) at 45^{o}C and then concentrating under reduced pressure to give a 95% ethanol extract (1.20 g). The extract was further purified on silica gel eluted with ethyl acetate–methanol gradients (19:1; 9:1; 2:1) and finally with methanol.

Nuclear magnetic resonance (NMR) analysis revealed that glabridin was the major flavonoid in the extract, with five other related flavonoids found. Fitness R Us Ltd (Kiryat Shmona, Israel) provided the extract as Licogen powder (Batch No: 14090023PWDR). Liquorice powder is sold as a natural antioxidant, flavouring powder and phytoestrogen for menopausal women.

Measurement of protozoal activity

The effect of liquorice extract on protozoal activity was measured *in vitro* as the breakdown of [^{14}C]-labelled bacteria by rumen protozoa as described by Wallace and McPherson (1987). Isotope-labelled bacteria were obtained by growing *Streptococcus bovis* ES1 in Wallace and McPherson media (Wallace and McPherson, 1987) containing [^{14}C] leucine (1.89 μCi/7.5 mL tube) as the sole nitrogen source, for 24 h. Cultures were centrifuged (3,000 g, 15 min), supernatant discarded and pellets re-suspended in 7 mL of simplex type salt solution (STS; Williams and Coleman, 1992) containing ^{12}C-leucine (5 mM). This process was repeated three times to prevent re-incorporation of released [^{14}C] leucine by bacteria.

Rumen digesta was obtained from four rumen-cannulated Holstein-Frisian cows fed at maintenance level (composed of perennial ryegrass hay and concentrate at 67:33 on a DM basis). Animal procedures were carried out in accordance with the Animal Scientific Procedures Act 1986 and protocols were approved by the Aberystwyth University Ethical Committee. Rumen digesta was obtained before the morning feeding and strained through two layers of muslin and diluted with STS (1:1) containing ^{12}C-leucine (5 mM). Diluted rumen fluid (7.5 mL) was then incubated with labelled bacteria prepared as described above (0.5 mL) in tubes containing no additive (control) or 0.25, 0.5, 1 or 2 g L^{-1} of liquorice extract. Incubations were carried out at 39ºC under a stream of CO_2 and tubes were sampled at time 0 and at 1 h intervals up to 5 h using a syringe with a 19 gauge needle. Samples (0.5 mL) were acidified (by adding 0.125 mL of 25% trichloroacetic acid (wt/vol) and centrifuged

(13,000 *g*, 5 min). Supernatant (0.200 mL), was diluted with 2 mL of OptiPhase HiSafe 2 scintillation fluid (Perkin Elmer, Seer Green, UK) to determine the radioactivity released by liquid-scintillation spectrometry (Hidex 300 SL, Lablogic Systems Ltd, Broomhill, UK). Bacterial breakdown at each incubation time was expressed as the percentage of the acid-soluble radioactivity released relative to the total radioactivity present in the initial labelled bacteria (Wallace and McPherson, 1987).

In vitro batch cultures

To measure the short term effect of liquorice extract on fermentation parameters, 24 h *in vitro* incubations were carried out. The experimental design consisted of a control (no additive) and liquorice extract added at 0.5, 1 or 2 g L^{-1}. The experiment was conducted in quadruplicate, using rumen fluid from the same four cannulated cows. Rumen contents were sampled before the morning feeding, filtered through a double layer of muslin and diluted 1:2 in artificial saliva solution (Menke and Steingass, 1988). Aliquots (30 mL) of the diluted strained rumen fluid were added anaerobically to 120 mL Wheaton bottles containing 0.3 g of diet composed of ryegrass hay and barley (40:60), previously ground to pass through a 1-mm^2 mesh screen. Bottles were sealed and incubated at 39 ºC receiving a gentle mix before sampling at 24 h.

Fermentation pattern, in terms of pH, ammonia and VFA was determined after 24 h of the incubation. A subsample (4 mL) was diluted with 1 mL of deproteinising solution (200 mL L^{-1} orthophosphoric acid containing 20 mmol L^{-1} of 2-ethylbutyric acid as an internal standard) for the determination of VFA using gas chromatography, as described by Stewart and Duncan (1985). Another subsample (1 mL) was diluted with 0.250 mL of 25% trichloroacetic acid (wt/vol) for analysis of ammonia using a colorimetric method (Weatherburn, 1967).

Rumen Simulation Technique

The Rumen Simulation Technique (RUSITEC; Czerkawski and Breckenridge 1977) was used to study the effect of a control diet alone or supplemented with liquorice extract at 1 g L^{-1} (liquorice 1; 0.66 g d^{-1}, 3.3% inclusion rate in DM) or 2 g L^{-1} (liquorice 2; 1.32 g d^{-1}, 6.6% inclusion rate in DM), doses that were selected based on the results obtained in the 24 h batch culture trial described above. The experimental diet was the same one used in the batch culture trial (40:60, ryegrass hay and barley grounded to pass through 1 mm^2 sieve size).

Rumen digesta was obtained from four rumen-cannulated Aberdale x Texel sheep, fed at maintenance level (diet composed of perennial ryegrass hay and concentrate at 67:33 on DM basis). Animal procedures were carried out in accordance with the Animal Scientific Procedures Act 1986 and protocols were approved by the Aberystwyth University Ethical Committee. Rumen digesta was obtained before the morning feeding, strained through two layers of muslin and stored anaerobically at 39^{o}C.

The trial consisted of a single incubation period using 12 vessels which were considered as experimental units. Each dietary treatment was randomly allocated to the vessels which were inoculated with rumen fluid from four different sheep (four replicates). Vessels had an effective volume of 800 mL and were kept at 39^{o}C under permanent vertical agitation.

On day 1, vessels were inoculated with strained rumen fluid mixed with artificial saliva (McDougall, 1948) and demineralized water in a 1:1:1 ratio. Then artificial saliva was continuously infused at a rate of 660 mL d^{-1} (dilution rate of 3%/h) using a multichannel peristaltic pump (Watson–Marlow 200 series, Cornwall, UK). Squeezed rumen solids (20 g FM) were placed in nylon bags (110 × 60 mm, pore size 100 μm^2) and incubated in each vessel for 1 day to provide solid-associated bacteria, while experimental feed (20 g DM) was supplied in a second bag. On subsequent days, the feed bag that had remained 2 days in each

vessel was squeezed, returning the liquid to the vessel, and discarded; a new bag, containing 20 g DM was then inserted to the vessel.

The trial lasted for 18 days, using the first 12 days for adaptation and the last 6 for sampling. Dry matter degradation, total gas and methane production and outflow of fermentation products were measured on days 13, 14, 15 and 16. Nylon bags were collected, rinsed with cold water for 20 min, and DM disappearance after 48 h incubation was calculated from the loss in weight. The residue was then analysed for organic matter (OM), nitrogen (N), Neutral-detergent (NDF) and Acid-detergent fibre (ADF) to determine nutrient disappearance. 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 methane production. Daily production of ammonia and VFA were measured in the overflow flasks with 10 mL of saturated $HgCl_2$ (diluted 1:5) added to stop the fermentation.

To describe diurnal changes in the fermentation pattern, on days 17 and 18 the content of the vessels was sampled (25 mL) by aspiration at 0, 2, 4 and 8 h after feeding. The pH was immediately recorded, and five subsamples were collected as follows: for microbial characterization and enzymatic activity, 16 mL were collected and immediately frozen in liquid N prior to long term storage at -80^{o}C. For VFA determination, 1.6 mL of sample was diluted with 0.4 mL of deproteinising solution (200 mL L^{-1} orthophosphoric acid containing 20 mmol L^{-1} of 2-ethylbutyric acid as an internal standard). For ammonia analysis, 0.8 mL of sample was diluted with 0.2 mL of trichloro-acetate (25% wt:vol). For lactate determination, 1 mL sample was collected and snapped frozen in liquid N prior to long term storage at -80^{o}C. For protozoa counts, 0.5 mL of sample were added to 0.5 mL of saline formaline solution (4% formaldehyde and 0.9% NaCl in distilled water) and stored at room tempreture.

Sample analyses

For feed analysis, DM and OM content were determined by drying in an oven at 105ºC for 24 h and heating at 550ºC for 6 h in a muffle furnace, respectively. Nitrogen concentration was measured by the Dumas combustion method (Elementar analyser, Vario MAX cube, Hanau, Germany). For NDF and ADF determination, the Automated Fiber Analyzer (ANKOM 2000, Macedon, USA) was used. Methane concentration was determined by directly injecting 0.5 mL of gas sample into a gas chromatograph (ATI Unicam 610 Series, Cambridge, UK) fitted with a 40 cm Porapak N metal packed column (Agilent, Cheshire, UK) and flame ionization detector. Ammonia and VFA concentrations in vessels and overflows were determined as described by Weatherburn (1967) using an automated spectrophotometer (ChemWell T,Astoria Pacific, Oregon, USA) and Stewart and Duncan (1985) using gas chromatography, respectively. Protozoa were quantified by optical microscope following the procedure described by Dehority (1993) and adapted by de la Fuente et al. (2006). Concentrations of L-lactate and D-Lactate were measured using the Enzytec D/L-Lactic Acid kit (r-biopharm, Darmstadt, Germany); total lactate was calculated as the sum of both. Enzymatic activities in vessels content were measured according to the procedure described by Giraldo *et al*. (2008) and Belanche at al. (2016). Endoglucanase (EC 3.2.1.4.), xylanase (EC 3.2.1.8.) and amylase activities (EC 3.2.1.1.) were measured in triplicate and expressed as mmol of sugar released from the corresponding substrates in 1 min per gram DM of sample (or gram of protein).

DNA extraction and quantitative PCR

Genomic DNA was extracted from vessel samples withdrawn at different time points. Freeze dried samples (25 mg DM) were bead-beaten in 4% SDS lysis buffer for 45 s and DNA was extracted using a CTAB/Chloroform method (adapted from Yu and Morrison 2004).

Concentration and quality of genomic DNA was assessed by spectrophotometry (Nanodrop ND-100, Thermo Scientific, USA). Absolute concentrations of DNA from total

bacteria, methanogens and fungi were determined by qPCR and serial dilutions of their respective standards (10^{-1}–10^{-5}) as previously described (Belanche *et al.* 2012 and 2016). Quantitative PCR (qPCR) was conducted in triplicate using a LightCycler 480 System (Roche, Mannheim, Germany).

Ion Torrent Next Generation Sequencing

Rumen bacteria and methanogenic archaea communities were studied using Next Generation Sequencing (NGS) (de la Fuente *et al.* 2014). For bacterial profiling, amplification of the V1–V2 hypervariable regions of the 16S rRNA gene was carried out using bacterial primers (27F and 357R) followed by Ion Torrent adaptors. For methanogens profiling, amplification of the V2-V3 hypervariable region of the 16S rRNA gene was performed using archaeal primers (86F and 519R) also followed by Ion Torrent adaptors.

Forward primers were barcoded with 10 nucleotides to allow sample identification. PCR was carried out on a 25 μL reaction containing DNA template (1 μL), 0.2 µL reverse primer, 1 µL forward primer, 5 µL buffer (PCR Biosystems Ltd., London, UK), 0.25 µL bio HiFi polymerase (PCR Biosystems) and 17.6 µL molecular grade water. Amplification conditions for bacteria and methanogens were 95ºC for 1 min, then 22 cycles of 95ºC for 15 s, 55ºC for 15 s and 72^{o}C for 30 s. To assess quality of amplifications, resultant amplicons were visualized on a 1% agarose gel. PCR products were then purified using Agencourt AMpure XP beads (Beckman Coulter Inc., Fullerton, USA) and DNA concentration was determined using an Epoch Microplate Spectrophotometer fitted with a Take 3 Micro-Volume plate (BioTek, Potton, UK) to enable equimolar pooling of samples with unique barcodes.

Libraries were further purified using the EGel system with 2% agarose gel (Life Technologies Ltd., Paisley, UK). Purified libraries were assessed for quality and quantified on an Agilent 2100 Bioanalyzer with High Sensitivity DNA chip (Agilent Technologies Ltd.,

Stockport, UK). Library preparation for NGS sequencing was carried out using the Ion Chef system (Life Technologies UK Ltd) and the Ion PGM HiQ Chef kit, and sequencing using the Ion Torrent Personal Genome Machine (PGM) system on an Ion PGM Sequencing 316 Chip v2 BC. Due to the lower abundance of methanogens than total bacteria, methanogens library was sequenced using a smaller chip (Ion PGM Sequencing 314 Chip v2).

Following sequencing, data were processed as previously described (de la Fuente *et al.* 2014). Briefly, sample identification numbers were assigned to multiplexed reads using the MOTHUR software package . Data were denoised by removing low-quality sequences, sequencing errors and chimeras (quality parameters: maximum 10 homopolymers, qaverage 13, qwindow 25, for archaea the qwindow was set at 30, and erate = 1; Chimera check, both de novo and database driven using Uchime).

Sequences were clustered into OTUs using the Uparse pipeline at 97% identity. Bacterial taxonomic information on 16S rRNA gene sequences was obtained by comparing against Ribosomal Database Project-II (Wang *et al* 2007), while the methanogens were compared with the RIM-DB database (Seedorf *et al.* 2014). The number of reads per sample were normalized to the sample with the lowest number of sequences. To exclude potential bacterial sequences from the methanogens dataset, methanogens sequences were blasted with the Ribosomal Database Project-II, and those annotations which matched with bacterial sequences were removed. Raw sequences reads from the bacterial and methanogens libraries were deposited at the EBI Short Read Archive of the European Nucleotide Archive (accession number PRJEB22945 and PRJEB22960, respectively).

Statistical analysis

Linear regression was conducted to model the relationship between the percentage of radioactivity released (relative to the ^{14}C-bacterial inoculum) and the time (from 0 h to 5 h), as well as its correlation coefficient. The slope of this trend-line indicated the bacterial

degradation rate (as % h^{-1}) by the rumen protozoa and ultimately their activity. Trend line slopes, 24 h fermentation parameters, daily productions of VFA and ammonia in the RUSITEC system together with nutrient disappearance and methane data were analysed statistically by randomized block ANOVA, with individual cows/sheep as a blocking term. For the rates of bacterial degradation and 24 h fermentation parameters, polynomial contrasts were also used to determine linear (L) and/or quadratic (Q) responses to the treatments. Rumen fermentation and qPCR data in the RUSITEC were analysed using a repeated-measurements procedure (REML) including the different time-points (0, 2, 4, and 8). The effect of treatment, time and treatment x time interaction on the relative abundance of different bacteria and archaea taxa was analysed by split plot ANOVA (3 treatments x 4 time points). P values were adjusted for multiple testing using the method proposed by Benjamini and Hochberg (1995) to decrease the False Discovery Rate. When effects were detected, treatment means were compared by Fisher's protected LSD-test. Findings with $P<0.05$, $P<0.10$ when applying Benjamini and Hochberg (1995) correction, were regarded statistically significant. Genstat 15th Edition (VSN International, Hemel Hempstead, UK) was used.

Permutation multivariate analysis of variance (PERMANOVA) was used to determine overall significant differences in bacterial and archaea community and was performed in PRIMER 6 & PERMANOVA+ (versions 6.1.18 and 1.0.8 respectively; Primer-E, Ivybridge, UK). Abundance percentage data were subjected to square root transformation and Bray-Curtis distance matrices calculated. PERMANOVA was carried out using default settings with 9999 unrestricted permutations and the Monte Carlo P value was calculated. Analysis of Similarity (ANOSIM) was carried out in PRIMER 6 & PERMANOVA+ using the Bray-Curtis distance matrix calculated above. This analysis was used to provide a metric of the degree of divergence between communities as given by the R statistic.

To calculate the contribution of environmental data on bacteria and archaea communities, distance based linear modelling was used to calculate which environmental variables had a significant correlation with the community data. Significant variables were used in distance based redundancy analysis (dbRDA) (Legendre and Anderson, 1999) as implemented in PRIMER 6 & PERMANOVA+.

RESULTS

Acute antiprotozoal activity and effect on fermentation parameters (in vitro batch incubations)

Bacterial degradation by protozoa increased linearly (R^2>0.99) over the 5 h incubation with the control treatment. Increasing levels of liquorice extract resulted in a linear and quadratic decrease (P<0.001) in the breakdown of bacteria by protozoa (Supplemental Table S1). Whereas the rate of bacterial breakdown was not affected by the addition of 0.25 g L^{-1} of the flavonoid-rich extract, it was reduced by 55.6% (P<0.001) in the presence of 0.5 g L^{-1}. Doses of 1 and 2 g L^{-1} of liquorice extract caused a dramatic reduction in protozoa activity (P<0.001) with no bacterial breakdown observed.

Based on these results, doses of 0.5, 1 and 2 g L^{-1} of the extract were tested over 24 h in *in vitro* incubations (Table 1). Liquorice extract added at 1 and 2 g L^{-1} of the incubation caused only a moderate decrease in pH, albeit significant (P=0.001), compared with the control. No effect on the concentration of total VFA or on the molar proportion of acetate was observed at any of the concentrations tested (P>0.05). Doses of 1 and 2 g L^{-1} resulted in a decrease in ammonia concentration (P=0.010) and in an increase in the molar proportion of propionate (P=0.002). A reduction in the molar proportions of butyrate (P=0.013) and branched chain volatile fatty acids (BCVFA) (P=0.024) was only observed with the highest dose of liquorice.

Feed degradability and fermentation pattern (Rumen simulation technique)

Because the batch culture experiment showed no effect on fermentation with liquorice added at 0.5 g L^{-1}, only doses of 1 and 2 g L^{-1} were further tested in the RUSITEC system.

The addition of liquorice to the diet did not have any detrimental effect on feed disappearance after 48 h of incubation (Table 2); although a trend (P=0.069) to decreased OM disappearance was observed with the highest dose of liquorice tested. When liquorice was added at 1 g L^{-1} no negative effects on fermentation were observed whilst ammonia production decreased (-51%; P<0.001). The addition of 2 g L^{-1}, however, had a strong effect decreasing total VFA concentration (P=0.014), shifting fermentation towards propionate (P=0.012) at the expense of acetate (P=0.003), as well as dramatically decreasing ammonia production (-77%; P<0.001). Although total gas production was not affected by the inclusion of 2 gL^{-1} liquorice in the diet, methane production decreased (P<0.05) by 35% (Table 3). Theoretical metabolic hydrogen production based on the VFA stoichiometry (Moss et al., 2000) was also lower (P=0.002) with 2 g L^{-1} of the flavonoid-rich extract.

The study of the fermentation pattern in the vessel over a 24 h period (days 17 and 18; Supplemental Table 2) showed the same differences between treatments as those described when studying the daily fermentation products in the overflow. Concentrations of D-, L- and total lactate were unaffected by the treatments. Sampling time had a strong effect on fermentation parameters with decreased ammonia concentration and increased propionate and butyrate concentrations after feeding (P<0.01). D-, L- and total lactate concentrations decreased (P<0.001) in samples taken after feeding.

Absolute and relative enzymatic activities (Table 4) increased (P<0.05) when liquorice was added at 2 g L^{-1}. Sampling time also had an effect, with decreased xylanase and endoglucanase activities after feeding (P<0.01). Quantitative PCR revealed decreases in only the relative abundance of anaerobic fungi (P<0.001) with 2 g L^{-1} liquorice. Protozoa

concentration decreased (P<0.001) in vessels fed liquorice, with the highest dose having a stronger effect. The addition of liquorice, at all the doses tested, caused the elimination of the holotrich protozoa.

Bacterial 16S rRNA gene sequencing

Quality filtering resulted in 1,684,022 high quality sequences (320 bp long) which clustered in 1811 different OTUs with 6,195 reads per sample after normalization.

Permutational analysis of variance (Table 5) showed a strong effect of both doses of liquorice on the structure of the bacterial community (P=0.0001). However, no effect of time was observed (P=0.986). Pairwise comparison showed that the structure of the bacterial community differed between control and liquorice treatments (P=0.0001) and between liquorice 1 and liquorice 2 treatments (P=0.001). This was confirmed by ANOSIM (P=0.001), with the greatest differences found between control and liquorice 2 and liquorice 1 and liquorice 2 treatments.

To detect possible correlations between the structure of the bacterial community and rumen fermentation parameters, a distance-based redundancy analysis (dbRDA) was performed. The primary axis accounted for 55.4% of the variation and a clear separation by treatment was observed (Fig. 1). Ammonia and BCVFA concentrations in the vessel (P<0.001) and bacterial richness (P=0.024) were positively and negatively correlated to the structure of the bacterial community of control and liquorice 2 samples, respectively.

Regarding bacterial diversity (Table 6), the addition of liquorice decreased Shannon and Simpson indexes (P=0.001 and P<0.001, respectively) with the highest dose of liquorice having a stronger effect. Bacterial richness also decreased (P<0.001) in the presence of liquorice extract as compared with the control.

No differences in bacterial abundances because of the addition of liquorice were observed at phylum (Table 7; P>0.1) or family level (Supplemental Table 3; P>0.1). At

genera level (Table 8), only changes in less abundant genera were detected. The greatest change observed was the increased amount of Rikenella with liquorice as compared to the control treatment (P corrected value=0.186).

Methanogens 16S rRNA gene sequencing

Quality filtering and removal of bacterial sequences resulted in 370,221 high quality methanogen sequences (average length of 380 bp) that were clustered in to 33 unique OTUs with 3,733 sequences per sample after normalization.

Permutational analysis of variance (Table 5) showed an effect of liquorice addition on the structure of the archaeal community (P=0.0001) but no effect of time was observed (P=0.993). Pairwise comparison showed differences in the structure of the archaea community between control and liquorice treatments (P=0.0001) and between liquorice 1 and liquorice 2 treatments (P=0.001). ANOSIM analysis also showed these differences (P=0.001), with the largest separation detected between the archaeal communities corresponding to control and liquorice 2 treatments. Distance-based redundancy analysis (Fig. 2) primary axes displayed 71.3% of the variation and a separation between treatments. Several variables (total and molar proportions of VFA, ammonia and archaea diversity and richness) were positively correlated (P<0.001) with the structure of the archaeal population in vessels corresponding to the liquorice 2 treatment.

Contrary to the effects on bacterial community structure, methanogens richness and diversity was unaffected by the addition of liquorice (P>0.05). Based on the RIM-DB database, three families (Methanomassiliicoccaceae, Methanosarcinaceae and Methanobacteriaceae) made up the archaeal population in this experiment. The addition of liquorice extract influenced the abundance of the main methanogen groups with the highest dose having a stronger effect. When added at 2 g L^{-1}, the flavonoid-rich extract dramatically decreased Methanomassiliicoccus Group 12 (P=0.035) and it also reduced Group 3a

(P=0.092). On the contrary, the highest dose of liquorice promoted an increased in the abundance of Methanomassiliicoccus Group 10 (P=0.035), Methanobrevibacter (P=0.092) and Methanosphaera (P=0.053).

DISCUSSION

Flavonoids have received interest as promising alternatives to antibiotics in ruminant feeding because of their antimicrobial activity (Cheng et al., 2014). Indeed, *in vitro* studies have shown that flavonoid-rich plant extracts reduce methane production in the rumen (Bodas et al., 2008; Patra and Saxena, 2010; Oskoueian et al., 2013) which has been associated with its effect on the methanogen (Patra and Saxena, 2010) and protozoal populations (Kim et al., 2015). Furthermore, recent *in vivo* studies have reported changes in the bacteria community as a consequence of supplementing the diet with flavonoids (Kasparovska et al., 2016; Nardi et al., 2016; Zhan et al., 2017).

Liquorice, the root of the *Glycyrrhiza* species, has long been used worldwide in herbal medicine and as a natural sweetener (Asl and Hosseinzadeh, 2008; Damle, 2014). More than 20 triterpenoids and nearly 300 flavonoids have been isolated from liquorice (Wang et al., 2015). Glycyrrhizin, a triterpenoid saponin, is considered as the bioactive constituent of liquorice (Asl and Hosseinzadeh, 2008). However, it has been shown that many biological activities of liquorice, including estrogenic, anti-cancer, anti-microbial, skin whitening and metabolic syndrome preventive, could be ascribed to its isoflavonoid constituents (Vaya et al., 1997). Isoflavonoids (3-phenyl benzopyrans) differ from other classes of flavonoids due to their greater structural variability, their presence mainly in free form, rather than as a glycoside, and by the greater frequency of isoprenoid substitution (Munke et al., 2011).

Our results showed that liquorice extract had a strong antiprotozoal effect *in vitro* when measuring protozoal activity based on the amount of released [^{14}C] from labelled bacteria. Incubations for 24 h revealed that doses of 1 and 2 g L^{-1} decreased ammonia

concentration by 11 and 21% and increased propionate molar proportion by 14 and 32%, respectively, without impairing the overall fermentation process. The highest dose of liquorice tested also decreased butyrate molar proportions by 21%. Stoichiometrically, and based on the equation of Moss et al. (2000), the shift in the fermentation pattern observed with 1 and 2 g L^{-1} of liquorice extract should have resulted in a reduction in methane of 3 and 13%, respectively. When these doses of isoflavonoid-rich extract were tested for their long-term effects in the RUSITEC system, similar effects on fermentation, without negative effects on nutrient digestibility were observed. The addition of 2 g L^{-1} of the extract also caused a decrease in methane per gram of disappeared OM (-27%). A decrease in total VFA and a substantial shift in the fermentation pattern from acetate towards propionate was observed, leading to a decrease in the theoretical metabolic hydrogen production (-13%).

Despite the inherent difficulty of maintaining high numbers of protozoa in the RUSITEC system (Hillman et al., 1991), protozoal numbers in our study were reasonable (3775 cells mL^{-1} for the control), allowing the assessment of the effect of the treatments on the protozoal community. The effects of our liquorice extract on methane emission could have been associated to a decreased protozoa population (-79% and -94% for doses of 1 and 2 g L^{-1}, respectively) since protozoa provide hydrogen as a reducing substrate to methanogens (Newbold et al., 2015). The elimination of holotrich protozoa, which play a disproportionate role in supporting methanogenesis (Newbold et al., 2015), would be in line with the reduction in methane reported. Although 2 g L^{-1} of liquorice also caused a great reduction in anaerobic fungi, which together with protozoa play a significant role in the degradation of ingested plant cellulosic fibers, the digestibility of the fiber resulted unaffected. Possibly an increase in bacterial activity, as reflected in a greater xylanase activity with liquorice, might have compensated for the decrease in protozoal and fungal activity. The greatest effect observed in the presence of liquorice was the reduction in ammonia production (-51 and -77% with 1 and

2 g L^{-1} of liquorice, respectively) which could have also been related to the decrease in protozoa as they are involved in the turnover of bacterial protein due to their predatory activity (Newbold et al., 2015). It is also possible that the formation of isoflavonoids-protein complexes could have reduced the availability of nitrogen to rumen microorganisms, as has been previously reported for other polyphenolic compounds (Ozdal et al., 2013). Isoflavonoids may also have other effects on rumen fermentation and microbial activities: some authors have suggested that derivatives from the microbial degradation of flavonoids can be used as alternative carbon source for rumen microbial activities (McSweeney et al., 2001; Smith et al., 2005; Ouskoueian et al., 2013) whilst others have proposed that flavonoids could act as a hydrogen sink via cleavage of ring structures and reductive dihydroxylation (Becker et al., 2013).

Although the total number of bacteria were unaffected by the addition of liquorice, the isoflavonoid-rich extract promoted a less diverse bacterial community. ANOSIM analysis showed that the bacterial community structure was highly separated between treatments. Only changes in the relative abundance of less abundant genera were however observed. The greatest change was observed for Rikenella which are thought to be involved in structural carbohydrates degradation (Pitta et al., 2010). Its increase in presence of liquorice would be in line with the observed increase in xylanase activity. Contrary to previous studies (Oskoueian et al., 2013; Seradj et al., 2014), no major effects on archaea numbers were observed with the addition of liquorice extract. The isoflavonoid-rich extract did not significantly affect archaea diversity. Liquorice extract had an effect on the structure of the methanogen community which differed between treatments, although not to the same extent as that of the bacterial communities. A shift in the methanogen community towards one less effective in producing methane could be suggested to explain differences in methane emissions. Although it has been reported that methane emission can be related to the

concentration of archaea in rumen digesta (Wallace et al., 2014), it seems that it is the metabolic activity of individual species rather than the number of archaea what is essential for the level of methane production (Shi et al., 2014). Methanomassiliicoccus Group12 and Group 3a were replaced by Methanomassiliicoccus Group 10, Methanosphaera and Methanobrevibacter. Methanobrevibacter, theoretically less active in methane production (Kang et al. 2013) increased by 0.27 log units with 2 g L^{-1} of the extract, as compared to the control. This observation was also reported by Belanche et al. (2016) when using ivy saponins in RUSITEC.

Liquorice extract added at 1 g L^{-1} decreased ammonia production without affecting the overall fermentation process. When added at 2 g L^{-1}, decreases in not only ammonia production but also methane and total VFA production were observed. These effects in fermentation were probably related to decreases in protozoa numbers, a less diverse bacteria population as well as changes in the structure of both bacteria and archaea communities. The inclusion of an isoflavonoid-rich extract from liquorice in the diet could potentially improve the efficiency of the feed utilization by ruminants. While we speculate that the observed effects could be attributed to the high content of isoflavonoids, and particularly glabridin, the contribution of other phytochemical to the reported effects cannot be ruled out.

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Table 1. Effect of liquorice extract at 0.5, 1 and 2 g/L on pH, NH_3-N and VFA profile in ruminal digesta after 24 h of incubation.

	Dose (g/L)						
	0	0.5	1	2	SED	P	Contrast
pH	6.44[b]	6.44[b]	6.37[a]	6.35[a]	0.017	0.001	L***
NH3-N (mg/L)	20.5[c]	19.5[bc]	18.2[ab]	16.2[a]	1.00	0.010	L**
Total VFAs (mmo L^{-1})	77.5	77.7	79.4	77.9	4.10	0.965	-
Molar proportions							
Acetate	59.2	59.0	59.6	57.1	3.14	0.858	-
Propionate	17.4[a]	18.0[ab]	19.8[b]	22.9[c]	1.03	0.002	L***
Butyrate	12.0[b]	11.8[b]	11.4[b]	9.42[a]	0.666	0.013	L**
BCVFA	2.49[b]	2.42[b]	2.34[b]	1.95[a]	0.149	0.024	L**

[a-c]Means with different superscript differ ($P<0.05$); L: linear response;**:$P<0.01$;***:$P<0.001$. BCVFA = Branched chain volatile fatty acids.

Table 2. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g/L, respectively) on feed disappearance in the RUSITEC system

Diets	C	L1	L2	SED	P
Disappearance (%)					
DM	43.4	43.4	42.2	1.42	0.653
OM	47.2	45.4	42.3	1.67	0.069
N	43.2	43.3	43.3	1.77	0.998
NDF	38.7	37.0	37.2	1.01	0.257
ADF	44.2	43.5	43.2	0.76	0.451

[a-b]Means with different superscript differ ($P<0.05$).

Table 3. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g/L, respectively) on fermentation products and methanogenesis in the RUSITEC system.

Diets	C	L1	L2	SED	P
Fermentation products (mmol d^{-1})					
Total VFA	33.7^{b}	34.1^{b}	28.5^{a}	1.056	0.003
Acetate	17.2^{b}	17.0^{b}	12.8^{a}	0.795	0.003
Propionate	3.55^{a}	4.06^{a}	4.94^{b}	0.313	0.012
Butyrate	8.08	7.95	7.56	0.333	0.332
BCVFA	3.51^{b}	3.74^{b}	0.482^{a}	0.213	<0.001
Ammonia	1.37^{c}	0.674^{b}	0.315^{a}	0.106	<0.001
Gas emissions					
Total gas (L d^{-1})	1.20	1.23	1.30	0.069	0.384
Methane (mM)	3.92^{b}	3.58^{b}	2.37^{a}	0.175	<0.001
Methane (mmol d^{-1})	4.67^{b}	4.35^{b}	3.06^{a}	0.445	0.024
Methane (mmol $gDOM^{-1}$)	0.510^{b}	0.492^{b}	0.374^{a}	0.043	0.039
2H produced (mmol d^{-1})	70.2^{b}	69.8^{b}	60.8^{a}	1.59	0.002

$^{a-b}$Means with different superscript differ ($P<0.05$). BCVFA = Branched chain volatile fatty acids.

Table 4. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g/L, respectively) and the sampling time (0, 2, 4 and 8 h after feeding) on rumen enzymatic activity and microbial numbers in the RUSITEC system.

	Diets				P-Value	Time after feeding					P-Value	
	C	L1	L2	SED	Diet	0 h	2 h	4 h	8 h	SED	Time	Interaction
Absolute enzymatic activity (mmol of sugar gDM^{-1} min^{-1})												
Amylase	0.060^{a}	0.069^{a}	0.138^{b}	0.016	0.005	0.083	0.097	0.092	0.083	0.011	0.435	0.464
Xylanase	0.081^{a}	0.086^{a}	0.103^{b}	0.006	0.034	0.123^{b}	0.083^{a}	0.083^{a}	0.071^{a}	0.006	<0.001	0.166
Endoglucanase	0.062	0.067	0.074	0.004	0.097	0.085^{b}	0.064^{a}	0.062^{a}	0.060^{a}	0.003	<0.001	0.031
Relative enzymatic activity (mmol of sugar $gProtein^{-1}$ min^{-1})												
Amylase	0.306^{a}	0.315^{a}	0.664^{b}	0.031	<0.001	0.405	0.444	0.401	0.463	0.061	0.567	0.391
Xylanase	0.42^{a}	0.396^{a}	0.493^{b}	0.022	0.012	0.606^{b}	0.392^{a}	0.357^{a}	0.390^{a}	0.039	<0.001	0.437
Endoglucanase	0.326^{a}	0.314^{a}	0.356^{b}	0.010	0.013	0.421^{b}	0.304^{a}	0.267^{a}	0.337^{a}	0.030	0.006	0.940
Microbial numbers												
Bacteria (log copies gDM^{-1})	11.4	11.4	11.4	0.033	0.093	11.3	11.4	11.4	11.4	0.029	0.068	0.135
Methanogens (log copies gDM^{-1})	9.66^{a}	9.82^{b}	9.61^{a}	0.051	0.016	9.83^{c}	9.73^{b}	9.62^{a}	9.62^{a}	0.032	<0.001	0.182
Anaerobic fungi (log copies gDM^{-1})	7.99^{b}	7.47^{b}	4.71^{a}	0.277	<0.001	7.04	6.75	6.69	6.41	0.250	0.160	0.690
Protozoa (log cells mL^{-1})												
Total	3.57^{c}	2.89^{b}	2.30^{a}	0.114	<0.001							
Holotrichs	2.91^{b}	0^{a}	0^{a}	0.062	<0.001							
Entodinomorphs	3.46^{c}	2.89^{b}	2.30^{a}	0.126	<0.001							

Table 5. Effects of the supplementation with liquorice extract (1 and 2 g/L) on the structure of the bacterial communities in the rumen simulating fermenter RUSITEC.

	Bacteria		Archaea	
	P(MC) (Permanova)	R-value (ANOSIM)	P(MC) (Permanova)	R-value (ANOSIM)
Treatment effect	0.0001	0.981	0.0001	0.744
Pairwise comparison				
Control vs Liquorice 1	0.0001	0.924	0.0001	0.573
Control vs Liquorice 2	0.0001	1	0.0001	0.971
Liquorice 1 vs liquorice 2	0.0001	1	0.0001	0.867
Time effect	0.9863	-0.161	0.993	-0.194
Pairwise comparison				
t0 vs t2	0.9179	-0.163	0.943	-0.208
t0 vs t4	0.82	-0.149	0.948	-0.125
t0 vs t8	0.4681	-0.087	0.578	-0.146
t2 vs t4	0.9986	-0.26	0.999	-0.240
t2 vs t8	0.8258	-0.167	0.935	-0.212
t4 vs t8	0.9309	-0.146	0.891	-0.222

Table 6. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g/L, respectively) and the sampling time (0, 2, 4 and 8 h after feeding) on the structure of the bacteria and methanogen communities in the RUSITEC system.

	Diets				P-Value	Time after feeding					P-Value	
	C	L1	L2	SED	Diet	0 h	2 h	4 h	8 h	SED	Time	Interaction
Bacteria												
Richness	497[c]	387[b]	231[a]	23.8	<0.001	387	369	387	343	12.6	0.018	0.160
Simpson index	0.959[b]	0.944[b]	0.923[a]	0.008	0.010	0.942	0.944	0.952	0.931	0.006	0.039	0.577
Shannon index	4.35[c]	3.95[b]	3.48[a]	0.115	<0.001	3.98	3.94	4.04	3.76	0.067	0.012	0.454
Archaea												
Richness	18.9	20.8	20.3	0.885	0.155	19.0	19.6	21.1	20.3	0.616	0.042	0.550
Simpson index	0.525	0.725	0.645	0.090	0.162	0.648	0.629	0.637	0.612	0.021	0.369	0.516
Shannon index	1.26	1.77	1.49	0.212	0.135	1.54	1.51	1.53	1.45	0.053	0.296	0.315

Table 7. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g/L, respectively) and the sampling time (0, 2, 4 and 8 h after feeding) on relative abundance of bacteria phyla present at an average of more than 0.5% (false discovery rate for Benjamini-Hochberg: 0.25)

	Treatment			Time				SED			Uncorrected P			Benjamini-Hochberg P-value		
	C	L1	L2	T0	T2	T4	T8	Trt	T	TrtxT	Trt	T	TrtxT	Trt	T	TrtxT
Proteobacteria	0.181	0.218	0.179	0.200	0.189	0.166	0.217	0.034	0.020	0.045	0.478	0.118	0.307	0.598	0.393	0.512
Bacteroidetes	0.482	0.473	0.517	0.470	0.496	0.502	0.495	0.032	0.029	0.054	0.406	0.623	0.472	0.580	0.811	0.590
Firmicutes	0.257	0.257	0.259	0.265	0.256	0.268	0.242	0.043	0.025	0.057	0.999	0.629	0.263	0.999	0.811	0.512
Spirochaetes	0.021	0.017	0.015	0.021	0.019	0.020	0.011	0.003	0.003	0.005	0.213	0.029	0.213	0.456	0.145	0.512
unclassified	0.021	0.009	0.007	0.013	0.011	0.014	0.012	0.007	0.003	0.009	0.217	0.649	0.251	0.456	0.811	0.512
Tenericutes	0.012	0.013	0.012	0.013	0.013	0.013	0.010	0.003	0.003	0.005	0.900	0.580	0.359	0.999	0.811	0.513
Verrucomicrobia	0.012	0.001	0.000	0.004	0.004	0.005	0.005	0.004	0.003	0.006	0.049	0.813	0.905	0.430	0.813	0.905
Fibrobacteres	0.006	0.003	0.001	0.005	0.004	0.003	0.001	0.002	0.001	0.002	0.086	0.020	0.104	0.430	0.145	0.512
Synergistetes	0.001	0.004	0.006	0.003	0.004	0.005	0.002	0.002	0.001	0.003	0.228	0.389	0.627	0.456	0.811	0.697
Elusimicrobia	0.006	0.003	0.001	0.004	0.003	0.003	0.003	0.003	0.002	0.004	0.356	0.746	0.047	0.580	0.813	0.470

Table 8. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g/L, respectively) and the sampling time (0, 2, 4 and 8 h after feeding) on relative abundance of bacteria genera present at an average of more than 0.2% (false discovery rate for Benjamini-Hochberg: 0.25)

	Treatment			Time				SED			Uncorrected P			Benjamini-Hochberg P-value		
	C	L1	L2	T0	T2	T4	T8	Trt	T	TrtxT	Trt	T	TrtxT	Trt	T	TrtxT
Ruminobacter	0.054	0.055	0.035	0.035	0.035	0.047	0.076	0.016	0.027	0.043	0.400	0.355	0.764	0.777	0.880	0.882
Prevotella	0.250	0.274	0.277	0.264	0.279	0.280	0.246	0.035	0.030	0.057	0.721	0.606	0.343	0.901	0.880	0.828
unclassified	0.226	0.203	0.192	0.216	0.198	0.219	0.195	0.018	0.023	0.039	0.237	0.551	0.205	0.631	0.880	0.828
Christensenella	0.018	0.012	0.017	0.014	0.011	0.016	0.023	0.007	0.006	0.011	0.624	0.245	0.273	0.901	0.880	0.828
Anaerovorax	0.021	0.004	0.012	0.013	0.013	0.012	0.013	0.009	0.011	0.018	0.228	0.991	0.401	0.631	0.991	0.828
Vampirovibrio	0.002	0.009	0.003	0.003	0.004	0.005	0.007	0.002	0.004	0.006	0.029	0.683	0.882	0.186	0.880	0.882
Selenomonas	0.010	0.017	0.014	0.013	0.019	0.011	0.012	0.005	0.004	0.008	0.467	0.196	0.539	0.830	0.880	0.828
Roseburia	0.003	0.006	0.003	0.005	0.006	0.003	0.002	0.004	0.003	0.007	0.731	0.600	0.401	0.901	0.880	0.828
Paraprevotella	0.042	0.044	0.039	0.057	0.029	0.050	0.029	0.024	0.025	0.045	0.975	0.547	0.570	0.975	0.880	0.828
Treponema	0.011	0.012	0.012	0.014	0.013	0.011	0.010	0.003	0.003	0.005	0.937	0.481	0.222	0.975	0.880	0.828
Anaeroplasma	0.007	0.010	0.011	0.009	0.010	0.009	0.009	0.002	0.002	0.004	0.241	0.890	0.602	0.631	0.949	0.828
Subdivision5_genera_incertae_sedis	0.009	0.002	0.001	0.006	0.005	0.005	0.002	0.005	0.003	0.007	0.276	0.529	0.792	0.631	0.880	0.882
Fibrobacter	0.005	0.003	0.002	0.006	0.004	0.003	0.002	0.002	0.001	0.003	0.413	0.059	0.156	0.777	0.880	0.828
Acidaminococcus	0.019	0.013	0.018	0.022	0.016	0.016	0.013	0.001	0.004	0.006	0.008	0.163	0.799	0.186	0.880	0.882
Solobacterium	0.002	0.003	0.002	0.002	0.003	0.002	0.002	0.000	0.001	0.001	0.259	0.604	0.864	0.631	0.880	0.882
Pyramidobacter	0.003	0.003	0.004	0.003	0.005	0.003	0.003	0.002	0.002	0.003	0.970	0.427	0.670	0.975	0.880	0.833
Anaerovibrio	0.020	0.017	0.017	0.015	0.020	0.017	0.021	0.008	0.007	0.014	0.919	0.690	0.198	0.975	0.880	0.828
Streptococcus	0.057	0.033	0.040	0.044	0.038	0.043	0.048	0.010	0.016	0.026	0.111	0.887	0.433	0.507	0.949	0.828
Pseudobutyrivibrio	0.015	0.017	0.018	0.017	0.018	0.015	0.016	0.006	0.007	0.012	0.856	0.955	0.356	0.975	0.986	0.828
Succinivibrio	0.121	0.145	0.139	0.134	0.152	0.118	0.137	0.027	0.026	0.048	0.670	0.603	0.252	0.901	0.880	0.828
Succiniclasticum	0.021	0.025	0.024	0.020	0.026	0.024	0.021	0.005	0.009	0.014	0.732	0.852	0.621	0.901	0.949	0.828
Coprococcus	0.003	0.005	0.006	0.004	0.005	0.005	0.005	0.001	0.001	0.002	0.142	0.285	0.323	0.568	0.880	0.828
Phocaeicola	0.009	0.004	0.006	0.007	0.005	0.006	0.008	0.001	0.003	0.005	0.044	0.692	0.582	0.235	0.880	0.828
Lactobacillus	0.014	0.017	0.016	0.019	0.013	0.012	0.019	0.003	0.005	0.008	0.552	0.392	0.436	0.901	0.880	0.828
Rikenella	0.012	0.022	0.021	0.013	0.019	0.019	0.022	0.003	0.005	0.009	0.027	0.341	0.336	0.186	0.880	0.828
Sphaerochaeta	0.004	0.004	0.006	0.005	0.005	0.005	0.004	0.001	0.001	0.002	0.396	0.539	0.879	0.777	0.880	0.882
Asteroleplasma	0.002	0.002	0.003	0.002	0.003	0.003	0.002	0.001	0.001	0.002	0.583	0.884	0.677	0.901	0.949	0.833
Candidatus Endomicrobium	0.001	0.003	0.005	0.002	0.003	0.004	0.004	0.002	0.002	0.004	0.188	0.715	0.599	0.631	0.880	0.828
Eubacterium	0.003	0.004	0.004	0.004	0.004	0.003	0.003	0.000	0.001	0.001	0.023	0.054	0.290	0.186	0.880	0.828
Butyricimonas	0.002	0.003	0.003	0.002	0.003	0.002	0.002	0.001	0.001	0.002	0.631	0.488	0.573	0.901	0.880	0.828

Mucinivorans	0.002	0.002	0.002	0.002	0.002	0.002	0.003	0.001	0.001	0.002	0.786	0.629	0.409	0.932	0.880	0.828
Anaerocella	0.012	0.009	0.028	0.012	0.016	0.014	0.023	0.005	0.009	0.015	0.018	0.604	0.501	0.186	0.880	0.828

Table 9. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g/L, respectively) and the sampling time (0, 2, 4 and 8 h after feeding) on relative abundance of archaea genera present at an average of more than 0.2% (false discovery rate for Benjamini-Hochberg: 0.25)

	Treatment			Time				SED			Uncorrected P			Benjamini-Hochberg P-value		
	C	L1	L2	T0	T2	T4	T8	Trt	T	TrtxT	Trt	T	TrtxT	Trt	T	TrtxT
Methanomassiliicoccus G12	0.588	0.171	0.009	0.231	0.256	0.269	0.268	0.109	0.012	0.111	0.005	0.034	0.327	0.035	0.170	0.483
Methanomassiliicoccus G9	0.222	0.423	0.258	0.260	0.310	0.299	0.335	0.164	0.026	0.168	0.470	0.114	0.337	0.470	0.380	0.483
Methanomassiliicoccus G10	0.002	0.018	0.309	0.126	0.115	0.112	0.086	0.068	0.011	0.070	0.007	0.017	<0.001	0.035	0.170	<0.011
Methanomassiliicoccus G11	0.023	0.099	0.001	0.042	0.043	0.041	0.038	0.057	0.004	0.057	0.267	0.522	0.452	0.297	0.522	0.483
Methanomassiliicoccus G3a	0.039	0.012	0.001	0.013	0.018	0.018	0.019	0.012	0.003	0.012	0.042	0.252	0.140	0.092	0.407	0.483
Methanomassiliicoccus G3b	0.017	0.024	0.001	0.020	0.011	0.013	0.012	0.011	0.005	0.013	0.180	0.260	0.483	0.225	0.407	0.483
Methanomassiliicoccus G8	0.023	0.056	0.004	0.041	0.024	0.021	0.024	0.017	0.013	0.026	0.062	0.326	0.464	0.103	0.407	0.483
Methanimicrococcus	0.002	0.040	0.004	0.023	0.014	0.014	0.011	0.020	0.009	0.024	0.176	0.425	0.402	0.225	0.472	0.483
Methanobrevibacter	0.083	0.151	0.354	0.218	0.186	0.192	0.189	0.086	0.019	0.091	0.046	0.324	0.112	0.092	0.398	0.466
Methanosphaera	0.0004	0.006	0.059	0.025	0.021	0.021	0.020	0.015	0.002	0.016	0.016	0.155	0.482	0.053	0.388	0.483

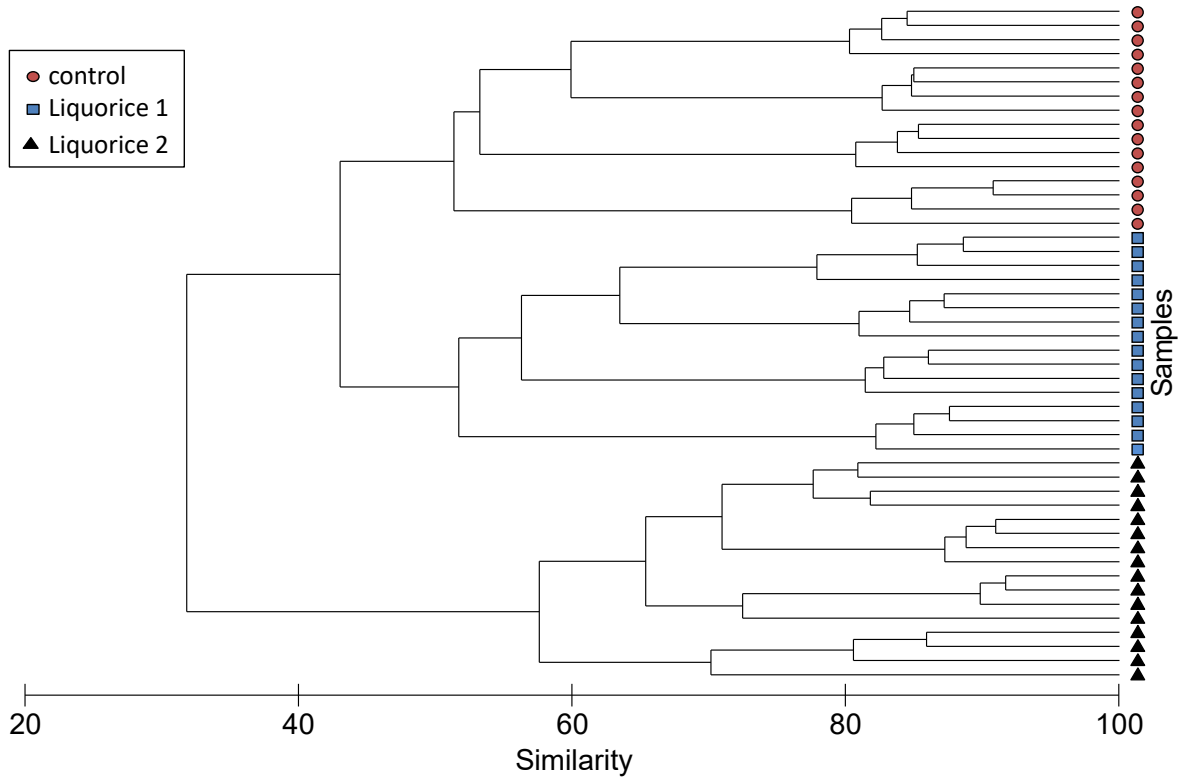

Figure 1. Bacterial population structure based on Bray-Curtis distance matrices calculated of square root transformed data.

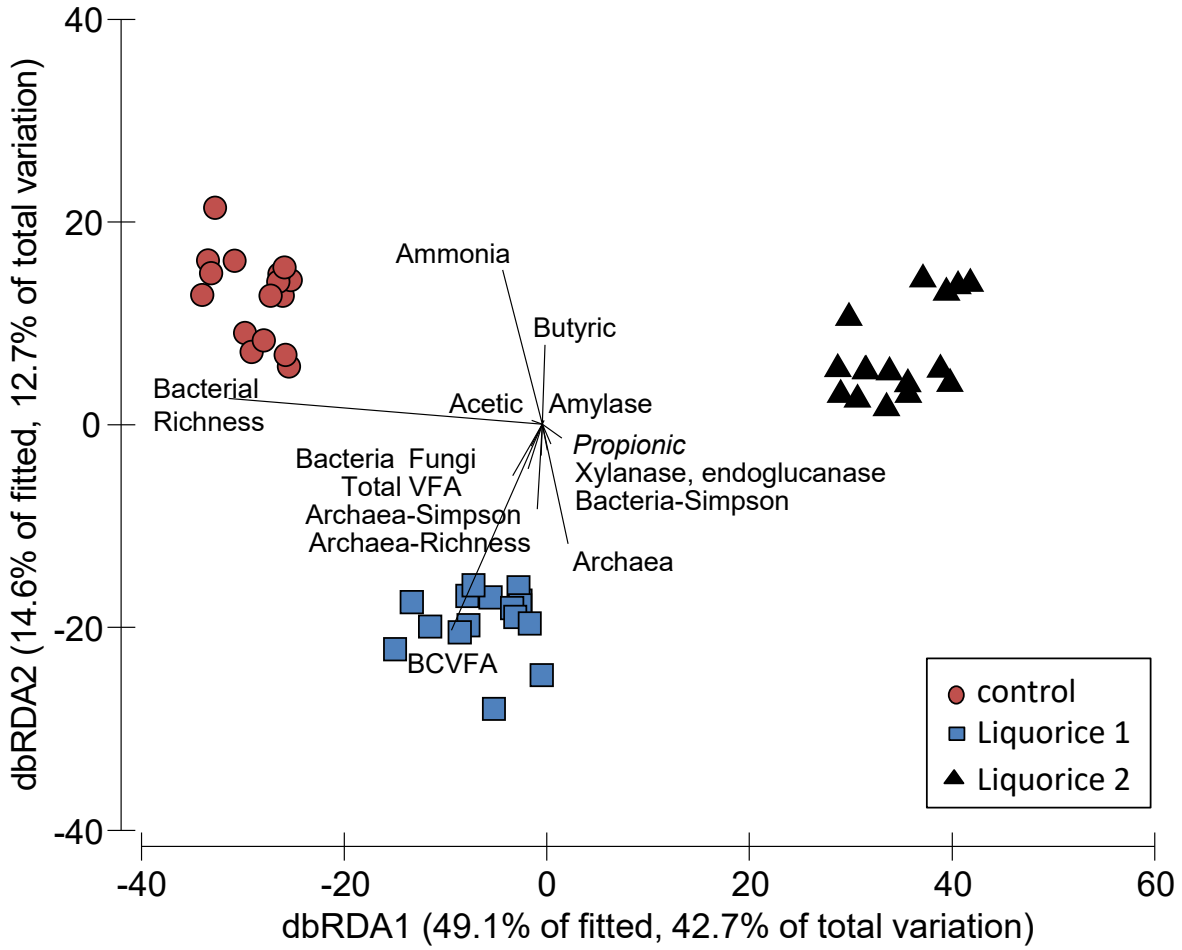

Figure 2. DbRDA illustrating the relationship between the structure of the bacterial community with the rumen fermentation pattern and microbial numbers and diversity in the RUSITEC system.

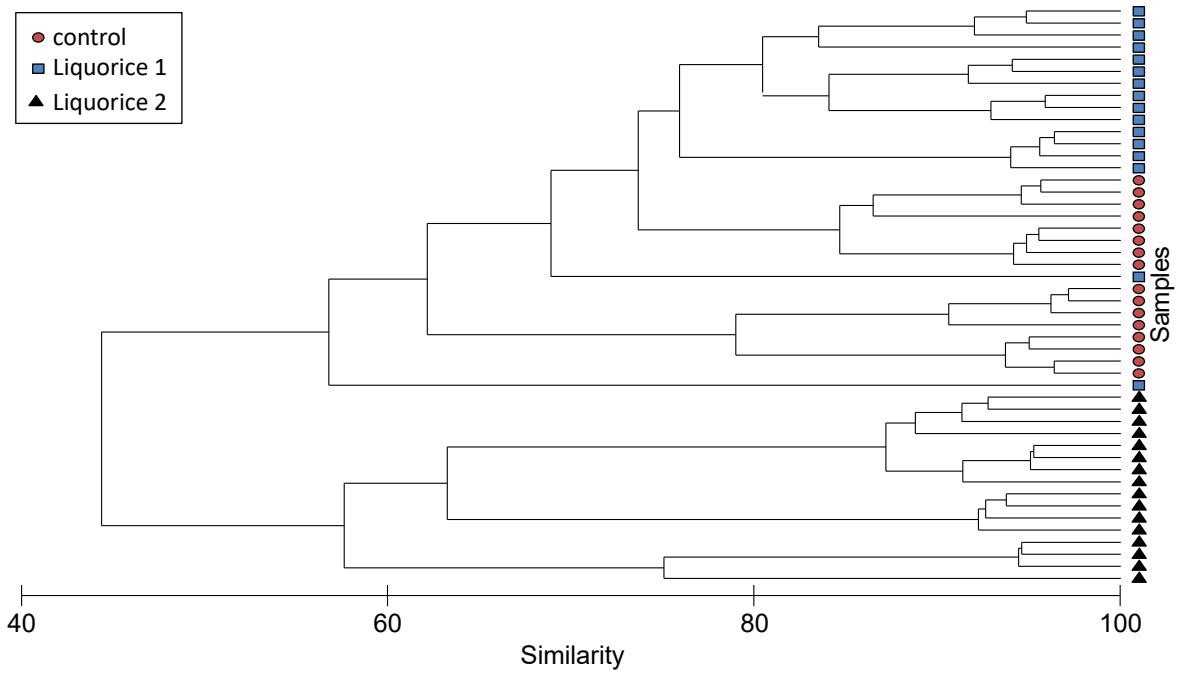

Figure 3.Archeal population structure based on Bray-Curtis distance matrices calculated of square root transformed data.

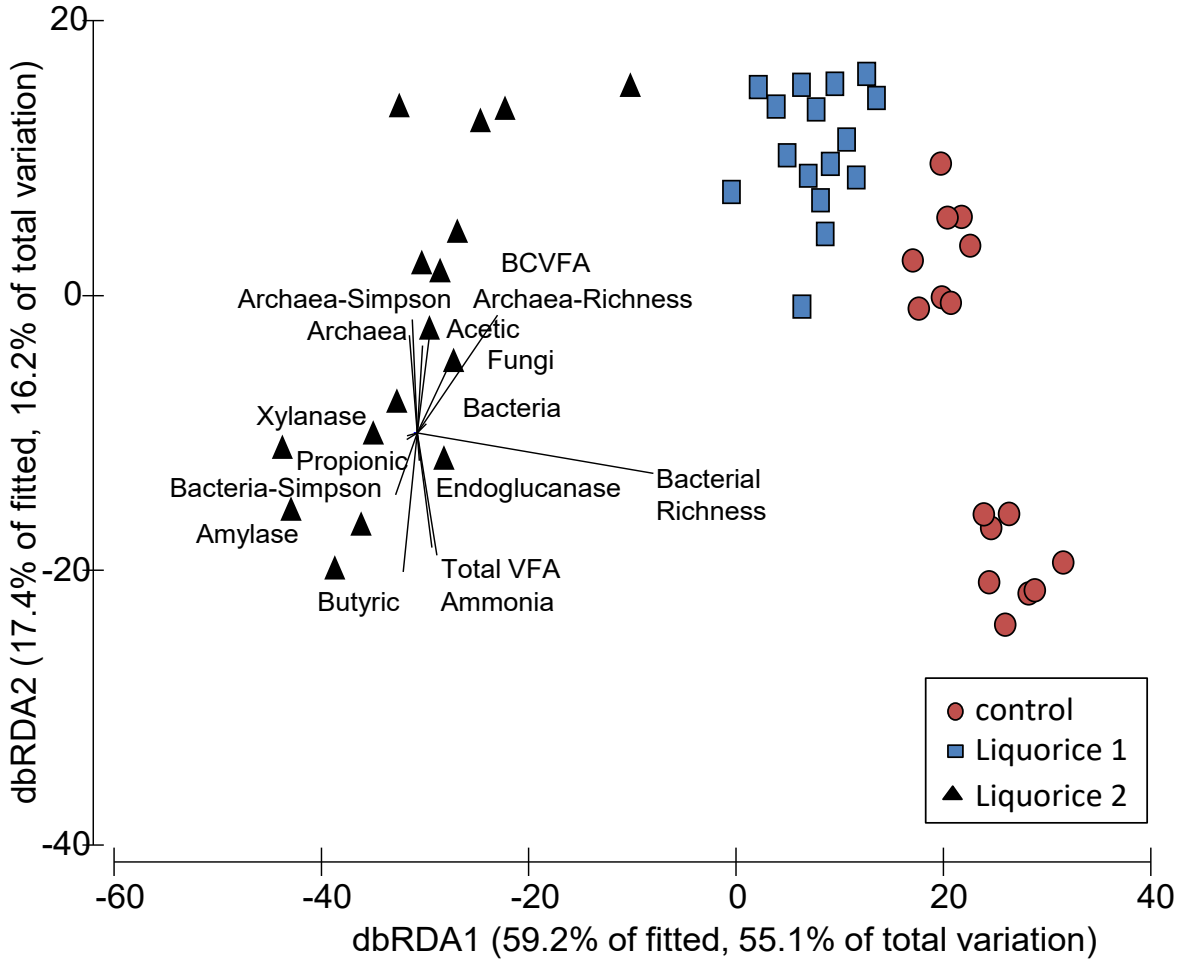

Figure 4. dbRDA illustrating the relationship between the structure of the archaeal community with the rumen fermentation pattern and microbial numbers and diversity in the RUSITEC system.

Supplemental Table 1. Effect of liquorice extract, added at 0.25, 0.5, 1 or 2 g/L, on rumen protozoa activity assessed *in vitro* as the amount of 14C-labelled bacteria broken down by rumen protozoa (% of the initial radioactivity released per hour)

	Dose (g/L)							
	0	0.25	0.5	1	2	SED	P	Contrast
Bacteria breakdown rate	3.47[c]	3.17[c]	1.54[b]	0[a]	0[a]	0.162	<0.001	L***Q***

[a-c]Means with different superscript differ (P<0.05); L: linear response; Q: quadratic response; ***:P<0.001

Supplemental Table 2. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g/L, respectively) and the sampling time (0, 2, 4 and 8 h after feeding) on rumen fermentation parameters in the RUSITEC system.

	Diets				P-Value	Time after feeding					P-Value	
	C	L1	L2	SED	Diet	0 h	2 h	4 h	8 h	SED	Time	Interaction
pH	6.64	6.65	6.66	0.013	0.320	6.44[a]	6.74[c]	6.72[bc]	6.71[b]	0.011	<0.001	0.096
Ammonia (mM)	2.78[c]	1.33[b]	0.749[a]	0.206	<.001	1.76[c]	1.77[c]	1.68[b]	1.27[a]	0.034	<0.001	0.017
Total VFA (mM)	52.4	51.7	47.6	1.932	0.093	51.1	50.2	49.8	51.2	0.923	0.351	0.506
Molar proportions												
Acetate	51.2	50.0	48.3	1.047	0.076	50.9[b]	49.3[a]	49.8[a]	49.4[a]	0.436	0.012	0.117
Propionate	11.3[a]	12.5[a]	15.5[b]	0.698	0.003	12.5[a]	13.3[b]	13.4[b]	13.2[b]	0.155	0.001	0.039
Butyrate	23.0	23.0	25.4	1.075	0.103	23.3[a]	24.0[b]	23.7[ab]	24.2[b]	0.264	0.032	0.054
BCVFA	9.63[b]	9.72[b]	1.55[a]	0.477	<.001	7.04	6.97	6.77	7.10	0.129	0.102	0.358
Lactate (mM)												
Total	0.583	0.651	0.566	0.046	0.224	1.796[b]	0.174[a]	0.227[a]	0.201[a]	0.042	<0.001	0.103
D-lactate	0.385	0.432	0.345	0.033	0.098	0.979[b]	0.171[a]	0.208[a]	0.191[a]	0.026	<0.001	0.157
L-lactate	0.198	0.219	0.221	0.024	0.593	0.816[b]	0.003[a]	0.020[a]	0.011[a]	0.025	<0.001	0.199

[a-c]Means with different superscript differ (*P<0.*05).

Supplemental Table 3. Effect of supplementing a control diet (C) with liquorice extract (L1 and L2, 1 and 2 g/L, respectively) and the sampling time (0, 2, 4 and 8 h after feeding) on abundance relative of bacteria families present at an average of more than 0.5% (false discovery rate for Benjamini-Hochberg: 0.25)

	Treatment			Time				SED			Uncorrected P			Benjamini-Hochberg P-value		
	C	L1	L2	T0	T2	T4	T8	Trt	T	TrtxT	Trt	T	TrtxT	Trt	T	TrtxT
Rikenellaceae	0.035	0.045	0.033	0.045	0.035	0.038	0.033	0.021	0.009	0.025	0.827	0.455	0.042	0.858	0.858	0.522
unclassified	0.153	0.095	0.123	0.136	0.116	0.116	0.128	0.030	0.018	0.040	0.238	0.502	0.112	0.685	0.858	0.522
Christensenellaceae	0.022	0.012	0.014	0.017	0.013	0.015	0.019	0.011	0.004	0.012	0.646	0.460	0.155	0.827	0.858	0.522
Clostridiales_Incertae Sedis XIII	0.014	0.020	0.005	0.013	0.015	0.012	0.012	0.017	0.007	0.020	0.717	0.831	0.184	0.827	0.858	0.522
Streptococcaceae	0.057	0.036	0.036	0.041	0.045	0.047	0.040	0.027	0.010	0.031	0.683	0.697	0.208	0.827	0.858	0.522
Spirochaetaceae	0.021	0.016	0.015	0.020	0.019	0.020	0.011	0.003	0.003	0.005	0.205	0.028	0.212	0.685	0.420	0.522
Ruminococcaceae	0.023	0.015	0.012	0.016	0.016	0.017	0.017	0.006	0.002	0.007	0.237	0.858	0.249	0.685	0.858	0.522
Succinivibrionaceae	0.168	0.207	0.175	0.191	0.180	0.156	0.207	0.031	0.019	0.042	0.461	0.102	0.284	0.768	0.765	0.522
Anaeroplasmataceae	0.011	0.012	0.012	0.012	0.012	0.012	0.010	0.003	0.003	0.005	0.858	0.611	0.313	0.858	0.858	0.522
Lachnospiraceae	0.021	0.038	0.054	0.036	0.039	0.040	0.035	0.019	0.007	0.021	0.274	0.746	0.415	0.685	0.858	0.623
Porphyromonadaceae	0.023	0.012	0.027	0.021	0.021	0.022	0.018	0.012	0.023	0.027	0.235	0.757	0.466	0.685	0.858	0.635
Prevotellaceae	0.318	0.306	0.348	0.284	0.341	0.344	0.328	0.033	0.028	0.054	0.458	0.169	0.624	0.768	0.845	0.778
Marinilabiliaceae	0.011	0.045	0.011	0.023	0.018	0.024	0.024	0.025	0.015	0.033	0.343	0.787	0.674	0.735	0.858	0.778
Erysipelotrichaceae	0.013	0.010	0.012	0.011	0.011	0.013	0.013	0.002	0.002	0.004	0.515	0.404	0.825	0.773	0.858	0.884
Veillonellaceae	0.018	0.034	0.044	0.033	0.034	0.033	0.027	0.013	0.007	0.017	0.184	0.664	0.897	0.685	0.858	0.897

Chapter 7

4th Contribution

Garlic (*Allium sativum L.*) fed to dairy cows does not modify the cheese-making properties of milk, but affects the color, texture and flavor of ripened cheese

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INTERPRETIVE SUMMARY

By Rossi *et al.*

Garlic has been advocated as a rumen modifier to reduce enteric methane emissions from dairy cows, but little is known of its influence on the sensorial and rheological characteristics of milk and cheese. We added 100 or 400 g/d of garlic cloves or 2 g/d of diallyl sulfide to the feed of lactating cows in a 4 x 4 Latin square experimental design. Neither supplement affected production performances or cheese-making properties of milk, but diallyl sulfide slightly influenced cheese quality. Four-hundred g/d of garlic cloves affected milk and cheese aroma, and modified cheese texture by reducing its shear force.

ABSTRACT

Garlic and garlic components have recently proposed as ruminal activity modulators to reduce the enteric methane emissions of ruminants, but little is known of its influence on the milk coagulation properties, nutrient recovery, cheese yield, and sensorial and rheological characteristics of milk and cheese. The present study assessed the effects of garlic and diallyl sulfide supplements on DMI, productive performances, milk coagulation properties, cheese yield, milk and cheese sensory profiles, and rheological characteristics. Four dairy cows were fed a total mixed ration (TMR) either alone (control) or supplemented with 100 or 400 g/d of garlic cloves or 2 g/d of diallyl sulfide in 4 consecutive experimental periods in a 4×4 Latin square design. The diallyl sulfide dose was established to provide approximately the same amount of allyl thiosulfinate compounds as 100 g of fresh garlic cloves**.** The TMR was composed of 0.29 corn silage, 0.23 corn-barley mixture, 0.17 sunflower-soybean mixture, 0.12 alfalfa hay, 0.12 grass hay, 0.04 sugar beet pulp and 0.02 other additives, and contained 0.253 starch, 0.130 crude protein and 0.375 neutral detergent fiber, on a dry matter basis. Each experimental period consisted of 7 d of transition and 14 d of treatment. On days 18 and

21 of each period, milk samples (10 L) were collected from each cow for chemical analysis and cheese-making. The organoleptic properties of the milk and 63 d ripened cheeses were assessed by a panel of 7 trained sensory evaluators. The experimental treatments had no effects on DMI, milk yield, feed efficiency (milk yield/DMI), milk coagulation properties, nutrient recovery and cheese yield. Garlic-like aroma, taste and flavor of milk and cheese were significantly influenced by the treatments, particularly the highest dose of garlic cloves, and we found close exponential relationships between milk and cheese for garlic-like aroma (R^2 = 0.87) and garlic-like flavor (R^2 =0.79). Diallyl sulfide and 400 g/d of garlic cloves resulted in lower pH, shear force and shear work of ripened cheeses compared with the other treatments. Garlic cloves and diallyl sulfide had opposite effects on cheese color indices. We conclude that adding 400 g/d of garlic to the feed of lactating dairy cows highly influences the sensory and rheological characteristics of cheese.

Keywords: cheese yield, diallyl sulfide, garlic clove, milk coagulation properties, sensory analysis.

INTRODUCTION

Methane produced in the rumen is a potent greenhouse gas, and the FAO (Steinfeld et al., 2006) has asserted that 18% of total anthropogenic greenhouse gases are directly or indirectly related to the livestock industry. Others have estimated that the contribution of ruminants to global warming is more likely in the order of 3% (Pitesky et al., 2009). However, as methane represents a 2 to 12% loss of energy in the conversion of feed to animal products, many strategies have been proposed to lower emissions of this gas without impairing rumen fermentation and animal performance (Hristov et al., 2013a, b).

Garlic (*Allium sativum*), and particularly its sulfur compounds, inhibits *in vitro* methanogenesis (Blanch et al., 2016), and consistently increases the acetate:propionate ratio and butyrate concentration in the rumen fluid (Yang et al., 2007; Klevenhusen et al., 2011).

The effect is similar, but not identical, to that of the antibiotic monensin (Calsamiglia et al., 2007), and reflects the anti-microbial and anti-protozoal properties of organosulfur garlic compounds (Reuter et al., 1996). Busquet et al. (2005) suggested that the anti-methanogenic action mechanism of garlic could be related to direct inhibition of archaea rumen bacteria through inhibition of the 3-hydroxy-3-methyl-glutaryl coenzyme A (HMG-CoA) reductase of its organosulfur compounds. Macheboeuf et al. (2006) reported dose response effects of garlic oil and some sulfur garlic compounds (Diallyl sulfide, diallyl disulfide, allicin, and allyl mercaptan) on ruminal fermentation and *in vitro* methane production. Garlic in various forms (cloves, powder, oil or pure sulfur compounds) was found to have antiparasitic, anticancer, antioxidant, immunomodulatory, anti-inflammatory and hypoglycemic activities in ruminants (Kamra et al., 2012).

A few experiments investigated the effect *in vivo* of garlic, or its sulfur constituents, on digestion, ruminal fermentation, milk production and quality in dairy cows (Van Zijderveld et al., 2011; Oh et al., 2013; Blanch et al., 2016). There is little or no evidence available of the effect of garlic as a feed additive on the milk coagulation properties, nutrient recovery, cheese yield, and sensorial and rheological characteristics of milk and cheese. In humans, volatile metabolites of garlic were detected in milk a few hours after ingestion of garlic cloves, and the sensory characteristics of the milk were consistently modified (Scheffler et al., 2016). By analogy, we could hypothesize that the sensory characteristics of bovine milk and cheese may also be influenced by adding garlic or its sulfur compounds to the cows' feed.

The current experiment aimed to evaluate the influence of increasing doses of garlic cloves and of diallyl sulfide on productive performance, the coagulation/cheese-making properties of milk, and the sensory and rheological characteristics of milk and cheese.

MATERIALS AND METHODS

Animals, rations and experimental design

The project was approved by the University of Padua's "Ethical Committee for the Care and Use of Experimental Animals", in accordance with Italian law on the care of research animals. To minimize the use of animals, we adopted a Latin square design and used only one cow per treatment.

Four Holstein-Friesian, second parity, dairy cows were housed in individual pens on the University of Padua's experimental farm (Legnaro, Italy). At the start of the experiment the cows had an average 119 ± 17 d in milk and 30.8 ± 3.4 kg/d milk yield, and an average body weight of 621 ± 26.1 kg and body condition score of 2.69 ± 0.32 on a 5-point scale. The cows were assigned to four feeding treatments in a 4 × 4 Latin square design. Each experimental period lasted 14 d - 7 d for adaptation and 7 d for recording and sample collection - and was followed by a 7 d transition period to reduce possible carryover effects. Feeds were distributed to each cow as a low protein total mixed ration (**TMR**), the ingredients and nutritional composition in accordance with NRC (2001) and Schiavon et al. (2015) for a milk yield of 30 kg/d (Table 1). The 4 experimental treatments were: a "control" diet, a supplement of 2 g/d of pure (0.97) diallyl sulfide (Allyl sulfide, Sigma-Aldrich Chemical, Milan, Italy), and 100 or 400 g/d of fresh peeled garlic cloves (cultivar Aglio Bianco Polesano). The garlic supplements were devised to provide approximately 4.35 or 17.39 g/kg DM intake, compared with the 10 g/kg DM used by Patra and Saxena (2010) for sheep. The 2 g/d of diallyl sulfide corresponded, in terms of total sulfur content, to approximately 100 g/d of fresh garlic cloves.

At 8 a.m., before TMR distribution, the peeled garlic cloves were weighed and mixed with about 3 kg of feed in a bowl to ensure they were completely consumed. Similarly, the diallyl sulfide was diluted in 150 mL of water and sprayed evenly over the 3 kg of feed in the bowl,

then distributed to the animal. The TMR was then distributed to the cows at about 9 a.m. and 1 p.m. every day.

Sampling and controls

The cows were weighed and assigned a body condition score (**BCS**) by the same trained technician at the beginning and end of each experimental period (Edmonson et al, 1989). DM intake (**DMI**) was calculated from the weight of the TMR distributed daily to each cow and the orts collected during the second experimental week. Samples of the base TMR and orts (1 kg) were collected on days 12 and 14 of each experimental period, and pooled by period before analysis. Feed efficiency was computed as the milk yield/DMI ratio.

Cows were milked twice a day, at 6 a.m. and 5 p.m., and daily milk yield was recorded during the second experimental week of each period. Milk samples (50 mL) were collected every day at the morning and evening milking for analysis of chemical composition, and milk coagulation and curd firming properties. Aliquots of milk produced by each cow (5 liters) were also collected in the morning and evening of days 12 and 14 of each experimental period for cheese making. Milk samples (1 liter) for sensory analysis were collected from each cow in the morning and evening of day 12 of each experimental period. The milk samples were immediately sent in sealed bottles to the sensory laboratory for analysis. Other milk samples were collected for the analysis of the milk fatty acid profile to achieve an indirect estimation of the *in vivo* enteric methane emission, but the result are not given in this paper (Negussie et al., 2017).

Chemical analyses

The TMR and garlic cloves used during the trial were analyzed in triplicate according to AOAC (2003) for DM (# 934.01), crude protein (**CP**; # 976.05), ether extract (**EE**; # 920.29) and ash (# 942.05). Neutral detergent fiber (**NDF**), expressed inclusive of residual ash, was determined with α-amylase and sodium sulfite in a neutral detergent solution (Mertens et al.,

2002) using an Ankom220 Fiber Analyzer (Ankom Technology® Corporation, Macedon, NY, USA). Acid detergent fiber, expressed inclusive of residual ash (**ADF**), and the sulfuric acid lignin (**ADL**) content were determined sequentially after NDF analysis (Robertson and Van Soest, 1981). Starch content was determined after hydrolysis to glucose by liquid chromatography (AOAC, 2003), and non-structural carbohydrates (**NSC**) were calculated as 1000 – EE – CP – NDF – Ash.

Garlic cloves and TMR samples (0.5 g of DM) were analyzed for their S content. The samples were digested in a microwave (Milestone Srl, Sorisole, Bergamo, Italy) with 7 mL of superpure HNO_3 67% and 2 mL of H_2O_2 30%. The operating conditions were: temperature increase from 25 to 200 °C in 15 min, held for 18 min, temperature decrease from 200 to 35 °C in 25 min. After cooling down to room temperature, the dissolved sample was diluted with ultrapure water to a final volume of 50.0 mL. Sulfur was determined using an Arcos EOP inductively coupled plasma-optical emission spectrometer (Spectro Analytical Instruments GmbH, Kleve, Germany).

Individual morning and evening milk subsamples, preserved with 2-Bromo-2-nitro-1,3-propanediol, were separately subjected to infrared analysis for fat, protein, casein, lactose and total solids contents using a Milko-Scan (FT2, Foss Electric A/S, Hillerød, Denmark). Somatic cell counts were obtained with a Fossomatic (FC automatic counter, Foss Electric A/S, Hillerød, Denmark) and log-transformed using the following equation: somatic cell score (**SCS**) = $3 + \ln_2$ (Somatic Cell Count × 10^{-5}), as reported by Ali and Shook (1980). Milk pH was measured using a Crison Basic 25 electrode (Crison, Barcelona, Spain). Milk protein fractions were analyzed by reverse-phase high-performance liquid chromatography, following Maurmayr et al. (2013).

Milk coagulation properties

Traditional milk coagulation properties (**MCP**) were measured with a Formagraph (Foss Electric A/S, Hillerød, Denmark), and expressed in terms of rennet coagulation time (**RCT**, min), curd-firming time ($\mathbf{k_{20}}$, min), and curd firmness 30 min after rennet addition ($\mathbf{a_{30}}$, mm), according to the method proposed by McMahon and Brown (1982). As, on average, the milk samples coagulated very late, curd firmness was also recorded 45 and 60 min after rennet addition (a_{45}, a_{60}, mm). Given the shortcomings of traditional MCPs demonstrated by Bittante (2011), all the 240 Formagraph measures (one every 15 s for 60 min) taken from each milk sample were retrieved from the instrument and modeled using the following equation:

$$CF_t = CF_P \times [1 - e^{-k_{CF} \times (t - RCTeq)}]$$

where CF_t is curd firmness at time t (mm); CF_P is the asymptotical potential value of CF at an infinite time (mm); k_{CF} is the curd-firming rate constant (%/min^{-1}); and RCT_{eq} is the rennet coagulation time estimated from all the information recorded. The parameter CF_P is conceptually independent from test duration and is not intrinsically dependent on RCT (unlike a_{30}). The parameter k_{CF} describes the shape of the curve from milk gelation to infinity and is conceptually different from k_{20}, as it is independent of CF_P, but uses all the available information.

Cheese making

The morning and evening milk samples collected from each cow were pooled by day. Two cheese-making session were carried out in each period giving a total of 32 cheese wheels produced in this experiment (8 per period). Cheeses from the 4 treatments were manufactured (Cattani et al., 2014) simultaneously in 4 small-scale experimental vats, without any preliminary heat treatment and skimming of milk samples. Briefly, milk from each cow was poured into a 10 L vat (Pierre Guérin Technologies, Mauze, France), and heated to 35° C by water circulation. The starter culture *Streptococcus thermophilus* (CSL Starter Cultures, Lodi, Italy) was added to facilitate milk acidification. After 20 minutes, 2.5 mL of liquid rennet

(Naturen tm Standard 215, Hansen, Pacovis Amrein AG, Bern, Switzerland) diluted in 40 mL of distilled water was added to the milk. After milk gelation, the curd was cut in 4 consecutive steps (at 3, 4.15, 5.30, and 10.45 min after gelation; each step 15 sec) to obtain curd cubes of about 0.5 cm^3, then cooked to 45°C. Following the syneresis phase (20 min), each individual curd was extracted from the vat and put into a cheese mold, pressed, and immersed in a brine solution (20% NaCl) for 24 h. Cheese yields and nutrient recoveries were computed for fresh curd according to Cipolat-Gotet et al. (2013). Finally, the cheeses were stored for 63 days at 15°C and 85% relative humidity, then weighed.

Sensory analysis

Sensory analyses were carried out by a panel of 7 trained evaluators with experience of the products in a testing room designed in accordance with ISO 8589 (1988) and equipped with individual booths and standard CIE white illumination D_{65} (Pinho et al., 2004). The panelists were seven women, 37 ± 2 years old, selected on the basis of their ability to identify the four basic tastes of sweetness, bitterness, saltiness, and sourness, and the aroma/flavor compounds, including diallyl sulfide and garlic cloves.

The panelists had previously attended 4 training sessions in which commercial fresh whole milk and caciotta cheese (50 d of ripening) were used to familiarize them with the products, and in which they discussed the vocabulary (Meilgaard et al., 1991) for the quantitative descriptive analysis of the milk and cheese. During these preliminary sessions, the evaluators and agreed upon a list of descriptors for the aroma, taste and flavor of milk and cheese, as suggested by Pagliarini et al. (1991). Garlic-like aroma and flavor were identified using crushed garlic cloves, fruit flavor was associated with almond and walnut, and grass aroma with green grass. The panel made their sensory assessment of milk in terms of two aromas (overall aroma intensity and garlic-like aroma), three tastes (sweet, salty and bitter), and two flavors (overall flavor intensity and garlic-like flavor). The descriptors for the cheese samples

included four aromas (aroma intensity, grass, fruit, garlic), four tastes (sweet, salty, sour, bitter), and two flavors (flavor intensity and garlic-like flavor). Attributes were scored on a scale of 1 (absence of perception) to 10 (very intense) anchored with standard food references (Lavanchy et al., 1999), which, in the current work, were represented by commercial milk and caciotta cheese.

Four milk evaluation sessions and four cheese evaluation sessions were carried out, in conformity with ISO 6658 (ISO,1985). In each milk testing session, the 4 individual milk samples of the evening and those of the following morning were pooled by cow into 2-liter bottles, kept at room temperature and scored in two replications. Ripened cheese samples were prepared according to Bàrcenas et al. (2007), and the wheels were cut into two and placed under vacuum conditions. Immediately before the evaluation, cheese samples 1.5 cm thick × 5 cm wide × 6-8 cm long were prepared, representing the whole cheese wheel without the rind. The cheese evaluations were also performed in replication.

All the milk and cheese samples, equilibrated at room temperature (21 ± 1° C), were identified by random three-digit codes and presented to assessors in randomized order (Suzzi et al., 2015). Water and breadsticks were provided to rinse the mouth after each taste.

Rheological analysis

The following analyses were performed on all the cheese wheels after 63 days of ripening.

The pH was measured in triplicate with a pH meter (HACH LANGE, Crison Instruments Sa, Carpi, Italy) equipped with a specific electrode (cat. 5053T, Crison Instruments s.a., Carpi, Italy).

Color was assessed on cheese carrots 1 cm in diameter and 1 cm long after exposure to the air (1 h, 21 ± 1 °C) on 5 consecutive sites from the rind to the center by a spectrophotometer (CM-600, Minolta Corp., Ramsey, NJ, USA) set on illuminant D65 (standard daylight), and with a 10° observer. Data were expressed according to the CIE L*a*b* colorimetric system

(CIE, 1986), where L* represents reflection (0 = black, 100 = white), and a* and b* have no specific numerical limits: a* ranges from green (negative values) to red (positive values) , and b* ranges from blue (negative values) to yellow (positive values).

The shear force and the corresponding shear work (the work done to cut the sample from the starting point to maximum shear force) were measured using an LS5 dynamometer (Lloyd Instruments Ltd, Bognor Regis, UK) equipped with a Warner-Bratzler probe, load cell 500N, and crosshead speed of 2 mm/sec^{-1}, as reported by Segato et al. (2007).

Statistical analysis

All the data were averaged by period and cow (15 d.f.) and analyzed according to a model of response for a classic 4 × 4 Latin square design using SAS PROC MIXED (SAS Institute, 2007):

$$y_{ilkl} = \mu + T_i + P_j + C_k + e_{ijkl},$$

where: y_{ijkl} is the observed trait; μ is the overall mean; T_i is the fixed effect of the feeding treatment (i = 1,…,4); P_j is the random effect of period (1,…,4); C_k is the random effect of cow (1,…,4); e_{ijkl} is the residual error term ~ N (0, σ^2e, 6 d.f.). The model used for statistical analysis of milk quality traits and milk sensory traits also included the SCS as a covariate. Orthogonal contrasts were run to test the significance of the treatment (control *vs*. treated samples), the kind of supplement (diallyl sulfide *vs*. fresh garlic), and the garlic dose (100 *vs*. 400 g/d of fresh garlic cloves). Differences were considered statistically significant at $P < 0.05$.

RESULTS

Dietary treatment had no influence on DMI (23.4 kg/d), milk yield (34.1 kg/d) and the milk yield/DMI ratio (1.62 kg milk/kg DMI; Table 2), and had a weak or null influence on milk quality traits compared with controls, with the exception of milk protein content ($P = 0.011$) and pH ($P = 0.033$), which were slightly lower, and ß-casein which was higher ($P < 0.001$).

The proportion of ß-casein exhibited a greater increase with diallyl sulfide than with garlic cloves ($P = 0.002$).

Rennet coagulation time (both RCT and RCT_{eq}) was very long, 28 min on average. The 32.5% of milk samples coagulating after 30 min were considered non-coagulating samples and therefore had no a_{30} values. CF_t modeling was not affected by these limitations and all parameters were obtained for all samples. However, the treatments did not influence any of the traditional MCP traits and curd firming equation parameters (Table 3). Similarly, dietary treatment had no influence on curd and solid cheese yields, retained water, recoveries of protein, fat, solids and energy, daily production of curd and cheese solids, and daily retained water.

Sensory and rheological characteristics of milk and cheese

The treatments increased the garlic-like aroma ($P < 0.001$) and the garlic-like flavor ($P < 0.001$) in the milk compared with controls, but not the other sensory traits (Table 4). However, the kind of supplement and the dose of garlic cloves had an impact on the milk sensory attributes. Perception of all the aroma and flavor traits, but not the taste traits, was significantly lower with diallyl sulfide than with garlic. Intensity of overall aroma ($P < 0.001$), garlic-like aroma ($P < 0.001$), saltiness ($P = 0.041$), and garlic-like flavor ($P < 0.001$) were notably greater with the 400 g/d dose of garlic cloves than with the lower dose.

The treatments increased the garlic-like aroma ($P < 0.001$), saltiness ($P = 0.004$), intensity of overall flavor ($P = 0.009$) and garlic-like flavor ($P < 0.001$) in the cheeses, and lowered the pH ($P = 0.007$) and the shear work ($P = 0.045$). Diallyl sulfide increased the fruit-like aroma ($P = 0.031$), the a* ($P < 0.001$) and the b* ($P < 0.001$) color traits, but lowered the garlic-like aroma ($P < 0.001$), garlic-like flavor ($P < 0.001$), pH ($P = 0.022$), shear force ($P = 0.032$) and shear work ($P < 0.001$) compared with garlic cloves. The highest dose of garlic cloves increased the intensity of overall aroma ($P < 0.001$) and garlic-like aroma ($P < 0.001$),

sourness ($P = 0.030$), and garlic-like flavor ($P < 0.001$), and lowered the L* ($P < 0.001$), shear force ($P < 0.001$) and shear work ($P = 0.003$) compared with the lowest garlic dose.

When the individual scores for garlic-like aroma (Figure 1; $R^2 = 0.874$) and garlic-like flavor (Figure 2; $R^2 = 0.796$) in milk and cheese were regressed, we found strong relationships with exponential coefficients less than unity. Furthermore, the garlic-like flavor of milk was positively correlated with the sour (r = 0.726) and bitter tastes (r = 0.685) of the cheese derived from it.

DISCUSSION

Interest in the use of bioactive plants or plant metabolites as an alternative to chemical substances to mitigate methane emission, has greatly increased in recent decades (Patra and Saxena, 2010). The use of garlic as a feed additive has been advocated due to its positive effects on rumen fermentation, digestive processes, and metabolic, anti-inflammatory and immune-stimulant activities, although the major interest is in its anti-methanogenic properties (Kamra et al., 2012). Additives may be in the form of fresh cloves, extracts, oil, or powder, or in the form of organosulfur metabolites derived from garlic cloves, such as allicin, diallyl sulfide, diallyl disulfide, and allyl mercaptan, either singly or in combination (Calsamiglia et al., 2007). These metabolites are produced from S-alk(en)yl-L-cysteine sulfoxides, allicin being the largest contributor, by allinase and other enzymes when the garlic cells are broken down. The S-alk(en)yl-L-cysteine sulfoxide content of garlic fresh weight is 0.53-1.3% (Patra and Saxena, 2010).

Garlic and dry matter intake, milk yield and milk quality

Different forms and doses of garlic have been found to have little or no influence on the DMI of sheep (Patra and Saxena, 2010; Klevenhusen et al., 2011), lactating cows (Oh et al., 2013; Blanch et al., 2016; Prayitno et al., 2016), and beef cattle (Staerfl et al., 2012). Patra and Saxena (2010) reported that the DMI of sheep decreased after the addition of 10 g/kg DM

of fresh garlic cloves, but the effect was transient, and no effects were observed after adaptation. Nolte and Provenza (1992) observed that garlic and onions influenced the feed preferences of lambs, although the effect was temporary. Garlic oil or garlic extracts were found to have a small influence on milk yield, milk composition and feed efficiency (Yang et al., 2007; Oh et al., 2013; Blanch et al., 2016). In the current study, the various treatments had no influence on DMI, milk yield, the milk yield/DMI ratio, and milk composition. Garlic cloves and diallyl sulfide supplements both reduced the milk protein content (N × 6.25) by about 2% compared with controls, but while the various protein fractions were lowered, the proportion of ß-casein increased. Such alterations can be, at least partially, explained by the possible influences that garlic can exert on the N metabolism at ruminal level (Calsamiglia et al., 2007). We suggest, therefore, that the use of garlic at a dose of up to approximately 17.4 g/kg DM would have little or no influence on nutrient utilization, milk yield and composition.

Cheese-making quality of the milk

The various garlic treatments had no influence on the milk coagulation properties evaluated by traditional procedures, although this result is of limited value due to the high incidence of milk samples coagulating later than 30 min after rennet addition, as is frequently observed with Holstein-Friesian cows (Bittante et al., 2012; Stocco et al., 2017a). CF_t modeling equation parameters, on the other hand, are not affected by the length of RCT_{eq} (Stocco et al., 2015), although also in this case dietary treatment had an almost null effect. The cheese yields and milk nutrient recoveries in cheese we obtained are also close to expectations for Holstein cows (Stocco et al., 2017b). Similarly, we found the treatments to have no influence on cheese yield traits nor on the recoveries of milk protein, fat, solids and energy in the curd. The absence of any effect of garlic cloves on these traits is consistent with the small variations we observed in the nutritional composition of the milk, and particularly in the milk protein profile. To our knowledge, this has not been previously reported in the

literature.

Sensory characteristics of milk and cheese

The sensory qualities of dairy products, flavor in particular, are known to have a strong influence on consumer acceptability and preferences, and market prices (Bittante et al., 2011a, b; Endrizzi et al., 2013). Flavor is a complex trait, composed principally of the sensations of aroma and taste (McSweeney et al., 1997). Milk and cheese flavors are influenced by a variety of volatile and nonvolatile substances (Drake, 2007; Bergamaschi et al., 2015), and as some of these are sulfur compounds (Sreekumar et al., 2009), the enrichment of milk and cheese with undesired flavors is of concern. In fact, many milk and cheese factories do not accept milk produced from cows fed garlic because of off flavors. Surprisingly, we found no mention of this shortcoming in papers reporting on the addition of garlic, or garlic compounds, to the feed of dairy cows. However, the presence of odorous metabolites in the cheese is not necessarily a defect, as many traditional cheeses containing garlic are produced in many parts of the world (Park, 1990; Regu et al., 2016).

A review of the scientific literature reveals a lack of information on the influence of garlic on the flavor of milk. In part, this is due to the difficulties of analyzing sulfur compounds, because of their volatility, sensitivity to oxidation and heat, and presence at very low concentrations (Sreekumar et al., 2009). The recent development of model cheese-making procedures has made it possible to carry out experiments on a large number of individual samples (Cipolat-Gotet et al., 2013), and offers new research opportunities in this field as it is now easier to study the influence of different feeding treatments on milk and cheese characteristics.

In our experiment, we found that garlic, particularly at high doses, influenced olfactory intensity and garlic-like aromatic notes in both milk and cheese. However, the sensory attributes of milk and cheese from cows fed supplements of diallyl sulfide or garlic cloves at

the lowest dosage were often no different than those of controls. It should be borne in mind that sulfur compounds, including diallyl sulfide, occur naturally in milk and cheese, conferring garlic flavor, and their concentrations increase with the aging of the cheeses to impart several typical aromatic notes (McGorrin, 2011). These sulfur compounds mainly arise from catabolism of L-methionine by bacteria and yeasts, but L-cysteine probably has a greater role in the release of volatile sulfur compounds imparting garlic-like flavor in cheese (Sutherland et al., 2003).

Garlic is considered to be a taste and flavor enhancer because of its high concentrations of organic sulfur compounds. In our research, 400 g/d of garlic cloves increased the salty taste of milk, the sourness of cheese, and the flavor intensity of both milk and cheese. Some garlic compounds, such as the ɣ-glutamyl derivatives of S-substituted cysteines found in garlic and in other plants of the Allium genus, may be responsible for these responses (Speranza and Morelli, 2012).

The garlic-like aroma and garlic-like flavor of the milk had close exponential relationships to the characteristics of the cheese derived from it. The intensity of these perceptions apparently decreased from the milk to the cheese with an exponential coefficient less than one (Figures 1 and 2). Sensorial perceptions are mediated by other compounds which are present in milk, and also in the cheese but in much greater amounts. The greater aromatic and flavor complexity in cheese may mask the olfactory notes of garlic and diallyl sulfide, and therefore suppress the effect of the garlic-like aroma/flavor (Speranza and Morelli, 2012). It should also be borne in mind that olfactory and flavor responses to sulfur molecules are generally non-linear, as perception differs at low concentrations and at higher concentrations, thereby altering the perception of garlic McGorrin, 2011).

Chemical and physical characteristics of milk and cheese

The properties of the sulfur molecules in garlic and their effect on microbial activity during cheese ripening may also be responsible for the observed alterations in the pH, color, shear force, and shear work of the cheese. Both diallyl sulfide and garlic at the highest dose reduced the shear force and shear work of the cheese compared with controls. This is consistent with results obtained by Regu et al. (2016) on cheese matrices with added garlic, and with results on other protein matrices obtained by Kim et al. (2009). Garlic compounds (polyphenols, such as flavonoids and sulfur-containing compounds) exert a strong antioxidant action on protein bonds, and intermolecular disulfide bridges may play a role in the protein strength of meat via oxidation of protein thiol (Wu et al., 2011). Although we did not look at the redox thiol/disulfide formation of cheese proteins in relation to the lowering of the rheological parameters, we might speculate that the samples with garlic at the higher dose and diallyl sulfide may have limited the protein thiol oxidation and the resulting disulfide formation in the cheese.

The results suggest that garlic at a high dose and diallyl sulfide affect cheese texture and limit the formation of disulfide bridges by reducing the cohesiveness of the protein network and the weakened cheese structure. The level of pH also seems to play a role in cheese texture. Lebecque et al. (2001) assert that “as the pH of cheese curds decreases, there is a loss of colloidal calcium phosphate from casein submicelles with a progressive dissociation of submicelles into smaller casein aggregates”. In addition, with the breakdown of casein micelles the protein surface is more exposed to the action of proteases, and the enzyme-substrate interaction is enhanced leading to a greater proteolytic effect (Upreti et al., 2006). Therefore, the low rheological values detected in the 400 g/d of garlic cloves and diallyl sulfide samples may also be due to the effect of pH, since these cheeses had lower pH values than the other samples.

CONCLUSIONS

We found that the use of garlic or diallyl sulfide has very little influence on DMI, milk yield and quality, cheese-making characteristics, nutrient recoveries, and cheese yields. However, garlic cloves in a dose of 400 g/d has a strong influence on the sensory characteristics of milk and cheese. Some rheological characteristics of the cheese, pH, color, and texture, appeared to be influenced by the administration of diallyl sulfide and garlic cloves. The current paper provided evidences that when garlic or garlic compounds are used to reduce enteric methane emissions, the sensory and rheological characteristics of milk and cheese are affected. However, the garlic-like aroma and the garlic-like flavor of milk and cheese are described by exponential relationships lower than 1.0, meaning that the increased sensory perception of garlic in milk is only partially reflected in cheese aroma and flavor.

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Table 1. Ingredients and chemical composition of the total mixed ration top dressed or not with garlic cloves or diallyl sulfide[1].

	Total mixed ration	Garlic cloves
Ingredients, g/kg DM		
Corn silage	291	-
Corn-barley based mixture	233	-
Sunflower-soybean based mixture	173	-
Alfalfa hay	123	-
Ryegrass hay	120	-
Sugar beet pulp	37	-
Mineral and vitamin mixture[2]	13	
Linseed	10	-
Chemical composition, g/kg DM		
DM (g/kg as fed)	462	744
Crude protein	130	192
Ether extract	34	5
NDF	375	62
ADF	219	25
ADL	37	3
Starch	253	-
NSC[2]	401	704
Ash	60	37
Total S, mg/g DM	1.27	8.45

[1] Diallyl sulphide, (Allyl sulfide, 0.97 pure, Sigma-Aldrich Chemical, Milan, Italy) with a stoichiometric S content of 0.281 ($C_6H_{10}S$), was top dressed in dose of 0 or 2 g/d, where garlic cloves were top dressed in doses of 0, 100 or 400 g/d.

[2] Milk H (Tecnozoo, Torreselle di Piombino Dese, Padova, Italy): 470,000 IU/kg vitamin A, 60,000 IU/kg vitamin D3, 2,000 mg/kg vitamin E, 200 mg/kg vitamin B_1, 150 mg/kg vitamin B_2, 100 mg/kg vitamin B_6, 0.3 mg/kg vitamin B_{12}, 12,000 mg/kg niacin, 40 mg/kg of biotin, 60 mg calcium (as calcium d-pantothenate), 2,000 mg/kg betaine (as betaine hydrochloride), 2,000 mg/kg betaine (as betaine hydrochloride), 1,000 mg/kg choline chloride, 300 mg/kg iron, 150 mg/kg iodine, 30 mg/kg cobalt, 300 mg/kg copper, 2,000 mg/kg manganese, 3,000 mg/kg zinc, 15 mg/kg selenium.

[3] Non-structural carbohydrates, computed as 1000 – EE – CP – NDF – ASH.

Table 2. Productive performance and milk quality of cows fed TMR supplemented or not with diallyl sulfide[1] (2 g/d) or with 100 or 400 g/d of garlic cloves.

	Treatments[2]				SEM[3]	Contrasts (*P*-values)		
	Control	Supplements				Control vs supplements	Diallyl sulfide vs garlic cloves	100 g/d vs 400 g/d
		Diallyl sulfide	Garlic cloves					
			100 g/d	400 g/d				
DMI[4], kg/d	23.1	23.8	23.6	23.0	1.17	0.78	0.72	0.70
Milk yield, kg/d	33.8	34.5	34.0	34.1	1.20	0.66	0.65	0.94
Milk yield/DMI	1.62	1.59	1.64	1.63	0.08	0.97	0.47	0.91
BCS[5]	2.62	2.69	2.69	2.62	0.04	0.35	0.51	0.27
Milk quality traits								
DM, %	13.0	12.9	12.9	13.1	0.37	0.80	0.80	0.35
Fat, %	4.17	4.05	4.06	4.27	0.33	0.86	0.61	0.40
Protein, %	3.43	3.39	3.35	3.34	0.15	0.014	0.14	0.91
Casein, %	2.63	2.63	2.58	2.59	0.13	0.14	0.09	0.67
Casein index, %	76.7	77.5	77.0	77.3	0.92	0.18	0.43	0.56
Lactose, %	4.89	4.96	4.93	4.95	0.04	0.10	0.54	0.70
SCS[6], units	1.74	2.76	1.93	1.64	0.74	0.54	0.13	0.70
pH	6.63	6.65	6.64	6.65	0.02	0.031	0.64	0.51
Milk protein profile								
α_{S1}-casein, %	34.0	33.6	33.7	33.8	0.41	0.47	0.71	0.86
α_{S2}-casein, %	7.06	6.94	6.77	7.16	0.43	0.80	0.95	0.40
β-casein, %	28.9	30.8	30.0	29.7	1.76	<0.001	0.002	0.41
κ-casein, %	9.28	8.62	8.95	8.74	0.48	0.26	0.62	0.67
α-lactalbumin, %	2.16	2.14	2.06	2.19	0.05	0.68	0.78	0.08
β-lactoglobulin, %	13.8	13.1	13.7	13.6	0.69	0.71	0.49	0.90

[1] Diallyl sulfide, 0.97 pure (Allyl sulfide, Sigma-Aldrich Chemical, Milan, Italy).
[2] n = 16 (4 periods × 4 cows).
[3] Standard error of the means.
[4] Dry matter intake.
[5] Body condition score (1-5 point scale).
[6] Somatic cell score = $3 + \ln_2$ (Somatic cell count × 10^{-5}).

Table 3. Milk coagulation properties (MCP), curd firming model parameters (CF_t), cheese yields and nutrient recoveries of cows fed TMR supplemented or not with diallyl sulfide[1] (2 g/d) or with 100 (or 400 g/d of garlic cloves.

	Treatments[2]				SEM[3]	Contrasts (*P*-values)		
		Supplements						
	Control	Diallyl sulfide	Garlic cloves			Control vs supplements	Diallyl sulfide vs garlic cloves	100g/d vs 400 g/d
			100 g/d	400 g/d				
MCP, traditional[4]								
RCT, min	27.8	27.2	29.9	27.8	1.48	0.66	0.21	0.15
k_{20}, min	6.82	6.75	7.27	7.54	0.57	0.43	0.20	0.63
a_{30}, mm	13.7	11.5	5.82	13.6	4.88	0.39	0.67	0.11
a_{45}, mm	35.9	36.3	30.4	34.9	3.46	0.47	0.23	0.19
a_{60}, mm	45.9	46.3	41.6	44.2	2.66	0.39	0.14	0.33
CF_t model parameters[5]								
$RCT_{eq,}$ min	28.0	27.5	30.1	28.0	2.03	0.66	0.16	0.25
$k_{CF,}$ min^{-1}	7.36	7.15	6.90	6.88	0.79	0.35	0.96	0.57
CF_P, mm	53.2	53.3	51.1	52.4	3.03	0.49	0.41	0.29
Cheese yield, g/kg								
CY_{CURD}	126	127	128	133	6.5	0.61	0.53	0.64
CY_{SOLIDS}	64.9	64.7	64.4	68.0	3.8	0.87	0.51	0.75
CY_{WATER}	61.2	63.7	63.0	64.8	4.1	0.43	0.66	0.94
Nutrient recovery[6], g/kg								
$REC_{PROTEIN}$	771	769	768	774	0.8	0.94	0.68	0.83
REC_{FAT}	866	870	862	869	16.2	0.95	0.73	0.82
REC_{SOLIDS}	511	507	506	521	15.4	0.99	0.53	0.75
REC_{ENERGY}	680	682	677	691	16.9	0.88	0.92	0.56
Production trait[7], kg/d								
dCY_{CURD}	4.17	4.14	4.29	4.47	0.15	0.47	0.42	0.25
dCY_{SOLIDS}	2.15	2.08	2.18	2.29	0.10	0.77	0.43	0.27
dCY_{WATER}	2.03	2.08	2.10	2.17	0.10	0.36	0.58	0.61

[1] Diallyl sulfide, 0.97 pure (Allyl sulfide, Sigma-Aldrich Chemical, Milan, Italy).
[2] n = 16 (4 periods × 4 cows).
[3] Standard error of the means.

[4] RCT = Rennet coagulation time, k_{20} = curd firming time; a_{30}, a_{45}, or a_{60} = curd firmness after 30, 45 or 60 min from rennet addition, respectively.
[5] RCT_{eq} = Rennet coagulation time, k_{CF} = curd-firming rate constant, CF_p = asymptotical potential value of CF at an infinite time (Bittante, 2011).
[6] REC = recovery of milk protein, fat, solids and energy in the curd.
[7] dCY = daily cheese yields of curd, solids and retained water (CY × daily milk yield).

Table 4. Sensory traits on raw milk, sensory traits and rheogical analysis on ripened cheese of cows fed TMR supplemented or not with diallyl sulfide[1] (2 g/d) or with 100 or 400 g/d of garlic peeled cloves.

	Treatments[2]					Contrasts (*P*-values)		
		Supplements						
			Garlic cloves					
	Control	Diallyl sulfide	100/d	400g/d	SEM[3]	Control vs supplements	Diallyl sulfide vs garlic cloves	100/d vs 400g/d
Milk:								
Aroma								
Overall intensity	2.80	2.46	2.95	4.36	0.23	0.09	<0.001	<0.001
Garlic-like	1.24	1.44	1.38	4.65	0.23	<0.001	<0.001	<0.001
Taste								
Sweetness	5.10	4.94	4.98	4.52	0.24	0.38	0.52	0.19
Saltiness	2.63	2.44	2.44	2.83	0.13	0.69	0.22	0.041
Bitterness	1.87	1.91	1.99	2.36	0.16	0.25	0.16	0.10
Flavor								
Overall intensity	4.69	4.63	5.04	5.39	0.23	0.97	0.033	0.28
Garlic-like	1.51	1.83	1.83	6.39	0.27	<0.001	<0.001	<0.001
Cheese:								
Aroma								
Overall intensity	3.96	4.20	3.66	4.53	0.16	0.37	0.62	<0.001
Grass-like	3.37	3.48	3.11	3.32	0.13	0.63	0.09	0.24
Fruit-like	2.77	3.07	2.73	2.68	0.14	0.71	0.031	0.78
Garlic-like	1.27	1.12	1.50	2.89	0.14	<0.001	<0.001	<0.001
Taste								
Sweetness	3.23	3.46	3.05	3.25	0.14	0.89	0.08	0.33
Saltiness	3.30	3.48	3.84	3.61	0.14	0.044	0.18	0.26
Sourness	2.45	2.48	2.57	3.11	0.17	0.17	0.09	0.030
Bitterness	2.14	1.89	2.09	2.52	0.19	0.91	0.09	0.12
Flavor								
Overall intensity	4.23	4.80	4.61	5.12	0.20	0.009	0.80	0.07
Garlic-like	1.73	1.27	2.59	4.50	0.24	<0.001	<0.001	<0.001
pH	5.33	5.21	5.30	5.27	0.02	0.007	0.02	0.31
Color[4]								
L*	66.7	66.5	67.0	64.9	2.53	0.12	0.12	<0.001
a*	0.25	0.39	0.16	0.18	0.11	0.81	<0.001	0.71
b*	25.1	25.4	24.5	24.7	2.23	0.16	<0.001	0.18
Shear force[5], N	32.7	30.1	34.7	29.6	6.21	0.17	0.032	<0.001
Shear work[6], $J \times 10^{-3}$	140	124	145	124	0.20	0.045	<0.001	0.003

[1] Diallyl sulfide, 0.97 pure (Allyl sulfide, Sigma-Aldrich Chemical, Milan, Italy).
[2] n = 16 (4 periods × 4 cows).
[3] Standard error of the means.
[4] CIElab = L* = reflexion index; a* green-red index; b* blue-yellow index.
[5] Maximum force required to cut the sample.
[6] Work done to cut the sample.

Figure 1. Relationship between milk and ripened cheese garlic-like aroma ($P < 0.01$) of cows fed TMR supplemented or not with 2 g/d of diallyl sulfide (Sigma-Aldrich Chemical, Milan, Italy) or with 100 (G100) or 400 (G400) g/d of garlic cloves.

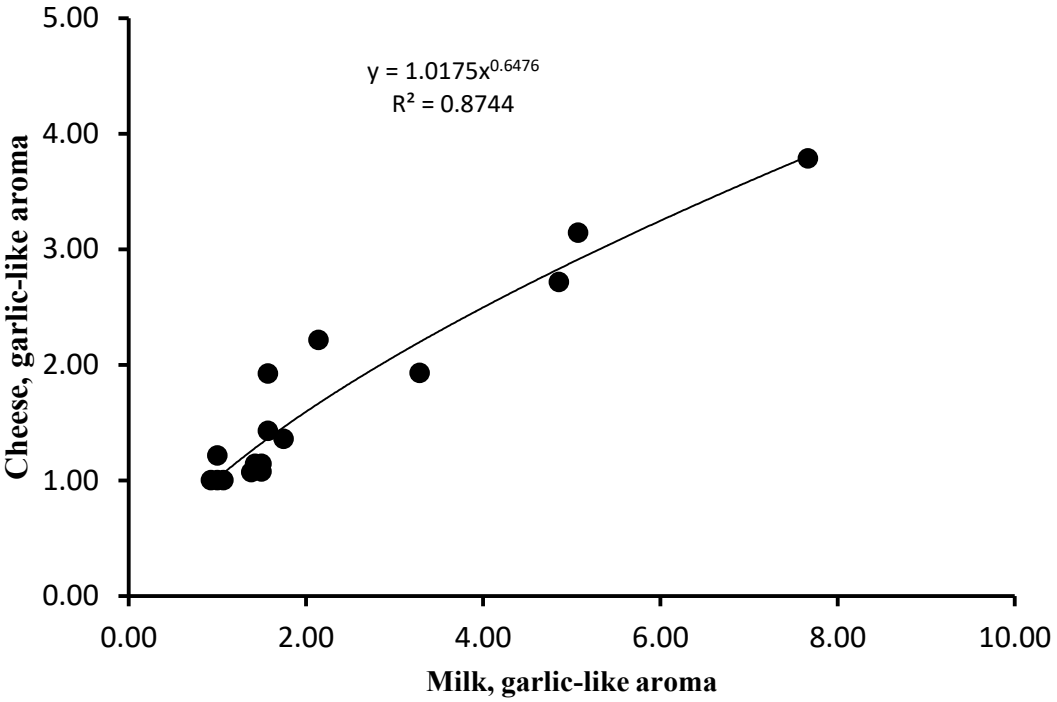
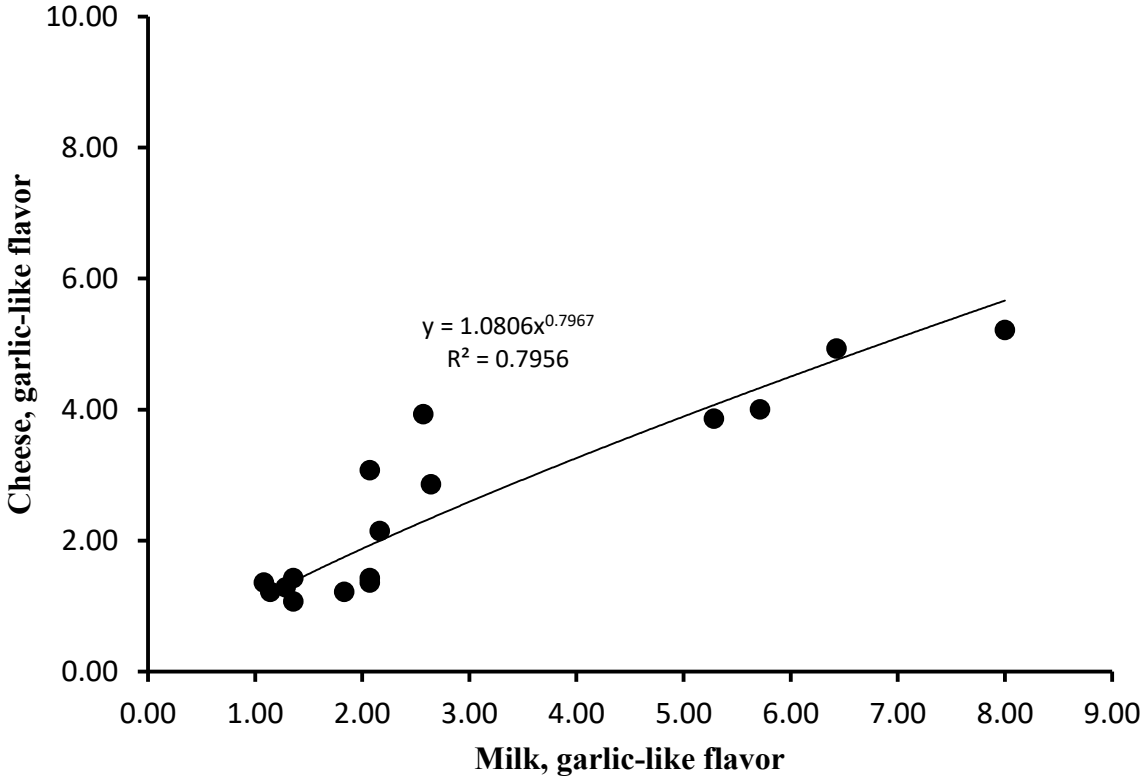


Figure 2. Relationship between milk and ripened cheese garlic-like flavor of cows fed TMR supplemented or not with 2 g/d of diallyl sulfide (Sigma-Aldrich Chemical, Milan, Italy) or with 100 (G100) or 400 (G400) g/d of garlic cloves.



8 General conclusion

Basing on the three years of research and on results obtained in the different experiments, the first screening study allowed better elucidate effects of pure extracts. The study onfirmed their effectiveness on *in vitro* fermentation and CH_4 production, however, different compounds lead to different effect. Indeed,. the pure compounds influenced *in vitro* fermentation and CH_4 production in a dose-dependent manner. The high dosage of limonene and allyl-sulfide showed to reduce CH_4 production notably *in vitro*, but such positive effect was accompanied by an overall impairment of rumen fermentation, especially in the case of limonene. The high dosage of allyl sulfide reduced *in vitro* GP and CH_4 production by about -15 and -32%, respectively, while manteining the feed degradability, showing an intermediate effect compared to limonene and cinnamaldehyde. Most promising results were observed for cinnamaldehyde that, when used at the high dosage, reduced gas and CH_4 production, without compromising the rumen degradability and VFA production. The high dosage of monensin determined the expected effects on rumen fermentation. However, the *in vitro* conditions and procedures are crucial. It then became necessary to verify if the adaptation of the rumen fluid, used as inoculum, to the extracts, may affect the fermentation and CH_4 production in batch culture system condition. In view of this, the second experiment included the active extracts in the short term on *in vitro* fermentations (allyl sulfide, cinnamaldehyde and limonene), but with a different procedure: the *in vivo* adaptation before the use of the rumen fluid as inoculum. It is clear that effectiveness of a given compound is real when it has a long-term effect on rumen fermentation, to highlight possible resistance by microbial population against the same compounds. In general, the use of adapted fluid did not affect any fermentation parameter compared to non-adapted fluid, but adding *in vitro* diet with allyl sulfide, the magnitude of the effects of this pure compound tended to be greater when incubated with adapted fluid compared to non-adapted fluid. These results suggest that the administration of

pure compounds to the cows can affect the rumen microbial activity and the response of *in vitro* short-term experiments.

The duration of *in vitro* incubation is very important to assess the real potential of the compounds. The long-term system aids in avoiding the discarding of compounds that may have needed a longer time to be effective on rumen fermentation. The RuSiTec is a continuous culture system, which maintain the rumen fluid vitality for several days. 1 and 2 g/L doses of isoflavonoid-rich extract were tested for their long-term effectiveness, a strong antiprotozoal effect, a decreased in ammonia concentration and increased propionate molar proportion have been found, without impairing the overall fermentation process. Liquorice extract added at 1 g/L decreased ammonia production without affecting the overall fermentation process. When added at 2 g/L, decreases in ammonia production, methane and total VFA production were observed. These effects in fermentation were probably related to decreases in protozoa numbers, a less diverse bacteria population, as well as changes in the structure of both bacteria and archaea communities. The inclusion of an isoflavonoid-rich extract from liquorice in the diet could potentially improve the efficiency of the feed utilisation by ruminants. While we speculate that the observed effects could be attributed to the high content of isoflavonoids, and particularly glabridin, the contribution of other phytochemical to the reported effects cannot be ruled out.

The use of plant secondary metabolites as a feed additive on rumen fermentation and methane emission is largely studied, however, the influence of this compounds on the production is very little, especially on the organoleptic profile and technology traits. Garlic (*Allium sativum*), and particularly its sulfur compounds, inhibits methanogenesis *in vitro,* and consistently increases the acetate: propionate ratio and butyrate concentration in the rumen fluid. The effect is similar, but not identical, to that of the antibiotic monensin, and reflects the anti-microbial and anti-protozoal properties of organosulfur garlic compounds. The last

experiment reveiled that the use of garlic or allyl sulfide has very little influence on DMI, milk yield and quality, cheese-making characteristics, nutrient recoveries, and cheese yields. However, garlic cloves in a dose of 400 g/d has a strong influence on the sensory characteristics of milk and cheese. Some rheological characteristics of the cheese, pH, colour, and texture, appeared to be influenced by the administration of allyl sulfide and garlic cloves. These results suggest that when garlic or garlic compounds are used to reduce enteric methane emissions, we can expect the sensory and rheological characteristics of milk and cheese to be affected. However, the garlic-like aroma and the garlic-like flavour of milk and cheese are described by exponential relationships lower than 1.0, meaning that the increased sensory perception of garlic in milk is only partially reflected in cheese aroma and flavour.

To date, no feed additives have demonstrated sustained reduction in CH_4 emissions without a negative effect on milk production or on the organoleptic profile of the dairy product in lactating dairy cattle. However, research should continue in identifying and developing rumen modifiers because of its value in elucidating rumen microbial interactions and increasing our knowledge of rumen function.

9 List of publications

9.1 Journal publications

Rossi, G., Tagliapietra, F., Pinloche, E., De La Fuente, G., Schiavon, S., Maccarana, L., Bittante, G., Newbold, C.J. 2015. The physical form of corn influences the rumen bacterial biodiversity–preliminary results, Poljoprivreda 21: (1) Dodatak, 93-96, DOI: 10.18047/poljo.21.1.sup.21.

Tagliapietra, F., Rossi, G., Schiavon, S., Ferragina, A., Gotet Cipolat, C., Bittante, G. 2015. Use of fourier transform infrared (FTIR) spectroscopy to predict VFA and ammonia from in vitro rumen fermentation, Poljoprivreda 21:(1) Dodatak, 130-134, DOI: 10.18047/poljo.21.1.sup.30.

Cattani, M., Maccarana, L., Rossi, G., Tagliapietra, F., Schiavon, S., Bailoni, L. 2016. Dose-response and inclusion effects of pure natural extractsand synthetic compounds on in vitro methane production, Anim Feed Sci Technol 218:2016, 100–109, DOI: 10.1016/j.anifeedsci.2016.05.014.

9.2 Oral presentations

The physical form of corn influences the rumen bacterial biodiversity– preliminary results - Oral presentation. 23rd International Symposium Animal Science Days (ASD) on "Utilization of local animal breeds and production systems in sustainable production of high quality animal products". Croatia, September 21-24th, 2015.

Effect of rumen fluid collected from cows exposed or not to pure extracts on the in vitro gas production kinetics, fermentations and methane production. - Oral presentation. 67th Annual Meeting of the European Federation of Animal Science (EAAP) on “Sustainable Food Production: Livestock's Key Role”, UK, 29st of August to 2th of September 2016.

Garlic as supplement for lactating cows: consequences on milk and cheese products. Oral presentation. 22nd Animal Science and Production Association (ASPA), Perugia, 12-16th June 2017.

APPENDIX 1

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THE PHYSICAL FORM OF CORN INFLUENCES THE RUMEN BACTERIAL BIODIVERSITY– PRELIMINARY RESULTS

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ABSTRACT

The aim of this study was to investigate the rumen bacteria in terms of genetic biodiversity and variation due to different physical form of corn in cow diet. A total of twenty dry cows were fed for 3 months with the same diet, only differed for corn physical form, ten received corn grains, white the other ones received corn flour. To investigate the biodiversity of the bacterial 16S rRNA gene clone library analysis has been built up and then the sequencing has been carried out using Ion Torrent PGM™ System. Bacterial population was tested using R statistical software. The Kruskal-Wallis one-way analysis of variance (Kruskal-Wallis, 1952) confirmed that the bacterial populations were different when the animals were fed with grain compared with flour corn. Both the OUT's abundance (Operational Taxonomic Unit) and the biodiversity indexes presented a significant difference among the two samples groups,

underlining the large changes that take place even with small diet modifications in ruminal environment. There is still the need to deepen how exactly the diet changes the rumen phylogenetic structure and the consequences on bacteria's activity.

Key words: rumen bacterial biodiversity, diet composition, physical form, OTUs.

INTRODUCTION

Dietary component and variation cause shifts in rumen bacterial ecology than can play a role in animal health and productivity. Because of this complexity further investigations are required. For several years the rumen has been studied for its role in nutrient digestion and to manipulate its microbial ecosystem to increase animal performance and efficiency.

Microbial population is not stable and changes according to ruminal environmental characteristics and diet (Biavati and Mattarelli, 1991; Tajima et al., 2000). Feeding large proportions of starch to ruminants increase rumen microbial activity and the animal productivity but on the other hand, can negatively affect the rumen environment and its functionality, the fibre digestibility, and the animal health (Theurer, 1985).

The use of grain instead of cereal flour in ruminant nutrition can affect the site of starch utilization leading to a shift from the rumen to the intestine with positive effect on efficiency of energy utilization and on rumen environment. For this reason there is a great interest to investigate the impact of the physical form of cereals on the rumen microbial population diversity. New technologies of DNA sequencing ("ultra-high throughput" Ion Torrent Personal Genome Machine, PGM) allow the simultaneous analysis of huge amounts of sequences at very low cost, improving accuracy in quantification, enabling the identification even of minor species. The PGM™ System has been used by Patel et al (2014) to described rumen microbiome of Indian cattle (Kankrej breed) under different dietary treatments, where cattle were gradually adapted to a high-forage diets. The study revealed significant differences

between all the diet treatments. The aim of this study was to investigate the rumen bacteria in terms of genetic biodiversity and its variation feeding finishing dry cows with corn grain and flour.

MATERIAL AND METHODS

A total of twenty dry cows of three different breeds (Holstein, Brown Swiss, and Simmental) reared in L. Toniolo experimental farm (Legnaro, PD, Italy) were fed for 3 months with a finishing diet composed by corn silage 44.6%, corn 34.2%, sunflower 8.3%, straw 5.8%, sugar beet pulp 3.7%, others additives 3.4% of DM (chemical composition: starch 38.8%, NDF (Neutral Detergent Fiber) 37.4%, crude protein 10.9%). The cows were divided in 2 homogeneous groups fed with the same diet that differed only for the corn physical form, ten received corn grains, while the other ones received corn flour. Rumen fluid samples were collected from each one using rumen probe and stored at -80°C. Subsequently, the DNA has been obtained using a method based on guanidine hydrochloride buffer and common DNA extraction columns (Yaffe et al., 2012) and then purified with silica and DNAse (Rohland and Hofreiter, 2007). After the isolation the bacterial 16S rRNA gene clone library analyse has been built up and then the sequencing of V1-V2 region has been carried out using Ion Torrent PGM™ System. After sequencing, data were combined and sample identification numbers assigned to multiplexed reads using the MOTHUR software environment (De La Fuente et al. 2014). Data were denoised, low quality sequences, pyrosequencing errors and chimeras were removed, then sequences were clustered into OTU's at 97% identity using the pipeline available from http://www.brmicrobiome.org/#!16s-profiling-ion-torrent/cpdg (Pylro et al., 2014). OTU's containing fewer than 5 reads were excluded due to the likelihood of them being a sequencing artifact. Samples were normalised by randomly resampling to the lowest number of sequences per sample using Daisychopper (De La Fuente et al., 2014). The OTUs' study was made using R software. A principal component analysis (PCA), and subsequently,

a k-mean cluster analysis, were performed to test the whole dataset without any prior information. The cluster analysis has shown that the cow breed did not affected the separation of samples in different groups so this factor has been removed from analysis. After this preliminary step the Kruskal-Wallis one-way analysis of variance (Kruskal-Wallis, 1952) has been applied to verify the difference between the two diets treatments. The number of sequences of each normalised sample was 14,289 sequences/sample and the number of sequences per each OTU was log transformed. Three indexes had been used to study the bacterial biodiversity. The Simpson's Index was computed as $D = \sum(n / N)2$, the Shannon's diversity index as $H = -\sum_{j=1}^{\%} p_j \ln p_j$ and Richness as mean of the number of OTUs of each sample.

RESULTS AND DISCUSSION

The 16S rRNA gene clone library analyses and the sequencing of V1-V2 region allowed to obtain 4.108 operational taxonomic units (OTUs) as sum of all samples analysed. As shown in Fig. 1 for grain and flour groups has been globally identified 3622 and 3089 different OUTs, respectively. The log transformation of the sequence number evidenced a different distribution of OTUs abundances among diet treatments. In almost all the OTUs, the grain group had an higher abundance of sequence, compared to flour group, with the exception of only 7 OTUs where flour groups showed a much higher abundance.

The k-means cluster analysis graphically and clearly explained the differences among the groups and allowed to identify two clusters. As shown in figure 2, the two ellipses divided the twenty animals in two clusters that clearly identify the diet treatments even if there is an intersection area where some samples were not assigned and there is also an error of attribution in corn flour ellipse. The Kruskal test, used to statistically analyse the biodiversity indexes, identified an outlier within the corn grain group, that behaviours differently from the

other and was excluded from the statistical analysis. As reported in table 1, the diversity indexes were significantly different for flour and grain groups.

The Shannon index increases as both the richness and the evenness of the community increase, and evidenced that animal fed with corn grain instead of corn flour were characterized for a more biodiverse rumen microbial population. The richness index suggest that the grain group has a much higher diversity in term of OTUs number compared to the flour (1692 vs. 1261 OUTs, respectively). Finally, also the Simpson index, that measure the microbial dominance, suggest also a slightly higher evenness of microbial population of grain group compared to flour group.

To our knowledge, there are no studies that investigate the specific effect of diet physical form on rumen bacterial dynamics, however, some Authors, who have worked on different levels of forages and concentrates, reported important variations in the rumen bacterial biodiversity. In particular, Fernando et al. (2010) found a reduction of biodiversity increasing the proportion of concentrates in the diet. This result suggests that manipulation of diet have an important role on rumen bacterial selection. In the present study these sensible effects of diet treatments on rumen diversity can be related to the different fermentative properties of corn fed to the animal as whole grains or after milling. The reduction of grain particle size is commonly associate to an increase of rumen fermentation rate and to a reduction of starch passage rate (Theurer, 1985). The rumen degradation of starch stimulate the microbial activity and the production of high proportions of VFA but, at the sometime, the increase of starch fermentation in the rumen is commonly related to a reduction of cellulolytic bacteria activity and fibre digestion (Russell, 2002). Indeed, when the rate of VFA production overcome the buffering and absorption capacity of rumen, their accumulation lead to fluctuation of rumen pH and may have a selective effect on microbial population (Tajima et al., 2001). The reduction of bacteria biodiversity can impair the fermentative activity of the rumen microbial consortium (Wang and McAllister, 2002). Indeed, rumen bacteria adhere and colonize feed particles in the rumen, however, not all bacteria are equipped with a complete array of digestive enzymes. Co-culture of different microbial species demonstrated the importance of cross-feeding among bacterial species in attaining greatest bacterial growth rates and complete digestion of feed (Huntington, 1997).

CONCLUSIONS

The present study confirmed the significant difference between rumen bacterial populations in cows fed corn with different physical form within the same diet. Thus underline the extreme dynamism of the bacteria and the susceptibility to even small changes in diet composition. There is a different rumen environmental equilibrium for the two theses that proved the variation in terms of bacterial diversity. The technology applied in this research does not allow to investigate the rumen bacterial activity. Anyway, this work represents a preliminary study that requires further investigations to understand the relation between the physical form of diet and the phylogenetic structure of the rumen population.

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Figure 1. OTUs and Sequences graphical distribution for both groups (Flour and Grain corn).

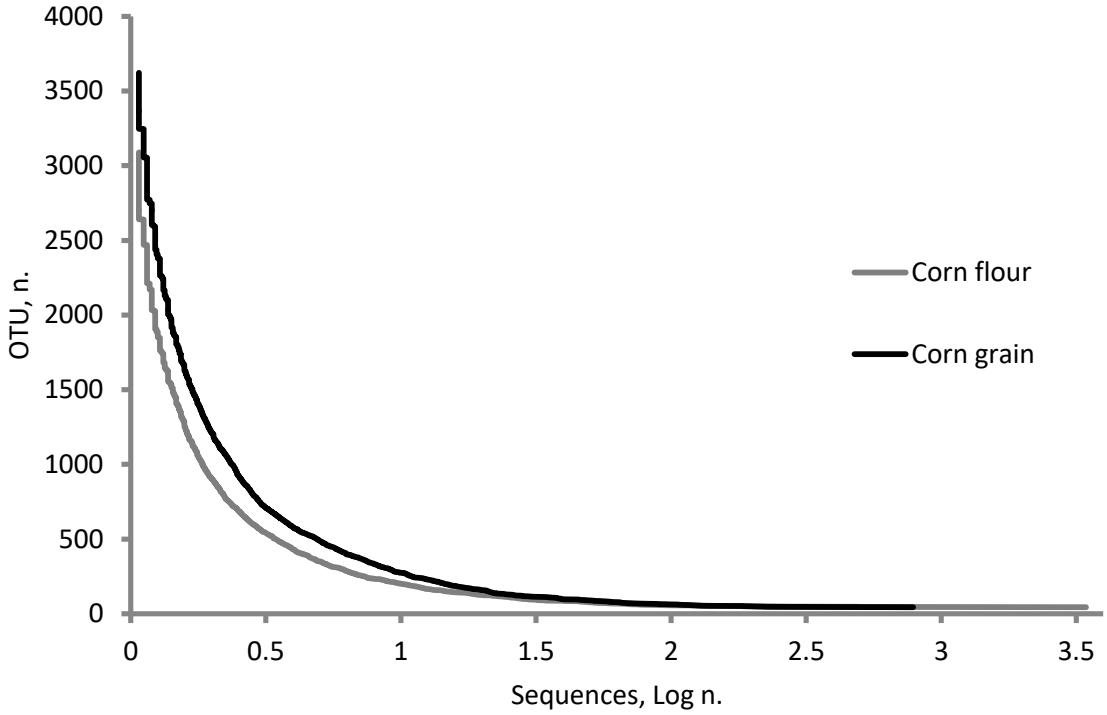

Figure 2. OTUs Cluster Analysis regarding principal component 1 (PC1) and principal component 2 (PC2).

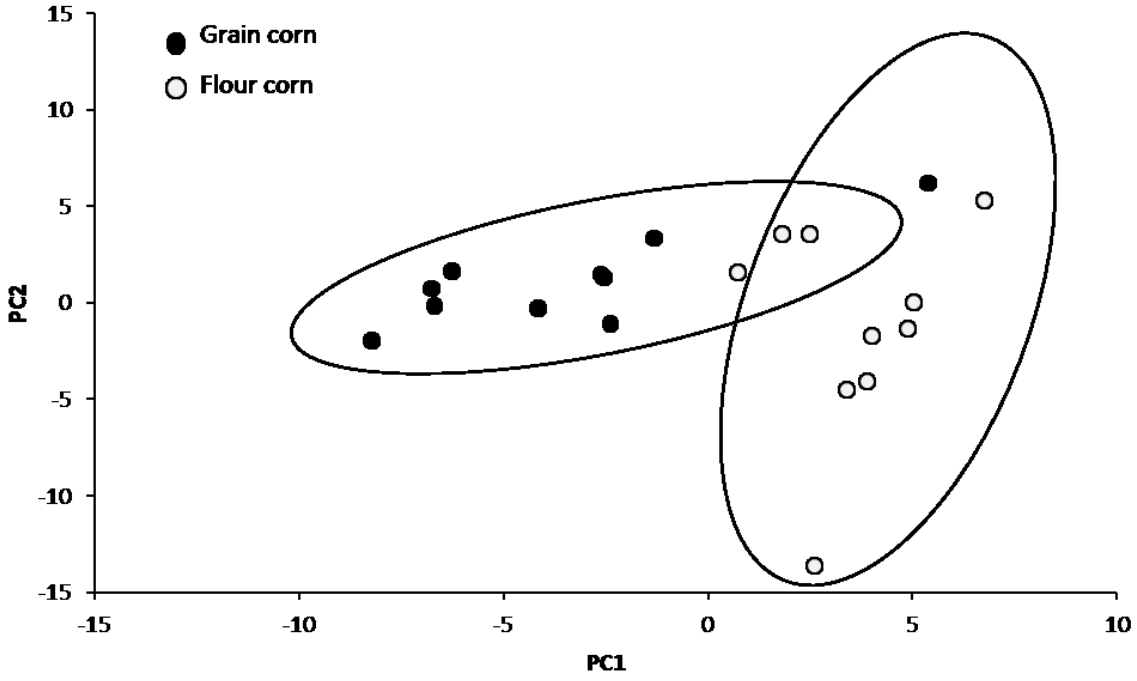

Table 1. X^2, mean of corn flour group (flour) and corn grain group (grain) and P-value of Simpson, Shannon and Richness diversity indexes.

Biodiversity Index	X^2	Corn treatment		*P-value*
		Flour	Grain	
Shannon	5.85	4.29	4.9	0.015
Richness	7.4	1261	1692	0.006
Simpson	3.72	0.91	0.89	0.053

APPENDIX 2

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USE OF FOURIER TRANSFORM INFRARED (FTIR) SPECTROSCOPY TO PREDICT VFA AND AMMONIA FROM *IN VITRO* RUMEN FERMENTATION

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ABSTRACT

The aim of present study was to develop a FTIR method to quantify amounts and proportions of volatile fatty acids (VFA) and ammonia nitrogen (N-NH3) in fermentation fluids collected in vitro using innovative Bayesian models as chemometric technique. A set of 170 fermentation fluids, collected before and after 4 in vitro incubations of 8 diets in 5 replication plus 5 blanks, were analysed for VFA, N-NH3 and scanned using the MilcoScan FT2 (Foss Electric, Hillerød, Denmark) in the spectral range between 5,000 and 900 cm-1. A Bayes B model was used to calibrate equations for each of the fermentative traits. The calibration equation predict well VFA and ammonia amounts in calibration and also in validation (R2VAL ranged from 0.93 to 0.83 for iso-valeric and n-butyric acid respectively). Whereas, the prediction of VFA expressed as proportions of total amount was much less accurate (R2VAL ranged from 0.81 to 0.52 for iso-valeric and N-butyric acid respectively). In

conclusion, the FTIR and Bayesian models can be used as simple, economic and rapid tool to accurately predict VFA amounts in vitro.

Key-words: mid infrared spectroscopy; in vitro rumen fermentation; Bayesian regression model

INTRODUCTION

Volatile fatty acids (VFAs) and ammonia nitrogen ($N\text{-}NH_3$) are the mean product of microbial fermentation and their production reflect the diet degradation in the rumen and the production of energy and nutrients for the animal (Tagliapietra et al., 2011). The Fourier Transform Infrared (FTIR) spectroscopy has been apply in many different fields because it is simple, rapid, economic and don't require sample pre-treatments. For these reasons, FTIR can be a useful tool to evaluate fermentative parameters of samples collected both in *in vitro* and *in vivo* study (Udén and Sjaunja, 2009; Bhagwat et al, 2012). In our knowledge, in literature, few attempt has been done to predict VFAs expressed as proportions of total VFA amount with FTIR. Different statistical approaches were used to calibrate FTIR equipment like the Partial Least Square Regression (PLS) that is a popular multivariate calibration technique used to analyse spectra data. Recently, Ferragina et al. (2005) compared the traditional PLS approach with diverse Bayesian regression models, commonly used for genomic selection, founding the "Bayes B" model as a powerful predictor of milk properties. Therefore, the present work aims to develop a FTIR method to quantity amounts and proportions of VFA and $N\text{-}NH_3$ in fermentation fluids collected *in vitro* using the Bayes B regression model.

MATERIAL AND METHODS

Data of 4 *in vitro* incubations were used to calibrate the FTIR equipment. Two incubations were stopped at 24 h, whereas the other two lasted 48 h. In each incubation were tested 8 diets in 5 replication plus 5 blanks, where the buffered rumen fluid (RF) was incubated without any

substrate. At the beginning of incubation were also collected 3 samples of RF, of buffer and of their mix. A total number of 54 samples per incubation were collected and stored for chemical and FTIR analysis. The tested diets were formulated for lactating cows and were differing for fibre, crude protein, lipids and starch content to be representative of Italian intensive dairy system (Dal Maso et al., 2009). Of each test diet, 1 g of sample was incubated with 100 ml of buffer (Menke and Steingass, 1988) and 50 ml of RF collected from 3 dry cows as described by Tagliapietra et al. (2012). The fermentations were monitored using a fully automatic gas production system described by Tagliapietra et al. (2010). RF, buffer and buffered RF at the beginning of incubation and fermentation fluid at the end of incubation were sampled for the immediate infrared (IR) analysis and others two aliquots were stored at −20°C with metaphosphoric acid (25%, w/v) until chemical analysis. The N-NH_3 content was measured using a FIAstar 5000 analyzer FOSS according a colorimetric method (Cataldo et al., 1975). The VFA profile 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). The Fourier transform equipment, designed for milk analysis (MilcoScan FT2, Foss Electic A/S, Hillerod, Denmark), was used for scanning the fresh samples within 3 h from collection over the spectral range of 5000 to 900 waves × cm^{-1}. Two spectral acquisitions were carried out for each sample, and the results were averaged before data analysis. For technical reasons, for 1 incubation, 3 of 5 sample replications and also the samples collected at the beginning of incubation were not scanned. The Mahalanobis distances were used for the detection of outliers, and the spectra showing a distance higher than three times the standard deviation were discarded. Data editing was done in R environment (R Core Team, 2013). Finally 170 spectra were available for the study. A Bayesian model (Bayes B), implemented in the open-source R-software BGLR (Pérez and De

Los Campos, 2014), was used to calibrate equations for each of the fermentative traits as recently described by Ferragina et al. (2015). The calibration was performed on a random dataset of 80% of data values available using as index of prediction accuracy the determination coefficient calculated as square of the correlation between observed and predicted values in the calibration dataset (R^2_{CAL}). The remained 20% of data values were used in validation, calculating the R^2 as square of the correlation between observed and predicted values in validation dataset (R^2_{VAL}); The calibration-validation procedure was repeated 20 times for each trait, this procedure guarantees that all the records are in either, the calibration and the validation set but never contemporarily.

RESULTS AND DISCUSSION

The mean values and the standard deviation of the fermentative parameters used in the study are given in table 1. On average the concentration of total VFA in fermentation fluid was about 3.06 g/L with a large variability (SD ±1.06 g/L) that reflect the different degradability of diets incubated but also the inclusion in the calibration set of RF (on average 3.77 g/L of total VFA), buffer (without VFA) and buffered RF (1.39 g/L of total VFA) collected at the beginning of incubation. The variability of VFA parameters expressed as proportion of total VFA was much lower. The SD of VFA proportions expressed as percentage of mean values ranged from 6.2% to 22.3% respectively for acetic acid and N-valeric acid. Also the N-NH_3 concentration in the calibration set showed a large variability both for different CP content of fermented diets and the inclusion in the data set of RF (74 mg/L), buffer (172 mg/L) and buffered RF (132 mg/L). Finally, the pH was on average close to 6.8 and rather stable among samples for the high concentration of bicarbonate in the medium used to buffer acids and ammonia produced throughout the fermentations. Therefore, except for pH, the variability of measurements was comparable to that reported by previous studies (Udén and Sjaunja, 2009)

and allows the development of robust calibrations. The sample spectra were homogeneous but some outliers were identified probably for the presence of small particles in suspension that could interfere with the instrument sensors design for milk analysis.

The determination coefficients between the measured and predicted values obtained in calibration (R^2_{CAL}) and validation (R^2_{VAL}) datasets are given in Table 1. The amounts of VFAs were accurately predicted as shown by the R^2 that on average ranged between 0.97 to 0.93 of respectively acetic acid and N-butyric acid. Moreover, the individual calibration, repeated 20 times, always exceed 0.90 R^2_{CAL}. In validation the performance of prediction remain high with mean R^2_{VAL} values always greater than 0.90 with the only exception of n-butyric acid (R^2=0.84) with a higher variability among individual validation runs. Also the values of $RMSE_{VAL}$, suggest an expected error analysing an external sample of about 0.15, 0.08 and 0.06 g/L for acetic acid, propionic acid and n-butyric acid respectively. Udén and Sjaunja (2009) reported comparable performance of calibration working with semi artificial rumen fluids, obtained removing the VFA naturally present in the samples by acidification and adding defined amounts of acetic, propionic, n-butyric acid and bicarbonate.

The prediction accuracy of VFA values, expressed as proportion of total amounts, was acceptable in calibration with R^2 that ranged between 0.92 to 0.69 for valeric acid and n-butyric acid respectively. Whereas the correlation between measured and predicted values decreased in validation but with large differences among VFAs. The ability of the model to predict the N-NH_3 in fermentation fluids was slightly lower compare to VFA amounts as evidenced by the lower R^2_{VAL} and the higher $RMSE_{VAL}$.

In Figure 1 the correlation coefficients between the trait and each wavelength absorbance, and the estimated coefficients of the Bayes B equations for the prediction of acetic acid are shown. For the prediction of the quantity of acetic acid (Figure 1a), it could be seen that the

absorbance recorded at several wavelength is correlated with the measured VFA, but only in two cases the correlation coefficient approached 0.50.

In both cases the spectral areas more correlated were close to the spectral regions characterized by the large variability of absorbance due to water bonds (SWIR-MWIR and MWIR2 regions as classify by Bittante and Cecchinato, 2013): the first, negative, in the SWIR region, and the second, positive, in the area between the MWIR1 and MWIR2 regions. The Bayesian method B selected one wavelength in this last area (1721 cm^{-1}) as the most important one for predicting acetate quantity. This specific wavelength correspond to the absorption peak of C=O bond of the carboxylic group (Bittante and Cecchinato, 2013). This result clearly evidences a direct relation between the prediction model and the chemical-physical properties of acetic acid. A similar condition was observed for the others VFAs and for $N\text{-}NH_3$. A different behaviour has been observed when VFA were expressed as proportion of their total amount. The pattern of correlations between absorbance at individual wavelengths and the acetate proportion is similar in shape, lower in extension and often opposite in sign than when acetate is expressed in g/L (Figure 1b). To predict the acetic acid proportion, the Bayes B model attributed a high coefficient to the wavelengths 4356, 3989 and especially 1644 waves × cm^{-1} with a negative, positive and negative sign respectively. These wavelengths are not directly linked to the absorbance properties of acetic acid and the prediction depend to the correlation between acetic acid proportion and other chemical compounds in the fermentation fluid. *In vitro* experiments aimed to evaluate the effects of different feed combination or additives (Cattani et al, 2012) would take benefits from contemporary measurements of FTIR predicting changes in VFA composition.

CONCLUSION

The FTIR technique, calibrated using a Bayesian regression approach, was able to predict accurately the VFA and ammonia amounts in fermentation fluids. Whereas, the prediction of VFA expressed as proportions of their total amount was much less accurate. This is due to the fact that FTIR absorbances are mainly related to the concentration of specific chemical bonds in the fluid and not to their proportions that seems to be predicted only in an indirect way on the bases of correlations with the amount of some compound present in the sample. For these parameters a greater number of samples in calibration set could be needed for a good prediction accuracy.

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Table 1. Statistics of samples used for the calibration, and prediction R-squared in calibration (R^2_{CAL}) and validation (R^2_{VAL}) and root mean square error in validation ($RMSE_{VAL}$).

	Mean	SD	R^2_{CAL}			R^2_{VAL}			$RMSE_{VAL}$
			Mean	Max	Min	Mean	Max	Min	Mean
VFA amounts, g/L									
- Acetic acid	1.76	0.60	0.97	0.98	0.95	0.92	0.97	0.83	0.15
- Propionic acid	0.71	0.26	0.96	0.96	0.95	0.90	0.96	0.76	0.08
- Iso-butyric acid	0.04	0.01	0.96	0.97	0.95	0.91	0.97	0.83	0.00
- N-butyric acid	0.43	0.17	0.93	0.94	0.91	0.84	0.91	0.65	0.06
- Iso- valeric acid	0.07	0.03	0.97	0.98	0.97	0.93	0.98	0.87	0.01
- N-valeric acid	0.06	0.02	0.96	0.97	0.95	0.91	0.96	0.81	0.01
Total VFA	3.06	1.06	0.97	0.98	0.97	0.93	0.97	0.85	0.26
VFA proportion, g/100 g									
- Acetic acid	58.1	3.60	0.87	0.91	0.85	0.64	0.80	0.43	2.1
- Propionic acid	22.9	2.27	0.81	0.87	0.76	0.55	0.82	0.36	1.5
- Iso-butyric acid	1.2	0.19	0.87	0.89	0.84	0.72	0.84	0.55	0.1
- N-butyric acid	13.7	1.92	0.69	0.75	0.65	0.52	0.75	0.36	1.4
- Iso- valeric acid	2.4	0.51	0.92	0.93	0.90	0.81	0.92	0.70	0.2
- N-valeric acid	1.8	0.39	0.92	0.95	0.90	0.77	0.89	0.55	0.2
Ammonia nitrogen, mg/L	176	47.3	0.86	0.89	0.84	0.73	0.86	0.50	23
pH	6.85	0.16	0.54	0.59	0.47	0.39	0.50	0.30	0.12

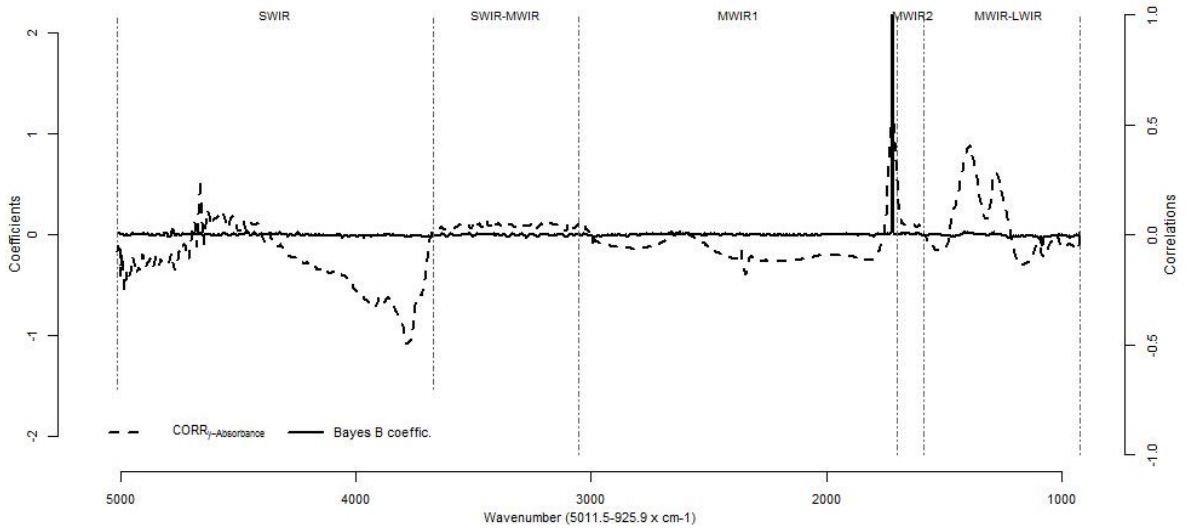
R^2_{CAL}= coefficient of determination calculated as the square of the correlation between observed and predicted values in calibration (80% of entire data set); Mean= mean of the R^2 of 20 replicas; Min= minimum value of R^2 obtained in 20 replicas; Max= maximum value of R^2 obtained in 20 replicas;

R^2_{VAL}= coefficient of determination calculated as the square of the correlation between observed and predicted values in validation (20% of entire data set); Mean= mean of the R^2 of 20 replicas; Min= minimum value of R^2 obtained in 20 replicas; Max= maximum value of R^2 obtained in 20 replicas;

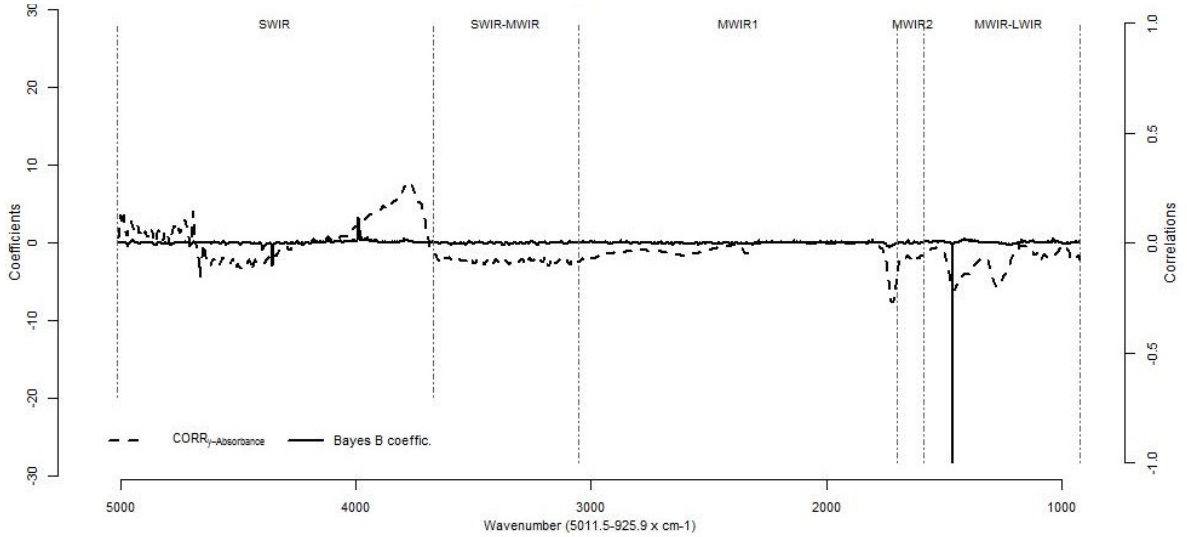
$RMSE_{VAL}$= mean of the root mean square errors in validation of 20 replicas.

Figure 1. Graphs of correlation coefficients (r) between *in vitro* chemical compounds (A: acetic acid mg/L; B: acetic acid, % VFA) and NIR spectrum wavenumber absorbance (dot line), and prediction equation coefficients of each spectrum wavenumber (solid line) between 5000 and 900 waves × cm^{-1}.

A) Acetic acid, g/L



B) Acetic acid, g/100 g VFA



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