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"Effect of plant compounds used as additives on *in vitro* rumen fermentation"

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Abstract

Farmed ruminants transform fibrous and nitrogen compounds of feeds into highly nutritive foods, such as milk and meat, and this ability is due to the presence of the rumen and its fermentation activity. The aptitude of ruminants to use feeds without competing with human food resources is a great help in terms of planet sustainability, given the expected increased demand for animal products in the next decades. However, the increase of livestock production can raise problems in the environment because of potential increased nitrogen pollution in water and enhanced greenhouse gas emissions (e.g. ammonia and methane).

The main purpose of the thesis was to test some plant compounds as modulators of the rumen fermentation towards greater nutritional efficiency and reduction of pollutants emissions. *In vitro* rumen fermentation systems (e.g. by using laboratory fermenters) were used as useful tools to study ruminal metabolism because the direct use of experimental animals (e.g. *in vivo* experiments) can raise ethical concerns and require long and expensive experiments.

The experimental section of the thesis is composed of four experiments.

The first experiment was held at the Veterinary Medicine in Vienna (Austria) and tested the dietary inclusion of an unexploited biorefinery by-product rich in fiber (an alfalfa silage cake, AC) and of a *Scrophularia striata* extract. Three diets with similar crude protein (CP) content were formulated; they contained the by-product AC, the original alfalfa silage, or a fiber-rich hay. The experimental diets were tested alone or added with the S. *Scrophularia striata* extract (6% of dry matter, DM). The inclusion of the biorefinery by-product reduced the rumen CP degradation ($P < 0.001$) and therefore it is an ingredient able to attenuate the rumen ammonia yield of diets. Sequencing of microbial DNA revealed several modifications of rumen microbiota due to the addition of *S. striata* extract, but no inhibitory effects on methanogens or depressions in rumen fermentation. Despite the absence of negative impacts on rumen fermentation the *S. striata* failed to show promising properties to mitigate rumen methane yield.

The second experiment studied the addition of chestnut tannin (CT) and a *Stevia rebaudiana* Bertoni (SB) extract on *in vitro* rumen fermentation. A control (CTR) substrate was fermented alone or added with 1.5% of CT or SB extracts in a batch culture system (Exp. 1) and in a subsequent continuous culture system (Exp. 2). Experiment 3 used the fermentation system of Exp. 1 and tested 7 doses of each extract added to CTR (from 0.2% to 1.4 % for 48 h). The addition of CT lowered ($P < 0.01$) the *in vitro* rumen ammonia concentration in all experiments and reduced the protozoa counts in Exp. 1 ($P < 0.05$). In contrast, the SB extract did not modify ammonia concentrations, but significantly lowered the protozoa counts in all 3 experiments (Exp. 1 and 2 $P < 0.05$; and quadratic reduction in Exp. 3, $P < 0.01$). Neither extracts affected the fermentation in terms of gas production

nor in volatile fatty acid (VFA) yield. Both studied substances had an impact on rumen metabolism, with SB reducing protozoa counts and CT lowering the rumen ammonia concentration and the effects of both extracts were appreciable at low dietary doses.

The previous study about SB and CT plant compounds was completed with a third experiment which was conducted at the University of Ljubljana (Slovenia). The specific aim was to confirm the antiprotozoal action of these plant products also in different *in vitro* experimental conditions (e.g. rumen fluid collected from cannulated sheep and small size batch fermentation system) and to evaluate any associate variation in CH_4 yield and in rumen fermentation. In this experiment both extracts were tested at 3 different levels of inclusion (0.75, 1.5 and 3% of incubated DM). The results showed that VFA concentration, their proportions and gas production were not modified by the plant extracts inclusion. Ammonia concentration decreased ($P < 0.05$) when 1.5 or 3% of CT were included into the diet. Rumen protozoa population was depressed by the SB inclusion ($P = 0.002$) with a maximum reduction of 40% at the highest SB dosage, whereas CT negatively affected total protozoa number (-19%) only at the dose of 3%. *In vitro* DM and NDF degradability were not affected by the supplementation of SB and CT, as well as the methane yield. Thus, the addition of SB and CT decrease with different intensity, the *in vitro* protozoa population of the rumen, without effects on fermentation parameters, apart from a reduction of nitrogen fermentability caused CT.

The last experiment was in collaboration with the University of Milan and examined the influence of essential oils extracted from *Achillea moschata* (three pure essential oils (camphor (CAM), bornyl acetate (BOR) and eucalyptol (EUCA) and two mixed essential oils) on rumen fermentation and CH_4 yield. In a first trial an *in vitro* batch fermentation system to measure gas production (GP) and CH_4 were used while in a second experiment the microbiota adaptation to pure oil components in a continuous culture system was considered. While there were no effects on the rumen fermentability measured as GP with the addition of the additives, a reduction of CH_4 as percentage on total GP at 48 h was recorded with EUCA and CAM addition ($P < 0.05$). In both experiments, all the compounds increased the total number of protozoa. Results of experiment 2 showed a total VFA decrease of about 20% for EUCA ($P = 0.029$) and consequently the stochiometric calculated CH_4 yield was lower ($P = 0.025$) for EUCA than the other treatments. The results of the present study showed that all the additives modulated rumen fermentation pattern and EUCA seems the most promising compound in terms of CH_4 reduction. Finally, all additives increased protozoa number in both experiments.

In present thesis we confirmed the role of tannins in reducing the rumen ammonia and their potential role as additives to mitigate N excretions by the ruminants. However, the most relevant and new result is the depression of rumen protozoa caused using *Stevia rebaudiana* Bertoni extract, which

was demonstrated in several independent experiments. On the contrary, in two experiments, protozoa were favorite by essential oil additions. Unfortunately, we were not able to find a close relationship between *in vitro* variations of protozoa population and methane yield, as suggested by most part of the literature. However, the important result is that we found some plant compounds able to modulate the presence of protozoa in the rumen. This can have some important implications because it is possible that rumen protozoa have an ideal dimension in the rumen microbiota to assure an optimal ruminal function.

In conclusion, further research efforts could be addressed to utilize such compounds as dietary additive suitable to adjust the development of the rumen protozoa population, according to the different dietary conditions.

Summary

Introduction 2

Thesis outline 5

Chapter 1. Literature review 8

1.1 Plant compounds used as additives in ruminant nutrition 8

1.2 Plants secondary metabolites 9

1.3 Tannins in ruminant nutrition 10

1.4 Saponins in ruminant nutrition 11

1.5 Essential oils in ruminant nutrition 12

1.6 Bibliographic review of the use of plant compounds on ruminant fermentation and microbiota 13

1.7 *In vitro* rumen fermentation systems 18

1.8 Batch systems 18

1.9 Continuous rumen fermenters (CCF) 23

Thesis aim 26

Chapter 2. A nutritional and rumen ecological evaluation of the biorefinery by-product alfalfa silage cake supplemented with Scrophularia striata extract using the rumen simulation technique 28

Appendix 1. Effects of *Scrophularia striata* extract on in vitro nutrient degradation and CH_4 formation when added to a diet containing a low-quality fibre source 52

Chapter 3. *In vitro* rumen fermentation of feed substrates added with chestnut tannins or an extract from *Stevia rebaudiana* Bertoni 56

Appendix 2. Effect of chestnut tannins and an extract from *Stevia Rebaudiana Bertoni* on in vitro rumen fermentation and microbiota 81

Chapter 4. Diets added with tannins from Chestnut wood or an extract from *Stevia rebaudiana* Bertoni and effects on *in vitro* rumen fermentation, protozoa count and methane production 85

Chapter 5. Dietary addition of two essential oils from *Achillea moschata* or their main pure components (camphor, eucalyptol and bornyl acetate) on *in vitro* rumen fermentation 100

Chapter 6. Comments and implications 124

References 134

Introduction

Farmed ruminants transform fibrous and nitrogen compounds of feeds into highly nutritive foods, such as milk and meat, and this ability is due to the presence of the rumen and its fermentation activity.

Fiber is not digestible by humans and monogastric animals, whereas it is a major component of ruminant diets (from 30 to 60 % of dry matter). Through rumen fermentation about 50 % of dietary fiber is transformed into absorbable nutritive compounds. However, rumen fiber degradation is associated with methane yield, because rumen methanogenesis is a physiological process to reduce hydrogen accumulation in the rumen. Methane is eliminated by eructation and it can contribute to about 25 % of the total anthropogenic greenhouse gas (GHG) production (Jones, 2016). Additionally, GHG emission represents an important dietary energy loss for the ruminant (Broderick, 2017).

Low-quality feed protein and nonprotein nitrogen compounds of ruminant diet are degraded by rumen microbiota to ammonia, which is then converted into proteins of microbes. The efficiency of ammonia conversion into microbial protein depends from several dietary factors and diets should be balanced to avoid the discharge of part of ammonia into urine as urea. However, the microbial protein is a protein source of high biological value for ruminant and protein transformation in the rumen places ruminants in a more efficient production position than other farm animals (such as swine and poultry). In fact, according to Wilkinson (2011), the output of animal protein in products (such as milk or meat) relative to the input of human-edible protein consumed by an animal is approximately 0.86 kg/kg for beef and milk production vs a mean of 0.43 for poultry, eggs and pork.

From the perspective of the increasing demand for animal products and a lack of food resources for humans in the next decades (FAO, 2019), the ability of ruminants to use feeds without competing with human resources is a great advantage in terms of sustainability. However, the increase of livestock production to support growing food demands can raise problems in the environment because of potential increased nitrogen pollution in water and enhanced GHG emissions.

Therefore, research on rumen fermentation is largely focused on two main goals: i) to increase the efficiency of rumen fermentation and maximize fiber degradation and microbial synthesis, and ii) to mitigate excretions of methane *in primis* but also to limit the rumen ammonia yield, which is then excreted in urine.

Ruminant nutritionists have been always fascinated by the idea of discovering a dietary compound that is able to improve the regulation and the optimization of rumen function. Research on new dietary additives are constantly growing (Martin et al., 2010), and promising results have been obtained with the use of several types of additives (e.g., probiotics, dietary lipids, organic acids,

enzymes, and plant secondary compounds) (Cobellis et al., 2016). Because of the concern about the utilization of chemicals as additives due to safety issues, natural compounds (such as plant secondary metabolites) are considered a promising alternative, and current research is focusing on this topic.

Hence, this thesis contributes to the potential use of some plant-derived additives (*Scrophularia striata* extract, chestnut tannins, *Stevia rebaudiana* extract, and three different essential oils) on several aspects of ruminal fermentation, microbiota and methane production.

Thesis outline

This thesis is composed of four research projects, which were performed at the University of Udine, during two visiting periods of the candidate in two foreign laboratories in Vienna (Austria) and in Ljubjana (Slovenia) and one in collaboration with a research group of the University of Milan.

The first part of this thesis (Chapter 1) presents a literature review on the use of secondary plant compounds as additives in ruminant nutrition and in *in vitro* rumen fermentation techniques.

In the second chapter the experimental work was performed at the University of Veterinary Medicine in Vienna (Austria) and aimed to study the use of *Scrophularia striata* extract on rumen fermentation parameters and microbiota modifications by using a rumen fermentation technique (Rusitec). The results were published as a research paper in the Journal of the Science of Food and Agriculture (Sarnataro et al., 2019). Part of the results were also presented as a poster at the 73. Jahrestagung der Gesellschaft für Ernährungs physiologie 2019 (13-15 March 2019 Göttingen, Germany).

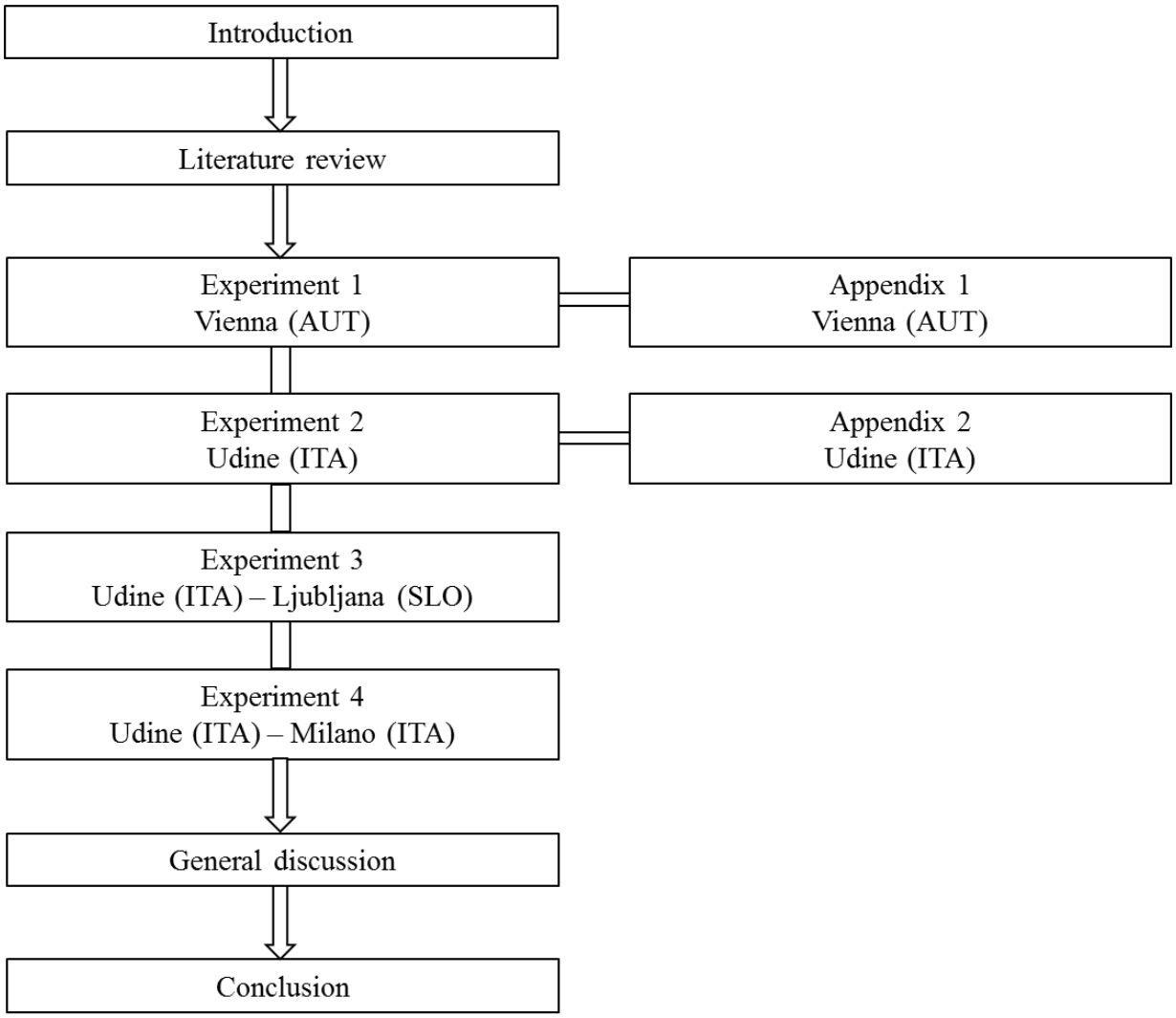
A second experiment was performed at the Department of Agricultural, Food, Environmental and Animal Sciences of Udine University and is discussed in the third Chapter. The aim was to evaluate the dietary addition of an extract of Stevia (*Stevia rebaudiana* Bertoni) and chestnut tannins on rumen metabolism by *in vitro* rumen culture systems. A research paper was published in Animal Nutrition Journal (Sarnataro and Spanghero, 2020) and an oral communication was presented at the 22nd Congress of the Animal Science and Production Association (ASPA) held in Sorrento in June 2019 (Sarnataro and Spanghero, 2019, in Appendix 2).

In Chapter 4, the effect of dietary supplementation with the extracts from chestnut wood tannins and *Stevia rebaudiana* Bertoni on *in vitro* rumen protozoa counts and methane production was analyzed. The experiment was conducted at the Biotechnology Department of the University of Ljubljana (Slovenia) and a research paper is submitted to the Archives of Animal Nutrition journal.

The experiment discussed in Chapter 5 was performed in collaboration with the University of Milan (Italy) and evaluated the effect of dietary addition of two essential oils from *Achillea moschata* and their main pure components (camphor, eucalyptol and bornyl acetate) on microbial adaptation and fermentation patterns. A research paper is submitted to the Animal Nutrition Journal.

General comments and implications of the scientific contributions are presented in Chapters 6.

Figure 1. Schematic representation of the contents of the thesis



Chapter 1. Literature review

1.1 Plant compounds used as additives in ruminant nutrition

Rumen microbiota is a complex community of several species within the main types of microorganisms (e.g., protozoa, bacteria and fungi) having numerous mutual interactions and relationships. In ruminant nutrition research, there is great interest for compounds able to modulate rumen fermentation towards greater efficiency and reduction of pollutants emission (e.g. methane from the rumen and/or ammonia from manure, Mendel et al., 2017).

Among the substances used in livestock production for rumen manipulation, antibiotics administered at nontherapeutic concentrations as growth promoters have been widely studied by nutritionists in the past (Reti et al., 2013). The problem of antimicrobial resistance and the possibility of transferring antibiotic resistance genes from animal to human microbiota (Mathur and Singh, 2005) has led the European Union to ban antibiotic use as growth promoters since January 1, 2006 (European Commission, 2003). Consequently, farmers must find another way to optimize the productivity of animals without any consequences to animal health and without increasing costs of livestock production. For this reason, the need to find a viable alternative has led to the possibility of using specific feed additives able to increase growth performance and welfare though the modulation of the gut microbiota (Gaggia et al., 2010). In fact, feed additives are used not only as growth promoters but also to improve the quality of the feed, breaking down anti-nutritive factors and absorbing toxins. In addition, feed additives are helpful in reducing energy-wasteful processes, such as methane and ammonia production (Mendel et al., 2017).

Plants contain several bioactive compounds that can enhance ruminant production and reduce polluting emissions. Moreover, it has been shown that many natural substances have antimicrobial activity, thus modifing the gut microbiota.

In a natural environment, such as a pasture, ruminants feed on different plant essences, which supply not only nutrients but also compounds and substances that are able to affect the activity of the rumen microbial populations (Figure 2). In more intensive feeding systems, the ruminant diet is based on a few vegetables raw materials (e.g., corn, soya, and alfalfa), on feeds that are often a selected part of plants (e.g., cereal meals and many byproducts), and on rations administered at high production levels. In such conditions, one can imagine a very low supply of natural compounds that can potentially influence the equilibrium among the rumen microbiota populations. Therefore, the dietary addition of plant substances for ruminants must not be considered only in terms of productive purpose (e.g. to increase efficiency and reduce excretions) but also in terms of maintaining the natural

ecological conditions in ruminant feeding with potential positive effects in terms of the animal welfare.

Figure 2. Plants from which the extracts tested in the thesis were obtained

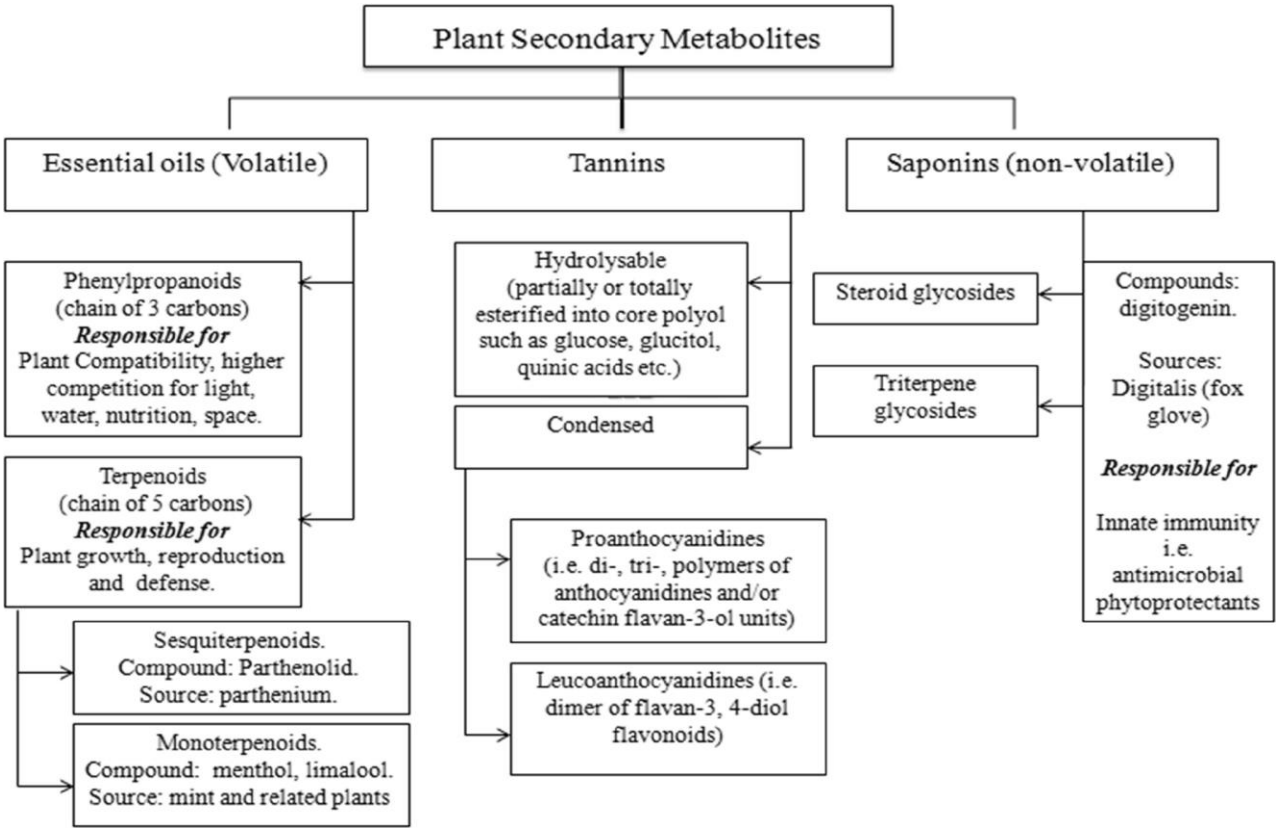
Scrophularia striata (A), *Achillea moscata* (B) and *Stevia rebaudiana* Bertoni (C)

1.2 Plants secondary metabolites

Plants synthetize a great amount of primary organic compounds, such as fibers, starch, sugars, protein, etc., that are needed for structural function, reproduction needs, storage and metabolic purposes. They also produce secondary metabolites, which are generally synthesized in low amounts and often related to sensorial properties (color, taste). Secondary metabolites can also have antinutritional or toxic actions used by vegetables as protection against biological attacks (e.g., pathogens, insects and animal predators, Iason, 2005). These metabolites are referred to as phytochemical feed additives or photobiotic, herbal and botanical compounds (Kumar et al., 2014).

The use of the term "plant secondary metabolite" (PSM) was introduced by Albrecht Kossel in 1891 (Hartmann, 2007) and describes numerous metabolites produced by plants not involved in the biochemical processes of plant growth and reproduction. A systematic work of scientific experiments to evaluate PSMs as medicines or as food preservation substances started approximately 200 years ago, and, currently, more than 200,000 PSMs have been identified (Hartmann, 2007). Their characteristics (e.g., activity and concentration) are influenced by the environmental conditions to which the plant from which they are derived is exposed. Additionally, the extraction protocol could influence the chemical characteristics of PSMs.

Figure 3. Classification of plant secondary metabolites by Arowolo and He, 2018



The classification of PSMs is not easy because their properties and mechanism of action often overlap and are difficult to distinguish among them (Bodas et al., 2012). Generally, PSMs are classified into three groups: tannins, saponins, and essential oils, as described by Arowolo and He (2018) and shown in Figure 3. In the following paragraphs, these groups will be briefly described, and their effects on ruminant nutrition will be underlined.

1.3 Tannins in ruminant nutrition

Tannins are a complex family of phenolic compounds produced by plants as a defense system against pathogens and insects (Huang et al., 2018), in addition to be a protective factor against predation by herbivores (Berbehenn and Constabel, 2011). Usually, tannins are divided into two different groups due to their chemical structure: hydrolysable tannins (HTs) and condensed tannins (CTs), also called protoanthocyanidins; a third group, composed of phlorotannins, coming principally from algae, can also be considered. The classification of tannins into two groups is the most common, and other classifications can be found in the literature.

Tannins are widely present in the plant kingdom and can be found in several plants, trees and fruits. While CTs are more common in forages, legumes, trees and shrubs, HTs are mostly in tropical vegetation (Min et al., 2003). Similar to saponins, tannins were considered in the past as

antinutritional factors (Kumar and Singh, 1984). Tannins show different adverse or beneficial effects, depending on the tannin source, concentration and chemical structure, in addition to animal species to which they are administered, their physiological state and diet (Piluzza et al., 2014). Traditional veterinary practice suggests the use of tannins for the treatment of internal parasites, especially on helminths (Bullitta et al., 2007); however, experimental tests have not been exhaustive.

The ability of tannins to act on protein digestibility in animal nutrition is well known. In fact, tannins can form a complex with proteins that is able to bypass the rumen and increase protein utilization at the small intestine level, with an improvement in animal performance. Moreover, it was suggested that tannins could inhibit the enzymatic activity of rumen protease and urease (Patra and Saxena, 2010; Patra and Aschenbach, 2018). Despite the possibility of improving protein supply, tannins are known to influence feed intake and digestibility: the use of tannins at a high dosage can decrease intake in ruminants (and also in other livestock production), reducing growth performance. The effect of tannins on the protozoa population is quite controversial in the literature (Patra and Saxena, 2010); while some authors did not find any effect on the protozoa population, Liu et al. (2011) reported a reduction of the number of protozoa with the addition of tannins to the diet.

Tannins have been used to mitigate methane emissions derived from fermentation processes (Lima et al., 2019). The reduction of methane is often linked to a general decrease in nutrient digestibility, particularly of fibrous components, as well as a decrease of the matter intake, as reported by Aboagye et al. (2018).

1.4 Saponins in ruminant nutrition

Saponins have been implemented for centuries in traditional medicine and used in place of soap due to their ability to produce a stable foam in water. The name originates from the Latin *sapo*, which means soap and derives from its foaming characteristic.

Saponins are produced by different plants, bacteria and lower marine animals (Yoshiki et al., 1998); they are chemically composed of saccharide chain units (1‑8 residues) glycosidically linked to a hydrophobic aglycone known as sapogenin. Saponins are a subclass of phytoanticipin and phytoprotectant metabolites that are found in plant parts that are the most vulnerable to fungal or bacterial attack or insect predation (Gusmayer et al., 1994; Morrissey and Osbourn, 1999).

Saponins have complex structures, and it was shown that variability of the aglycone structure, the nature of the side chains and the connection of the sugar moieties to aglycone influence the biological activity of the saponins (Jung et al., 2004).

Most studies on the use of saponins at the rumen level report an effect of reduction on rumen protozoa: cholesterol present on protozoa cells is complexed by saponins, and this complex causes irreversible

damage to the protozoa cell (Wina et al., 2005). However, protozoa seem to have different sensitivity to saponins due to the sterol composition of the membrane, suggesting a way to select the protozoa population (Patra and Saxena, 2009a). This effect is transitory because saponins are rapidly degraded by rumen microorganisms (Newbold et al., 1997; Patra and Saxena, 2009b).

In general, the prevailing biological effect of saponins is linked to their action at the membrane level and in particular the hemolytic effect of saponins: these substances attack the membrane of erythrocytes, causing a lytic effect.

In addition to the effects related to cell membranes, saponins seem to have an effect of reduction on protein degradation by complexing proteins, as suggested by Potter et al. (1993), thus making them unavailable for rumen bacteria. Furthermore, Patra and Yu (2012) show how the use of these substances leads to the selection of bacterial components and an increase of the *Prevotella* sp., *Ruminococcus flaviciens* and *F. succinogenes* population, thus suggesting an improvement of the digestibility.

1.5 Essential oils in ruminant nutrition

The term essential oil derives from the definition of an effective component of the drug "Quinta essential" given by the swiss alchemist Paracelsus von Hohenheim (Guenther, 1948). However, the term "essential" does not refer to a nonreplaceable and fundamental element at a nutritional level; in addition, "oil" does not indicate a compound that has a lipid component as a base. In fact, they are aromatic and volatile compounds, also called ethereal oils (Guenther, 1948), that are generally isolated by steam distillation processes from plant material. They can be classified as alcohol, ester or aldehyde derivatives of phenylpropanoids and terpenoids (Greathead, 2003).

Essential oils are used as fragrances and as preservatives and derive from different parts of the plant (e.g., roots, bark, flowers, leaves, and fruit). The concentration of the essential oil can vary depending on several factors, such as plant health, vegetation stage, environmental conditions and extraction method (Okoh et al., 2010).

Essential oils have different beneficial properties on health, and the antioxidant effect and free radical scavenging is well known, especially in human medicine (Cobellis et al., 2016). In addition, antiseptic and antimicrobial activities have been reported, even if the mechanism of interaction with the microbes is unclear and seems dependent on the possibility of acting on many cellular targets (Benchaar et al., 2007). For this reason, it is difficult to predict the effect of essential oils on the ruminal population because they can change depending on the oil and the susceptibility of the different microbes in the rumen (Pauli and Schilcher, 2010).

The use of essential oil in nutrition is well known, and some positive impacts on rumen fermentation have been reported (Joch et al., 2019). Among the effects, for example, the reduction of ammonia (Patra and Yu, 2012) and the slowing of starch-rich substrate degradation (Hart et al., 2008), are influenced by a shift of the microbial population in the rumen and can lead to a reduction of methanogenesis (Patra and Saxena, 2010). However, this reduction seems to be a consequence of a reduction of fiber degradation and VFA production and can be attributed to the nonspecific activity on rumen bacteria of essential oils (Cobellis et al., 2016).

1.6 Bibliographic review of the use of plant compounds on ruminant fermentation and microbiota

This section of the thesis contains a collection of recent review articles that consider the use of secondary plant metabolites (e.g., saponins, essential oils and tannins) in ruminant nutrition. The criteria used for the selection were the year of publication (within the last ten years, from 2009 to 2019) and scientific ranking of the journal (only Q1 journals). The keywords considered were "rumen", "essential oil", "plant compounds", "tannin", "saponin" and "extracts", and only review articles were selected. Scopus and ScienceDirect as search engines were used. A total of twelve studies were exanimated and reported in Table 1. In these papers, the effects on rumen metabolism of over 400 natural extracts have been considered, with the examination of over 1500 citations. To show that the interest in this topic is global, the inspection of the nationalities of the first author of each review showed that the articles are equally distributed throughout the world. Except for Huang et al. (2018), who underlined the effect of tannins only in *in vivo* experiments, all the reviews consider *in vitro* and *in vivo* experiments.

Among the papers, the most commonly analyzed effects of the use of natural additives were on protozoa, methane production, methanogens and fermentative parameters (e.g., volatile fatty acids (VFA) and ammonia). These parameters can be considered the most important to evaluate both fermentation and microbiota and can provide a screening of the possible effects of the additives. For this reason, the effect of tannins, essential oils and saponins on these parameters were analyzed. Considering the same reviews listed in Table 1, the percentage of articles where the increase, decrease or no effect were included in graphic form. It should be noted that bacteria are not included in this analysis. Although this aspect has been well studied in recent years, the great variability of the bacterial population in the rumen makes it difficult to discuss general considerations on this topic in a review. The results of this analysis are shown in Figure 4. Means and standard error in the graphs were obtained considering the percentage of articles per review that reported the variation (or nonvariation) of the parameter considered. In general, from the study of the reviews, it was seen that

there is good agreement among the results reported for the various parameters by different authors. Despite the great interest in the use of these additives for the increase of fermentation efficiency and the modification of fermentation patterns, the result concerning the VFA is fairly unanimous. For all three extracts, this parameter is not affected, demonstrating that with the addition of these substances, there is not a substantial modification of the fermentation profile. Analyzing the graphs (Figure 3), it is possible to see that in all three additives, there is a predominant decrease in methane production and protozoa population; furthermore, more than 80 % of the authors that included tannins in their review and almost 70 % including essential oils have reported a decrease of methanogens.

The role of protozoa in the rumen is controversial in the literature (Newbold et al., 2015). Although protozoa contribute up to 50 % of the total biomass in the rumen, studies of defaunation have shown their nonnecessity for rumen function (Gruniger et al., 2019). However, the association of protozoa and methane production seems to be certain (Balanche et al., 2015); in fact, defaunation trials have shown a reduction of methane of approximately 12 % (Morgavi et al., 2010, Hegarty, 1999). Protozoa can produce H_2, which can be used by methanogens that live on protozoa surfaces to produce methane (Ellis et al., 1994). For this reason, the reduction of the protozoa population could also be correlated with a reduction of the methanogen population, as reported in Figure 3. Another important parameter considered is ammonia production: this gas, in addition to being toxic to the animal at high concentrations, is polluting, so the interest of the scientific community is to reduce the excretions of this product. From these results, it can be seen that for tannins, there is a certain effect of reducing this pollutant. This result is expected, as it is known that tannins are involved in protein metabolism: due to their capacity to complex with proteins or inhibit the protease and urease in the rumen (Patra and Saxena, 2010, Patra and Aschenbach, 2018), tannins reduce protein utilization and, consequently, the production of ammonia. Concerning saponins and essential oils, their effect on ammonia is reported as being halfway between diminution and no effect. This result can be expected because these two groups include many compounds.

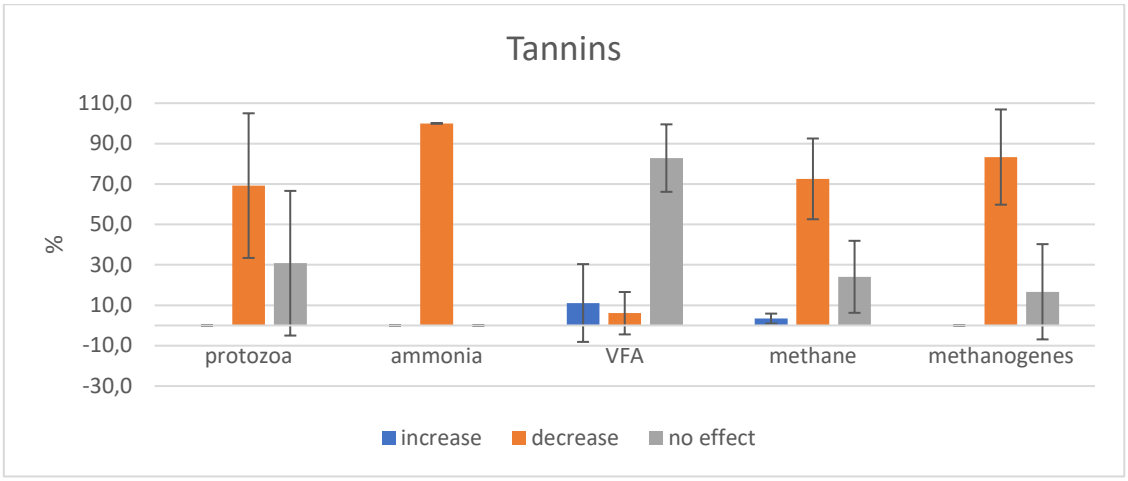
Table 1. Collection of 12 reviews published in selected top-rated Journals in the last decade (2009-2009) concerning plant secondary metabolites on ruminant nutrition.

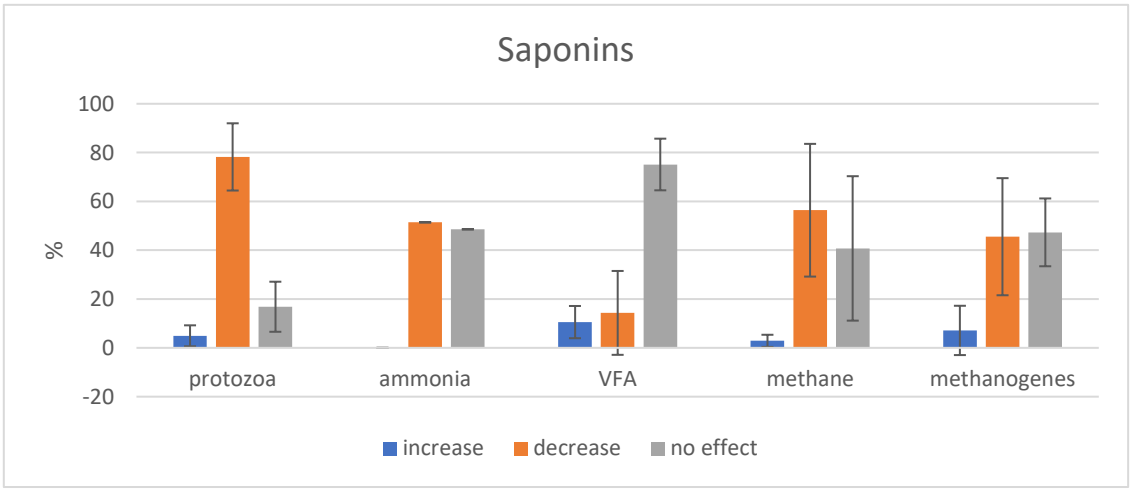
Year	Author	Country	Journal	*In vivo/in vitro*	Plant compound	Number of additives	Number of papers	Effects
2009[a]	Patra A.K.	India	Nutrition research reviews	*In vivo/in vitro*	Saponins	20	119	Protozoa, rumen fermentation, methane and performance
2010	Patra A.K.	India	Phytochemistry	*In vivo/in vitro*	Saponins Tannins Essential oils	31	173	Methane
2013	Cieslak A.	Poland	Animal	*In vivo/in vitro*	Saponins Tannins Essential oils	72	124	Methane, methanogens, potozoa
2013	Piluzza G.	Italy	Grass and forage science	*In vivo/in vitro*	Tannins	59	153	Feed intake, protein digestibility, rumen fermentation, animal production, animal disease, methane
2016	Cobellis G.	Italy	Science of the total environment	*In vivo/in vitro*	Essential oils	113	55	Methane, ammonia, VFA, DMD, DMI
2017	Mendel M.	Poland	Animal Feed Science and Technology	*In vivo/in vitro*	Saponins Tannins Essential oils	44	143	Gastrointestinal contractility
2018	Arowolo M.A.	China	Animal nutrition	*In vivo/in vitro*	Saponin Tannins	13	86	Microbiota, animal performance, rumen fermentation

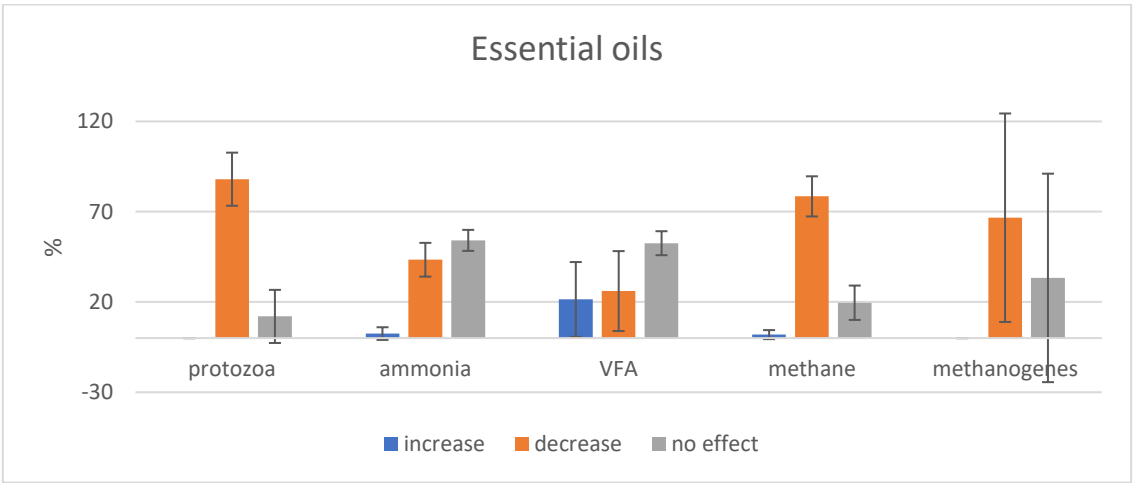
Table 1. (Continued) Collection of 12 reviews published in selected top-rated Journals in a last decade (2009-2009) concerning plant secondary metabolites on ruminant nutrition.

Year	Author	Country	Journal	*In vivo/in vitro*	Plant compound	Number of additives	Number of papers	Effects
2018	Min B.R.	USA	Journal of animal physiology and animal nutrition	*In vivo/in vitro*	Tannins	26	108	DMI, DMD, protein digestibility, animal performances, microbiome, methane, biohydrogenation
2019	Olagaray K.E.	USA	Animal Feed Science and Technology	*In vivo/in vitro*	Flavonoids	21	122	Rumen degradation, growth performances, metabolism in calf, feed intake and milk production, insulin resistance, lipolysis and ketogenesis, mastitis
2019	Ugbogu E.A.	Nigeria	Journal of Cleaner production	*In vivo/in vitro*	Saponin Tannins Essential oils	33	141	Methane, microbiota, rumen fermentation
2019	Vasta V.	Italy	Journal of Dairy science	*In vivo/in vitro*	Polyphenols	10	161	Microbiota, biohydrogenation, methane, VFA

Figure 4. Effect of tannins, saponins and essential oils on protozoa, ammonia, VFA, methane and methanogens in last ten years reviews.







1.7 *In vitro* rumen fermentation systems

The study of the ruminal environment and metabolism is essential to improve the efficiency of nutritional processes, minimizing energy losses and undesirable effects on animal welfare. Historically, the study of ruminant nutrition has been performed using experimental animals and was characterized by long performance times and high costs (*in vivo* experiments). Since the second half of the twentieth century, numerous *in vitro* ruminal fermentation systems have been developed. These techniques use laboratory systems that simulate the rumen environment without the direct use of experimental animals. In general, *in vitro* rumen fermentation systems are composed of laboratory glassware in which a rumen inoculum and a diet are included. Rumen inoculum consists of a mixture of rumen liquid collected from donor animals (e.g., from cannulated or intubated animals or at a slaughterhouse) with the addition of an artificial saliva (or buffer solution). Saliva is mainly composed of bicarbonate and is used to maintain an adequate environment for fermentation processes, to supply nutrients and to maintain a constant pH (Mould et al., 2005). The use of *in vitro* systems allows faster screening of many diets or additives and lower costs of execution compared to those of *in vivo* systems. Although these tests are increasingly sophisticated, they are not able to replace tests with animals yet. In fact, usually after an initial *in vitro* screening, an *in vivo* confirmation test is necessary. However, the public opinion is opposing to the use of experimental animals; therefore, the use of *in vitro* ruminal fermentation techniques will be increasingly implemented.

In vitro rumen fermentation techniques can be divided into two categories: discontinuous rumen fermenters and continuous rumen fermenters. The first category is essentially based on closed systems designed for short-term studies ("batch" systems). These systems include *in vitro* techniques able to measure the degradability of a substrate at the end of a fermentation process and to estimate the nutritional value of a feed. The second category includes systems that reproduce the ruminal metabolic processes of nutrient utilization and include continuous fermentation (CC) systems designed for long-term studies (Minson, 1998; Tamminga and Williams, 1998). An artificial rumen that was designed and set up in the Laboratory of Animal Nutrition of the Department of Agriculture, Food, Environmental and Animal Sciences of the University of Udine (Mason et al., 2015) is included in the second category.

1.8 Batch systems

Batch systems do not provide continuous infusion or removal of fermentation liquid; for this reason, they are called discontinuous systems or “batch” systems. To avoid metabolite accumulation, they use solution buffers and adopt a high fermentation ratio between the liquid and substrate. Most systems use simple laboratory equipment composed of laboratory glassware (tubes, vials, flasks and

syringes) with limited capacity (maximum 500 mL) in which small quantities of ground substrate are added (from 0.25 to approximately 1.0 g) together with rumen liquid and placed in a heated system at approximately 39°C with agitation. Most systems generally implement a single determination at the end of the fermentation ("end point" measure), while others obtain measurements during the entire duration of the fermentation, allowing for the study of kinetics. These systems allow the simultaneous perform of a high number of fermentation units and are suitable to quickly test a high number of substrates or additives. In this category, it is possible to distinguish different batch culture systems based on the type of experimental survey: gravimetric measurement of the undigested residue, measurement of the fermentation gas, or measurement of the fermentation metabolites.

Gravimetric measurement of the undigested residue

These systems provide a quantitative evaluation of rumen degradation of the substrate and are used to predict the nutritional value of feeds. The first method was proposed by Tilley and Terry in 1963 and consists of a two-step fermentation process that simulates ruminal and intestinal digestion. More recently, systems that estimate the degradation of the NDF have been implemented. This parameter is fundamental for estimating the nutritional value of feed. The most known and used technique for estimating NDF involves the use of a fermenter called “Daisy” (Ankom, Tech. Co., Fairport, NY, USA), which allows for rumen fermentation with a high work capacity (approximately 30 samples in triple per fermentation). This *in vitro* method consists of incubating porous bags (5 × 5 cm; porosity: 25 µm) containing the substrate (250 mg of dry, finely ground sample) in four glass vessels (jars) containing rumen fluid (400 mL) diluted in a special mineral solution (1600 mL). The containers are introduced into the "Daisy" equipment and kept at a temperature of 39 °C with continuous slow stirring. At the end of the incubation period, the bags are rinsed with water and then with a neutral detergent solution to remove the microbial residues and any soluble particles remaining inside; they are subsequently placed in a ventilated stove at 60 °C for one night to determine the rumen degradability of NDF. The critical point of this system is the porosity of the bags, which can limit the accessibility of the feed to the microbes, providing underestimated values (Adesogan, 2005). Another element of criticism can be ascribed to the limited precision of the system even though its accuracy, determined by comparison with *in situ* data, appears encouraging (Spanghero et al, 2010). These elements of weakness have stimulated the development of systems alternative to the “Daisy” fermenter. Hall and Mertens, in 2008, compared fermentation tubes of different volumes (50 and 125 mL) filled with quantities of substrate (0.2-0.5 g) maintained in fermentation for up to 48 hours with different gas release systems. Compared to the “Daisy” fermenter, the quantification of the undigested NDF residue in the abovementioned protocol is more laborious, as it requires recovering the

fermentation residue on a filter crucible and then proceeding with the neutral treatment; moreover, the analysis capacity for the fermentation run is limited.

Another gravimetric measurement of the residue system was suggested by Ross et al. (2013) and consists of a two-step degradation process of the substrate for the determination of nitrogen utilization. This method uses flasks (150 mL) in which substrate (0.5 g) and a mixture of rumen liquid and buffer solution are added (1:4 ratio). Samples are incubated in quadruplicate, half of them for the ruminal step and the other half for the intestinal process. After 24 h of incubation, half of the samples are filtered and used for nitrogen determination. The other bottles are acidified, and pepsin enzyme is added before subsequent incubation at 39 °C for one hour to simulate the gastric step. Afterwards, the intestinal step is simulated using pancreatin enzyme and with the modification of pH with a buffer solution and 16 h of incubation. For the ruminal step, samples for the intestinal step are filtered, and the nitrogen content is measured.

Measurement of gas production

The "gas production" technique is based on the production of gas as an indirect method to evaluate the nutritive value of the substrate. The gas originates mainly from the fermentation of carbohydrates but also from the fermentation of proteins and fats (Menke and Steingass, 1988; Getachew et al, 1998).

The production of gas originating from the fermentation of the feed (from acetate and butyrate) is defined as "direct gas production" and represents approximately 40 % of the total gas produced:

$$C_6H_{12}O_6 + 2H_2O \quad \text{acetic acid} + 2CO_2 + 4H_2$$

$$C_6H_{12}O_6 + 2H_2 \quad \text{propionic acid} + 2H_2O$$

$$C_6H_{12}O_6 \quad \text{butyric acid} + 2CO_2 + 2H_2$$

The remaining 60 % is produced by the buffer of volatile fatty acids (acetate, butyrate, and propionate) and called "indirect gas production" (Makkar, 2005). From each mole of production of volatile fatty acids, the bicarbonate buffer releases about a mole of carbon dioxide:

$$H^+ + HCO_3 \quad H_2CO_3 \; CO_2 + 2H_2O$$

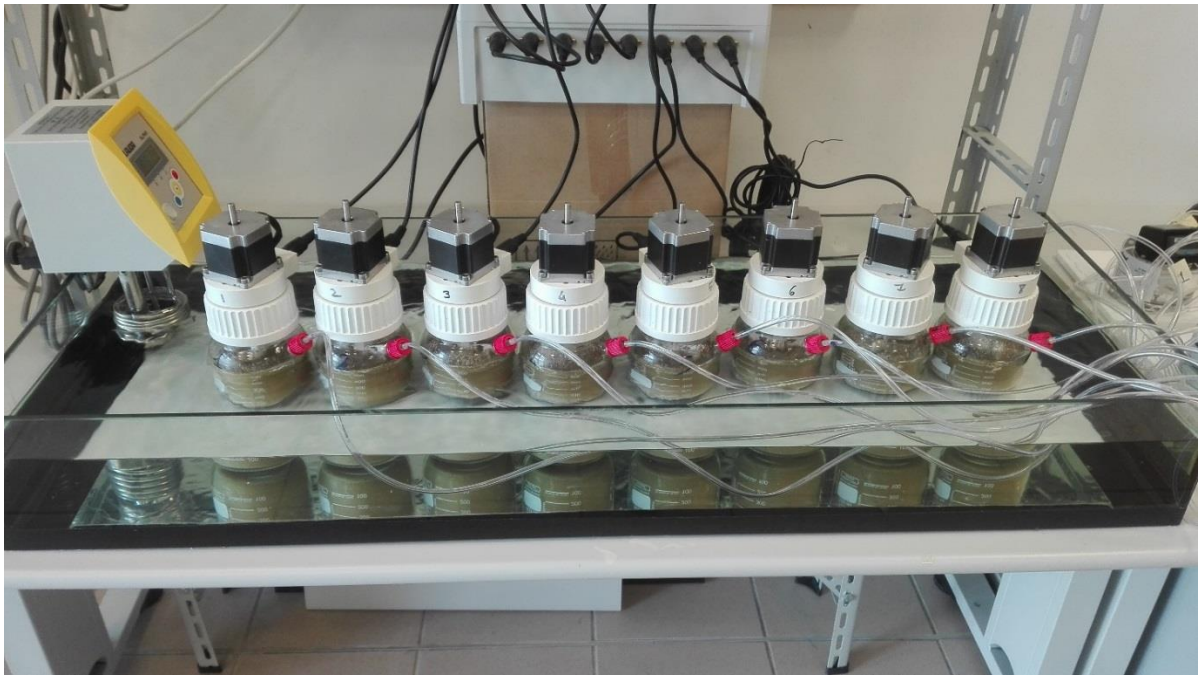
A research group at the University of Hohenheim proposed a technique (Menke and Steingass, 1988) based on the use of graduated glass syringes (100 mL, Haberle Labortechnik, Germany). Syringes are provided with closures in place of the needles. As a sample, of approximately 200 mg of dry feed (ground with a 1 mm grid, rebalanced in ambient air) is introduced, 30 mL of a solution consisting of rumen liquid diluted in a mineral solution (at a ratio of 1:2) is added. Immediately after loading, the syringe is positioned vertically on a special support, which allows almost complete immersion in water maintained at 39 °C in a thermostatic bath. Each substrate is carried out in triplicate, and in

each series, syringes without ingredients are utilized to estimate the production of gas coming from the rumen liquid. Measurements of the gas produced are usually made at 0, 1, 2, 4, 6, 8, 10, 12, 24, 48, 72, and 96 h to create a kinetic profile of gas production. Depending on the expected results, the measurement time can be modified.

This method has advantages, including its simplicity and the limited cost of the equipment used, and the results obtained in tests conducted in different laboratories highlighted good levels of precision (Pell and Schofield, 1993). In addition to the use of gas production for the evaluation of feed ingredients, Spanghero et al. (2018) suggested the use for the evaluation of slow-release urea products.

Recently, automated methods have become widespread, in which the fermentation bottles are equipped with pressure detectors and a wireless connection to a computer that greatly reduces the work of manual manipulation of the bottles and syringes for gas detection and allows a 'continuous' analysis of volumes (Ankom Technology, Macedon, NY, USA). This equipment is more expensive than the manual ones, but the continuous measurement allows to an interpolation of the kinetics of gas production according to very sophisticated and complex mathematical models. Another new kind of equipment based on the continuous measurement of gas production has been produced by the Ritter company (Dr.-Ing. Ritter Apparatebau GmbH & Co. KG) and consists of 500 mL fermentation bottles equipped with a stirring device as shown in Figure 5.

Figure 5. Ritter gas production system (Dr.-Ing. Ritter Apparatebau GmbH & Co. KG)



Measurement of the gas outflow is possible due to a hole connected by a rubber tube to a MilliGas counter (measuring range, 1 mL/h to 1 L/h; measuring accuracy, 3 %; Dr.-Ing. Ritter Apparatebau GmbH Co. KG). With this system, it is also possible to connect bags for gas analysis, which allows for the analysis of the composition of the gas produced by the fermentation process. This system, which was used by Sarnataro and Spanghero (2020) and is reported in Figure 5, allowed for continuous measurements with a very high precision. In addition, the possibility of using a high volume (500 mL) allows for the use of a great quantity of substrate, allowing for testing, for example, of low-additive inclusion with a high precision of weighing.

Measurement of fermentation metabolites (ammonia or volatile fatty acids)

These are generally simple fermentation systems that are inspired by the first stage of ruminal fermentation of the original Tilley and Terry technique of 1963. These techniques are usually applied to test the effect of the addition of additives potentially capable of modulating fermentation or to control the release of ammonia from feed substrates. Fermentation is carried out in 100 mL glass tubes fitted with a cap containing a valve for venting gases, in which 500 mg of substrate and 50 mL of inoculum consisting of rumen liquid diluted at a 1:1 ratio with a mineral solution (Cardozo et al., 2005) are introduced. The tubes are kept in a water bath (at 39 °C) provided with a slow stirring system, and after 24 h of incubation, the residual fermentation liquid is collected and stored for subsequent analyses of volatile fatty acids (VFA), lactic acid and ammonia.
Examples of applications of these procedures (Cardozo et al, 2005; Spanghero et al, 2008) tested the effect of different essential oils as potential modifiers of rumen populations and therefore of the type of fermentation products.

An interesting application of metabolic measurement is to determine the concentration of ammonia in the fermentation liquid to quantify the extent of the nitrogen degradation process. Cooke et al. (2009) used 250 mL (Erlenmeyer type) flasks closed with a stopper fitted with a valve to release fermentation gases. In each flask, a quantity of substrate equivalent to 155 mg of N and a fermentation solution of 200 mL is introduced, consisting of rumen inoculum and a buffer in a 1:4 ratio (v:v). The flasks are placed in a water bath at 39 °C, and a sample (10 mL) is collected at set times from the beginning of the fermentation (0, 1, 3, 5, 20 and 24 h) for the determination of NH_3-N. The determination is performed in triplicate (3 fermentation flasks per sample) using a spectrophotometer. This method was used to compare the release kinetics of different slow-release urea-based products and has an application outlined in the recent article by Nikulina et al. (2018), where the ammonia release of urea-treated barley and maize was determined.

1.9 Continuous rumen fermenters (CCF)

Continuous ruminal fermentation systems are the most complex and elaborate *in vitro* techniques and reproduce the rumen environment as closely as possible. The continuous influx of mineral solution, the daily addition of substrate and the constant removal of fermented liquid simulate what normally occurs in a rumen. For these kinds of techniques, large fermentation bottles are generally used (e.g., 1.0-2.0 L). These bottles are placed in heating systems at 39 °C, and a stirring system is provided. Miettinen and Setälä (1989) divide the continuous fermentation systems used in rumen metabolism studies into 3 categories:

-The system developed by Czerkawski and Breckenridge in 1977, in which the food is kept in polyester bags inside a vase and changed periodically (Rusitec).

-The system described by Hoover et al. in 1976, in which solid food is added to the fermenter and the output rates of the solid and liquid effluents are controlled separately.

-The system described by Slyter et al in 1964, in which solid food is added to the fermenter and the effluent exits by overflow.

More modern fermentation systems are described in literature: Teather and Sauer (1988) described a system with agitation through a helical blade at the exit of the effluent, while Muetzel et al. (2009) suggested a different position of the effluent outlet tube (oblique instead of horizontal) and the remote control of the system by a computer.

In addition to these systems, Mason et al. (2015) described a stratified continuous rumen fermenter system. This system (Figure 6) is composed of 8 vessels (2 L) maintained at 39 °C in a water bath and agitated by a magnetic stirrer.

Figure 6. Continuous culture system designed and set up at the University of Udine (Mason et al., 2015)

Artificial saliva is continuously infused by a peristaltic pump, and the outflow is possible through an exit at the bottom of the vessel. This position allows for the stratification of the material. Less dense particles form a layer of matter on the top of the bottle, allowing for the maintenance of anaerobic conditions.

Continuous fermentation systems should allow for the bacterial flora to stabilize in relation to the type of substrate or additive inserted or the specially created environmental conditions (e.g., liquid turn over, feed-buffer ratio, pH, etc.) and allow for the characterization of the fermentation. In discontinuous systems, the short duration of fermentation does not allow for the evaluation of changes in bacterial population relative to changes in substrate. One of the main critical points of continuous fermentation systems is the difficulty of maintaining the original microbial population, which generally degenerates, and, in particular, protozoa disappear. According to Coleman (1980), there are several reasons that lead to the disappearance of protozoa, including the difficulty of these to feed if the fermentation liquid is artificially stirred, the accumulation of products that are toxic and the need for protozoa to have one "dead space" in the fermentation vessel in which they can shelter. Recent work (Hristov et al., 2012) has compared, in a meta-analysis of 180 publications over the last 30 years, the fermentation conditions of continuous fermentation systems with the data obtained *in vivo*. The results showed that the *in vitro* systems provide concentrations of VFA, the density of protozoa and the lower digestibility values of the organic substances and NDF to those that are recorded *in vivo*. However, the method published by Mason et al. (2015) showed the possibility of maintaining a protozoa population until the end of fermentation due to its design and mild stirring, in addition to the layer of matter on the top of the system that maintains anaerobic conditions in the liquid.

Thesis aim

The experimental part of this thesis is composed of four experiments with the following aims:

1. The first experiment was an *in vitro* study using a rumen simulation technique (Rusitec) to evaluate the use of an extract of *Scrophularia striata* in three different diets based on fibrous components (i.e., alfalfa silage, alfalfa silage cakes (residue of a biorefinery) and a fibrous hay). The possible effect on degradability, methane production and microbiota has been studied in this experiment. This experiment was conducted at the Vetmeduni in Vienna (Austria).
2. In the second experiment, an extract of *Stevia rebaudiana* Bertoni and chestnut tannins were used in different rumen simulation techniques. The inclusion of the two extracts aims to evaluate the fermentative parameters and the modification of rumen microbiota.
3. The third experiment is related to the second experiment. The same extracts, Stevia and chestnut tannins, were used to confirm protozoa modification in the rumen. In addition, methane production was determined. This experiment was conducted at the University of Ljubljana (Slovenia).
4. Finally, the last experiment includes a study on three different essential oil-derived compounds (camphor, bornyl acetate and eucalyptol) that are the main components of *Achillea moschata* essential oil. The essential oil-based compounds were tested in different *in vitro* experiments, and the evaluation of their effects on methane production and on fermentative parameters was conducted. In addition, microbiota was analyzed and an adaptation test using a continuous culture system (Mason et al., 2015). The study was conducted in collaboration with the University of Milan (Italy), and only the microbiota adaptation test was performed at the University of Udine.

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Chiara Sarnataro,[a] Renee M Petri,[b] Mauro Spanghero,[a] Qendrim Zebeli[b] and Fenja Klevenhusen[b*]

Abstract

BACKGROUND: By-products of the food production chain are gaining importance as feedstuffs for ruminants. Alfalfa silage cake (AC) is an unexploited biorefinery by-product rich in fiber. The aim of this study was to test AC, using an *in vitro* rumen simulation technique (Rusitec), for its suitability as a fiber source for cattle. Three diets with similar crude protein (CP) content were formulated; they contained the biorefinery by-product AC, the original alfalfa silage (OA), or a fiber-rich hay. As fibrous feedstuffs are known to promote ruminal methanogenesis, we additionally tested a plant extract of *Scrophularia striata* (60 mg g^{-1} dry matter) for its methane mitigation and antimicrobial properties.

RESULTS: Diets containing AC displayed lower nutrient degradability, with the largest difference in CP degradation ($P < 0.001$). Sequencing of microbial DNA revealed several effects of the diet and of the addition of *S. striata* extract, but no inhibitory effect on methanogens. Likewise, methane production, which, in general, is lower with AC and OA diets, was not inhibited by *S. striata* extract, while the short chain fatty acid (SCFA) profiles were unaffected.

CONCLUSION: Although CP degradation of the AC diet was lower, degradation of the fiber fractions was similar among diets. According to the present results, AC can be used as fibrous feedstuff for ruminants. Supplementation with *S. striata* extract did not inhibit methane formation.

Keywords: forage; rumen; plant extract; methane; sequencing; microbiome

INTRODUCTION

During the last decades, the need for sustainable food and feed production has gained importance, which has led to significant growth in the biorefinery sector.[1] Biorefinery products are commonly used as added-value food and feed supplements[1] and their production requires improved utilization of the generated by-products to enhance sustainability.

Alfalfa is a perennial legume known for its high protein content and biological value in animal nutrition.[2] It can be produced even under organic conditions to supply protein for various uses. The biorefinery that uses alfalfa silage juice as a valuable source of amino acids generates a fibrous by-product, termed alfalfa silage cake (AC), which, potentially, could be used as a source of fiber for ruminants as they are adapted to utilize cellulose-based feed with low-quality protein.[3,4] During mechanical processing and aqueous extraction of the protein-rich fraction of alfalfa, the chemical, physical, and nutritive characteristics of AC are altered and a nutritional evaluation of AC as feedstuff is necessary. In general, fibrous feedstuffs, such as forages, are known to promote methane production in the rumen,[5,6] in contrast to easily degradable starch. Ruminal methane production reduces feed energy efficiency in ruminants[7] and contributes to greenhouse gas emissions from the agricultural production sector.[8] Therefore, there is a need to develop dietary strategies to decrease ruminal methanogenesis, while providing sufficient fiber for rumen health. A rewarding strategy consists in using feed additives that directly or indirectly inhibit methanogenic microorganisms in the rumen. In this regard, considerable efforts have been devoted to investigating the potential use of plant bioactive compounds including phenolic and flavonoid compounds.[9–11] In a previous study, we observed that *Scrophularia striata* extract, rich in phenolic and flavonoid compounds,[12] when added to a diet containing 50% concentrate feedstuffs with a neutral detergent

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Accordingly, in the present study, we hypothesized that AC can serve as a fiber source for ruminants with a rumen fiber degradability, influenced by previous mechanical processing, similar to that of fiber-rich hay (Hay). In addition, we expected fiber degradation to be improved by supplementation with *S. striata* extract because of the expected increased relative abundance of fibrolytic Fibrobacteres,[13] while methane formation was inhibited. The objectives of this *in vitro* experiment were to analyze the dietary effects on nutrient degradability, fermentation traits, and methane production by using the *in vitro* rumen simulation technique (Rusitec).[14] In addition, 16S rRNA gene-targeted sequencing of DNA in the liquid and the solid phase was performed to investigate the effects of the diet and *S. striata* extract on rumen microbiota.

MATERIALS AND METHODS

Experimental diets

Three different forages, namely, the biorefinery by-product AC, the original alfalfa silage (OA), and Hay as control were tested as part of a total mixed feed ration, with or without supplementation of *S. striata* extract (60mgg^{-1} of DM). The tested forages differed considerably in CP (in g kg^{-1} DM: AC=116, OA=187 and Hay=72.7) and NDF composition. While the NDF content was similar between AC and Hay (714 g kg^{-1} DM in comparison to 545 g kg^{-1} DM in OA), the lignin content (acid detergent lignin, ADL) was higher in AC (87.3 g kg^{-1} DM) compared to OA (66.6 g kg^{-1} DM) and Hay (48.1 g kg^{-1} DM). A mixture of wheat, rapeseed meal, and calcium caseinate was used as

concentrate (Table 1) to obtain similar CP content among diets (169± 6.2 g kg^{-1} DM). Forages and concentrates were dried (60°C for 48 h) and sieved through a 4-mm sieve for diet preparation.

Plant extraction and extract supplementation

S. striata was purchased from a local herbalist in Ilam in the west of Iran. Ground plant material was immersed in 60% aqueous ethanol solution at the ratio of 1:25 (w/v) for 1 h in a sonicator bath, after which the mixture was filtered through filter paper and concentrated at 40°C using a rotary evaporator consisting of waterbath B-480, Rotovapor R-124, and Vacum Controller B-72 (Büchi, Flawil, Switzerland). The concentrate was frozen at −20°C and freeze dried (Snijders, Tilburg, The Neederlands). Freeze dried *S. striata* extract (60mgg^{-1} DM) was mixed with diets shortly before being incubated.

Experimental design

The Rusitec procedure is described by Klevenhusen et al.[15] Briefly, two Rusitec systems consisting of six fermenters each were used. Each fermenter had an incubation volume of 800 mL. The experiment consisted in three runs, each lasting 10 days, with the first five days serving as the adaptation period and the last five days as the sampling period. For each run, ruminal fluid and solid digesta were collected from three non-lactating rumen-cannulated Holstein cows, housed at the Teaching and Research Farm (VetFarm) of the University of Veterinary Medicine, Vienna, Austria. Cows were fed ad libitum with a forage-only diet (2/3 grass silage and 1/3 hay) and were housed according to the Austrian guidelines for animal welfare.[16] Before use, the rumen liquid from the three cows was mixed and filtered through four layers of medical gauze (ca 1 mm pore size) and 600 mL of strained ruminal fluid were added to each vessel, in addition to 100 mL of artificial saliva.[17] A 12-channel peristaltic pump (Model ISM932, ISMATEC, IDEXHealth and Science GmbH, Wertheim, Germany) ensured a continuous infusion of artificial saliva at a rate of 336±27 mL day^{-1}. Daily effluent and fermentation gases were collected in a 1-L volumetric flask located in an ice-filled box and gas-tight aluminum bags, respectively (Tecobag 5L, Tesseraux Container GmbH, Burstadt, Germany). Anaerobic conditions were ensured by flushing nitrogen gas for 3 min after daily operations.

Daily measurements and sample collection

During the sampling period, daily before feeding, samples of fermenter fluid were collected from each fermenter using a syringe. One part of the sample was immediately frozen at −20°C for NH_3 and short chain fatty acid (SCFA) analyses, while another part was snap frozen in liquid nitrogen and stored at −80°C for DNA extraction. A further part was used immediately for determination of pH

and Redox potential (Seven Multi TM,Mettler-ToledoGmbH, Schwerzenbach, Switzerland), and for counting of protozoa by using a 0.1 mm depth Bürker counting chamber (Blau Brand, Wertheim, Germany). Samples for NH_3 kinetics were collected every 2 h after the feeding procedure (2, 4, 6 h) from Day 6 to Day 9. Feed bags, after 48 h of incubation, were washed until the water ran clear and were stored at −20°C for analysis of nutrient degradation. The effluent volume was recorded daily and gas composition was analyzed using an infrared detector (ATEX Biogas Monitor Check BM 2000, Ansyco, Karlsruhe, Germany). Gas volume was determined by the water replacement method. On the last day, feed samples that had been incubated for 24 h were snap-frozen in liquid nitrogen and stored at −80°C for DNA extraction.

Laboratory analysis

Prior to analysis, feed samples were oven-dried at 65°C for 48h, while feed residues after 48 h of incubation were freeze dried and pooled per fermenter over the five sampling days. All samples were ground to pass through a 1-mm sieve and used for analyses according to VDLUFA methods.[18] To obtain DM, the samples were dried at 103°C overnight and for ash samples they were combusted at 580°C overnight. Ether extract (EE) analysis was performed using a Soxhlet extractor (extraction System B-811, Büchi, Flawil, Switzerland) and CP content was determined with the Kjeldahl method. Contents of NDF, acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined with the Fibretherm FT 12 system (Gerhardt GmbH und Co. KG, Königswinter, Germany) using a heat-stable α-amylase in case of NDF. Contents of NDF, ADF, and ADL are reported to be devoid of residual ash. Non-fiber carbohydrate contents were calculated as follows: NFC=100−(CP+ash+EE+NDF). Nutrient degradability was calculated as the difference between the amount in the diet and the amount recovered in the feed residue in percent of original content of the diet. For NH_3 and SCFA analyses, samples of the five sampling days were pooled per fermenter. Hourly NH_3 kinetics from Day 6 to Day 9 were determined using samples pooled per hour and fermenter. The concentrations of SCFA (acetate, propionate, iso-butyrate, n-butyrate, iso-valerate, n-valerate, and capronate) were determined by gas chromatography (Simadzu/GC-2010 PLUS) as described by Qumar et al.[19] The concentration of ammonia was determined using the indophenol reaction.[20]

DNA extraction, sequencing, sequence processing, and bioinformatics analysis Fermenter fluid and solid samples were processed separately for DNA extraction. Total DNA was extracted from approximately 800 µL of fluid and 0.25 g of solid digesta using the Power Soil DNA extraction kit (MoBio Laboratories Inc., Carlsbad, CA, USA), with modifications according to previously published procedures.[21] This was followed by chemical removal of cell debris and PCR inhibitors by

centrifugation, and supernatants were transferred to fresh tubes for column-based isolation of total genomic DNA. The concentration of isolated DNA was determined with a Qubit 2.0 Fluorometer (Life Technologies, Carlsbad, CA, USA) using the Qubit dsDNA HS Assay Kit (Life Technologies). DNA was stored at −20°C until further analysis. Samples of DNA from the liquid and solid digesta fractions were pooled at the same concentration, and amplicon sequencing was performed using Illumina MiSeq paired-ends sequencing technology (Microsynth AG, Balgach, Switzerland). For sequencing, the hypervariable regions V3-V4 of 16S rRNA were used with the primer set 341F_ill (5′-CCTACGGNGGCWGCAG-3′) and 802R_ill (5′-GACTACHVGGGTATCTAATCC-3′) to produce an amplicon of ca 460 bp.[22] The sequencing procedure is described by Bagheri Varzaneh et al.[13] Using purified PCR products, libraries were constructed by ligating sequencing adapters and indices (Nextera XT Sample Preparation Kit, Illumina, CA) according to the manufacturer's recommendations. Equimolar amounts of each library were pooled and sequenced on an Illumina MiSeq Personal Sequencer. The resulting paired ends were stitched together by Microsynth (Balach, Switzerland) (in a total of 1.8 million unfiltered reads with an average length of 409 nucleotides). Quality control of the sequences was performed using the QIIME pipeline.[23] The sequence was filtered, screened, and filtered for chimeras usingUSEARCH24 (v8.1).Clustering and alignment were performed using UCLUST25 and operational taxonomic units (OTUs) were defined using the SILVA database 26, 27 (v128) (accessed January 23, 2018) and PyNAST. To obtain the OTUs, a 97% degree of similarity between sequences was defined. All clusters of OTUs with less than ten readings were removed manually. A relative abundance greater than 0.1% (122 OTUs) was taken into consideration for statistical analysis at the OTU level. For alpha diversity analysis, Chao1, Shannon, and Simpson indices were used. Beta diversity analysis was performed using weighted Unifrac dissimilarity metrics and principal coordinates analysis (PCoA) plotting in QIIME with rarefaction at 15 068 sequences, based on the lowest number of sequences in a sample. Sequences were deposited in the NCBI sequence read archive (SRA) under the temporary accession number SUB4286151.

Statistical analysis

Statistical analyses were performed using the mixed procedure of SAS (version 9.4, SAS institute Inc., Cary, NC). Mean values of the last five days per treatment and run were analyzed using a 3 × 2 factorial design (with three different diets and two treatments, i.e., no addition of *S. striata* extract or supplementation with 60 mg/g^{-1} DM *S. striata* extract). The experimental run was included as random factor. Multiple comparisons among means were made using Tukey's method. Data on NH3, obtained at different times for the same fermenter, were considered as repeated measures with an

autoregressive variance–covariance method to model time dependency. Differences at $P < 0.05$ were considered to be significant, a tendency was considered at $0.05 \leq P < 0.10$.

RESULTS

FERMENTATION TRAITS

Results of the fermentation traits are shown in Table 2. Fermenter fluid samples tended to differ in pH according to diet ($P = 0.071$) but did not differ in redox potential ($P = 0.385$). However, the redox potential increased when *S. striata* extract was included ($P < 0.001$). Concentrations of holotrich protozoa were greater in fermenter fluid samples incubated with the Hay diet ($P = 0.007$), whereas the inclusion of *S. striata* extract decreased the concentration of Entodiniomorphs ($P = 0.049$). Ammonia production was affected by diet ($P < 0.001$), being lowest with the AC diet. Supplementation of *S. striata* extract strongly decreased ammonia production with an interaction of diet and *S. striata* extract ($P = 0.007$), with greater effects of the AC and Hay diets compared to the OA diet (Fig. 1). Production of total SCFA was affected by neither diet nor S. striata extract (respectively, $P = 0.309$ and $P = 0.126$) but there were interactions between diet and inclusion of *S. striata* extract concerning the proportion of iso-butyrate, iso-valerate, and n-valerate (respectively, $P = 0.001$, $P < 0.001$, and $P = 0.005$). In general, the proportion of iso-butyrate, iso-valerate, and n-valerate increased with inclusion of *S. striata* extract when the OA diet was incubated, while it decreased when the other two diets were used (Table 2).

Nutrient degradability and gas production

The degradation of DM, OM, and CP was lower with the AC diet compared to the other diets (diet effect, $P < 0.001$ for DM, OM, and CP degradability) (Table 3). The largest difference was found in CP degradability, which was 20% to 24% lower with the AC diet compared to the Hay and OA diets, respectively (diet effect, $P < 0.001$). The inclusion of *S. striata* extract decreased degradation of OM ($P = 0.006$), CP ($P = 0.002$), EE ($P = 0.018$), NDF ($P = 0.002$), ADF ($P = 0.021$) with tendencies for a diet *S. striata* extract interaction in NDF ($P = 0.096$) and ADF ($P = 0.068$) degradability. In detail, *S. striata* extract tended to decrease NDF degradability only in the AC and Hay diets ($P = 0.063$ and $P = 0.089$, respectively), while ADF degradability decreased when *S. striata* extract was added to the AC diet ($P = 0.034$; data not shown). Variability of ADL degradability was large, showing no effect of *S. striata* extract supplementation, but it was larger with the OA and Hay diets compared to the AC diet ($P = 0.020$). The amount of CH_4 produced per day (mL day^{-1}) and the amount of CH_4 per gram of degraded OM and NDF differed among diets ($P = 0.013$, $P = 0.001$, and $P = 0.027$, respectively), with the greatest amount of CH_4 being produced with the Hay diet. The addition of *S. striata* extract had no effect on absolute CH_4 production, but the amount of CH_4 per g of degraded

NDF was increased (P = 0.036). Interestingly, the same was observed for CO_2, the production of which was increased per g of degraded NDF when *S. striata* extract was added (P = 0.005). The interaction between diet and *S. striata* extract tended to be significant for absolute (mL day−1) CH_4 and CO_2 (P < 0.1) and became significant for CH_4 and CO_2 produced per gram of degraded OM (P = 0.024 and P = 0.044, respectively). While the pairwise comparison of means using Student́ s t-test showed increased production of CH_4 g^{-1} degraded OM (P =0.043) and of CO_2 g^{-1} degraded OM (P =0.019) with the OA diet, differences were not significant when Tukeý s test was used.

Ruminal microbiota

No diet effect on α-diversity indices was observed (Table 4). However, the Chao 1 (P =0.034) and Shannon (P =0.070) indices were decreased or tended toward decrease by the inclusion of *S. striata* extract. Likewise, the numbers of OTUs tended toward decrease following supplementation with *S. striata* extract (P =0.056). In Fig. 2, the two-dimensional PCoA plots show phylogenetic clustering according to the different diets. Thus, samples obtained from fermenters incubated with the Hay diet, with or without *S. striata* extract supplementation, cluster closely together, indicating a distinct microbial composition, while there is no clear separation between the OA and AC diets, with and without *S. striata* extract. Sequencing allowed identification of 14 phyla and 26 genera.

Seven phyla were affected by diet (Table 5), with higher abundances of *Fibrobacteres*, *Lentisphaera*, and *Planctomycetes* with the Hay diet. *Proteobacteria* and *Verrucomicrobia* were highest with the AC diet (P <0.001 and P =0.018, respectively) and decreased with SE supplementation (P <0.001 and P =0.004, respectively). Abundances of *Spirochaetes* and *Tenericutes* were both highest with the OA diet (P =0.038 and P =0.001), while abundances of only *Tenericutes* decreased after *S. striata* extract supplementation (P =0.001). No effects of diet or of *S. striata* extract on *Actinobacteria*, *Cyanobacteria*, *Firmicutes*, *Synergistetis*, *TM7*, or *Euryarchaeota* were found. The effects of diet and *S. striata* extract on identified genera are presented in Fig. 3. The most abundant genus was *Prevotella*, with an approximate relative abundance of 7%, followed by *Bifidobacterium*, with approximately 5%. Neither genus was affected by diet or *S. striata* extract supplementation. The genus *Lactobacillus* was highly abundant in the fermenter samples of the Hay diet supplemented with *S. striata* extract; however, differences were not statistically significant. The genera *Anaerovibrio*, *Butyrivibrio*, *Fibrobacter*, *Methanobrevibacter*, *Methanosphaera*, *Oscillospira*, *Pseudobutyrivubrio*, *Pseudoramibacter*, *Shuttleworthia*, *Streptococcus*, *Succiniclasticum*, Succinivibrio, Treponema, and YRC22 were affected by diet. Supplementation with *S. striata* extract tended to increase the relative abundance of *Anaerovibrio*, and *vadinCA11* (respectively, P =0.092 and P =0.096) and to increase the relative abundance of *Ruminococcus*, *Streptococcus*, and *Succinivibrio* (P =0.016, P =0.006 and

P =0.002, respectively), while the relative abundance of *Butyrivibio*, *Clostridium*, *Pseudobutyrivibrio*, *Pseudoramibacter*, and *YRC22* was suppressed. No effect of *S. striata* extract was detected on *Methanobrevibacter*, *Methanoplanus* and *Methanosphaera*. An interaction between diet and *S. striata* extract supplementation affected the genera *Anaerovibrio* and *Pseudoramibacter* and tended to affect *Pseudobutyrivibrio*. In the case of *Anaerovibrio*, *S. striata* extract supplementation caused an increase in relative abundance only with the Hay diet (P =0.014), while this supplementation caused a significant decline in relative abundance of *Pseudoramibacter* only with the OA diet (P =0.016) and of *Pseudobutyrivibrio* only with the Hay diet (P =0.037).

DISCUSSION

As expected, the tested biorefinery by-product AC was rich in fiber and ADL, while the CP content was considerably lower than that in the original alfalfa silage. As the soluble protein fraction of AC was leached during the biorefinery process, the remaining CP can be expected to be bound to the cell walls[28] and, as such, difficult to digest. In addition, rapeseed meal was added as a CP source, which, in contrast to caseinate, is known to be of low rumen degradability.[29] Indeed, the CP degradability of the AC diet was lower than that of the OA and Hay diets, resulting in lower ammonia production. Supplementation with *S. striata* extract further decreased CP degradability and ammonia production, confirming the results of Bagheri Varzaneh et al.[13] who suggested an improved utilization of nitrogen by the host animal as the result of *S. striata* extract supplementation.

The decrease in degradation due to the inclusion of *S. striata* extract is related to a significant modification of the rumen microbiota. The Chao 1 and Shannon indices showed a decrease in richness and diversity of the microbial population when *S. striata* extract was added, supporting the notion of an antimicrobial effect of *S. striata* extract, as pointed out by Dixon et al.[30] and confirmed by Bagheri Varzaneh et al.[13] However, in contrast to the data presented by the latter authors,[13] no effect of *S. striata* extract was seen on *Fibrobacter*, which is considered to be one of the major fiber-degrading bacteria in the herbivore gut,[31,32] and which is currently the sole formal genus of the bacterial phylum *Fibrobacteres*.[33]

Thus, contrary to our hypothesis of improved fiber degradation due to an increased abundance of *Fibrobacter* with *S. striata* extract supplementation, NDF and ADF degradability were found to decrease significantly when *S. striata* extract was added to the fermenters, which resulted in only a numerically decreased production of SCFA. As the relative abundance of *Fibrobacter* was not affected by the *S. striata* extract, supplementation with this extract must have led to a decrease in the relative abundance or fibrolytic activity of other major fibrolytic bacteria. These include *Ruminococcus* and *Treponema*, which however, like *Fibrobacter*, were not decreased in abundance

by the *S. striata* extract. The inhibiting effects of flavonoid compounds,[11,34] as present in *S. striata* extract,[12] on fiber degradation have been shown before, and an explanation is required, considering that, in the present study, the abundance of major fibrolytic bacteria remained unaffected by *S. striata* extract. In contrast, relative abundance of the genus *Prevotella* increased with the addition of *S. striata* extract to AC and OA diets, which was also observed in the study of Bagheri Varzaneh et al.*13* *Prevotella* spp. are a group of bacteria with highly diverse metabolic capabilities;[35] however, their increased abundance as the result of *S. striata* extract supplementation did not improve nutrient degradability. Overall, addition of *S. striata* extract significantly decreased microbial diversity and richness.

The observed shifts in relative abundance of the identified genera, however, are not sufficient to explain the effects of *S. striata* extract on nutrient degradability. It is well known that CH_4 production is related to fiber degradation,[36] and, although fiber is necessary for maintaining rumen health, a high level of NDF generally promotes CH_4 emission. In support of our hypotheses, CH_4 production was found to be higher with the fiber-rich AC and Hay diets and CH_4 production (i.e., CH_4 g^{-1} of degraded NDF) increased when NDF degradability was lowered by *S. striata* extract. However, in comparison to the study of Bagheri Varzaneh et al.,[13] overall CH_4 production was generally low and *S. striata* extract supplementation did not further decrease methanogenesis. This was unexpected as, in the previous study, CH_4 production was decreased by approximately 30% when *S. striata* extract was supplemented. No difference in the relative abundance of *Euryarchaeota* was found. Likewise, methanogenic genera were unaffected by *S. striata* extract supplementation, with a diet *S. striata* extract interaction apparent only for *Methanosphaera*, which tended to be less abundant when the OA diet was supplemented, as opposed to unsupplemented, with *S. striata* extract. In conclusion, the present findings suggest that AC can be used as fibrous feedstuff for ruminants, as fermentability of the fiber fraction was comparable to that of the other diets.

However, depending on the livestock production type, additional protein supply is necessary, as the remaining CP in AC is hardly accessible for degradation. Unexpectedly, the inclusion of *S. striata* extract did not inhibit methane formation with the present diets.

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Table 1. Ingredients and chemical composition of diets containing OA (original alfalfa silage), AC (alfalfa silage cake) or Hay.

	no *S. striata* extract[a]			plus *S. striata* extract[a]		
	OA	AC	Hay	OA	AC	Hay
Diets components (g kg DM^{-1})						
Grass hay	-	-	600	-	-	567
Alfalfa silage	790	-	-	746	-	-
Alfalfa silage cake	-	600	-	-	567	-
Wheat	200	220	180	189	208	179
Rapeseed meal	-	170	160	-	160	151
Mineral feed	10	10	10	9	9	9
Ca-Caseinate	-	-	40	-	-	38
Plant extract	-	-	-	56	56	56
Chemical composition of diets (g kg DM^{-1})						
OM	89.9	94.3	93.4	90.4	94.5	93.8
CP	17.6	16.3	16.7	16.6	15.4	15.8
EE	2.09	2.35	1.64	1.97	2.22	1.55
NDF	45.4	50.6	49.7	42.9	47.8	47.0
ADF	33.9	38.9	29.4	32.0	36.7	27.7
ADL	5.50	7.07	4.59	5.19	6.68	4.33
NFC	24.8	24.9	25.4	28.9	29.1	29.5

[a]Diets were incubated either with or without supplementation of 60 mg g^{-1} DM of *Scrophularia striata* extract

Table 2. Ruminal fermentation parameters and short-chain fatty acids (SCFA) of fermenters incubated with diets containing OA (original alfalfa silage), AC (alfalfa silage cake) or Hay.

	no *S. striata* extract[a]			plus *S. striata* extract[a]				*P*-value		
Parameter	OA	AC	Hay	OA	AC	Hay	SEM	Diet	Extract	Diet×Extract
pH	6.88	6.89	6.85	6.89	6.85	6.85	0.02	0.071	0.396	0.311
Redox potential (mV)	-261	-271	-278	-232	-224	-234	10.4	0.385	<0.001	0.427
Protozoa (10^3 mL^{-1})										
Holotrichs	0.33	0.77	1.63	0.29	0.90	2.53	0.58	0.007	0.456	0.642
Entodiniomorphs	7.25	10.6	10.3	5.70	7.29	8.27	2.97	0.098	0.049	0.799
Ammonia (mmol d^{-1})	3.82^{a}	2.85^{bc}	3.99^{a}	3.21^{b}	1.44^{d}	2.48^{c}	0.201	<0.001	<0.001	0.007
Total SCFA (mmol d^{-1})	33.5	31.9	32.5	32.3	31.5	29.4	1.23	0.310	0.126	0.539
Individual SCFA (% proportion)										
Acetate	53.9	52.3	51.5	50.6	52.1	52.5	1.42	0.979	0.446	0.240
Propionate	26.7	25.7	26.7	27.0	27.0	28.2	1.05	0.394	0.152	0.710
iso-butyrate	0.813^{ab}	0.825^{ab}	0.85^{ab}	0.927^{a}	0.728^{b}	0.703^{b}	0.043	0.011	0.137	0.001
n-butyrate	11.7	12.1	12.6	12.6	12.0	11.4	0.737	0.982	0.749	0.221
iso –valerate	2.50^{b}	3.08^{a}	2.98^{ab}	3.17^{a}	2.93^{ab}	2.53^{b}	0.128	0.151	0.813	<0.001
n-valerate	3.21^{b}	4.30^{ab}	3.90^{ab}	4.31^{a}	3.82^{ab}	3.38^{ab}	0.371	0.265	0.875	0.005
Caproate	1.11	1.65	1.47	1.53	1.42	1.23	0.187	0.291	0.886	0.050

[a]Diets were incubated either with or without supplementation of 60 mg g^{-1} DM of *Scrophularia striata* extract

Means in the same row bearing different letters (a, b, c, d) indicate differences at P<0.05

Table 3. Nutrient degradability and gas production of diets containing OA (original alfalfa silage), AC (alfalfa silage cake) or Hay.

	no *S. striata* extract[a]			plus *S. striata* extract[a]				P-value		
Parameter	OA	AC	Hay	OA	AC	Hay	SEM	Diet	Extract	Diet×Extract
Nutrient degradability (%)										
DM	59.5	53.6	57.0	61.1	51.1	54.7	2.58	<0.001	0. 206	0.073
OM	59.8	53.9	56.1	58.8	50.1	53.7	2.57	<0.001	0.006	0.390
CP	75.2	57.8	71.0	73.9	51.0	61.6	5.18	<0.001	0.002	0.167
EE	59.8	55.9	53.3	59.8	46.3	45.2	8.05	0.002	0.018	0.216
NDF	29.5	28.7	29.9	29.1	19.7	21.3	2.51	0.068	0.002	0.096
ADF	27.9	29.2	23.0	28.4	21.9	20.2	2.41	0.001	0.021	0.068
ADL	13.5	6.73	14.0	14.0	3.33	7.75	2.85	0.020	0.207	0.498
Gas production										
CH_4 (mL d^{-1})	38.0	43.1	54.7	47.5	33.6	49.1	5.51	0.013	0.597	0.087
CO_2 (mL d^{-1})	256	276	298	323	244	287	30.8	0.290	0.657	0.086
CH_4 (mL g^{-1} of degraded OM)	6.18^{b}	7.50^{b}	10.4^{a}	8.14^{ab}	6.43^{b}	8.83^{ab}	0.75	0.001	0.671	0.024
CO_2 (mL g^{-1} of degraded OM	43.7^{b}	49.4^{ab}	59.9^{a}	55.7^{ab}	50.2^{ab}	54.0^{ab}	3.78	0.071	0.412	0.044
CH_4 (mL g^{-1} of degraded aNDFom)	27.6	27.6	37.2	34.7	35.2	60.1	7.24	0.027	0.036	0.451
CO_2 (mL g^{-1} of degraded aNDFom)	195	182	214	238	274	359	37.1	0.146	0.005	0.402

[a]Diets were incubated either with or without supplementation of 60 mg g^{-1} DM of *Scrophularia striata* extract

Means in the same row bearing different letters (a, b) indicate differences at $P < 0.05$

Table 4. Alpha diversity indices Chao 1, Shannon and Simpson and OTUs of fermenter samples incubated with diets containing OA (original alfalfa silage), AC (alfalfa silage cake) or Hay.

	no *S. striata* extract[a]			plus *S. striata* extract[a]				P-value		
	OA	AC	Hay	OA	AC	Hay	SEM	Diet	Extract	Diet×Extract
Chao 1	1806	1810	1772	1747	1681	1607	83.4	0.403	0.034	0.705
Shannon	6.30	6.36	6.87	6.09	6.10	6.26	0.317	0.234	0.070	0.648
Simpson	0.94	0.94	0.97	0.93	0.94	0.94	0.017	0.574	0.191	0.739
OTUs	1319	1306	1284	1246	1193	1168	80.5	0.666	0.056	0.929

[a]Diets were incubated either with or without supplementation of 60 mg g^{-1} DM of *Scrophularia striata* extract

Table 5. Effects of diet containing OA (original alfalfa silage), AC (alfalfa silage cake) or Hay on relative percent abundance of microbial phyla in pooled liquid and solid fermenter content.

	no *S. striata* extract[a]			plus *S. striata* extract[a]				P-value		
Phylum	OA	AC	Hay	OA	AC	Hay	SEM	Diet	Extract	Diet×Extract
Actinobacteria	5.81	5.47	5.02	8.82	5.13	4.69	3.585	0.576	0.701	0.740
Bacteroidetes	8.17	8.62	9.47	8.92	10.1	8.17	0.721	0.407	0.531	0.085
Cyanobacteria	0.63	0.24	0.45	0.94	0.47	1.56	0.444	0.344	0.142	0.142
Euryarchaeota	1.09	1.19	0.92	1.07	1.20	0.97	0.143	0.173	0.882	0.958
Fibrobacteres	0.24	0.53	2.10	0.42	0.65	1.27	0.345	0.001	0.538	0.278
Firmicutes	59.1	59.1	53.0	57.0	58.6	61.6	4.199	0.884	0.436	0.190
Lentisphaerae	0.18	0.23	0.46	0.21	0.19	0.36	0.054	0.001	0.427	0.538
Planctomycetes	0.08	0.11	0.27	0.04	0.11	0.17	0.051	0.010	0.237	0.569
Proteobacteria	0.41	1.22	0.59	0.70	1.81	1.09	0.114	<0.001	<0.001	0.238
Spirochaetes	3.21	2.25	2.18	2.73	2.35	1.77	0.404	0.038	0.396	0.705
Synergistetes	0.12	0.12	0.13	0.13	0.12	0.11	0.031	0.946	0.819	0.834
Tenericutes	4.35	2.22	2.34	2.18	1.68	1.15	0.417	0.001	0.001	0.124
TM7	0.32	0.21	0.32	0.25	0.20	0.22	0.073	0.348	0.203	0.748
Verrucomicrobia	0.23	0.36	0.24	0.18	0.22	0.14	0.079	0.018	0.004	0.404

[a]Diets were incubated either with or without supplementation of 60 mg g^{-1} DM of *Scrophularia striata* extract

Figure 1. Ammonia production in the fermenter fluid after providing fresh feed bags (0, 2, 4, 6 h). Diets comprised alfalfa silage cake (AC), original alfalfa (AO) or fiber-rich hay (Hay), with or whitout 60 mg g^{-1} DM of *Scrophularia striata* extract.

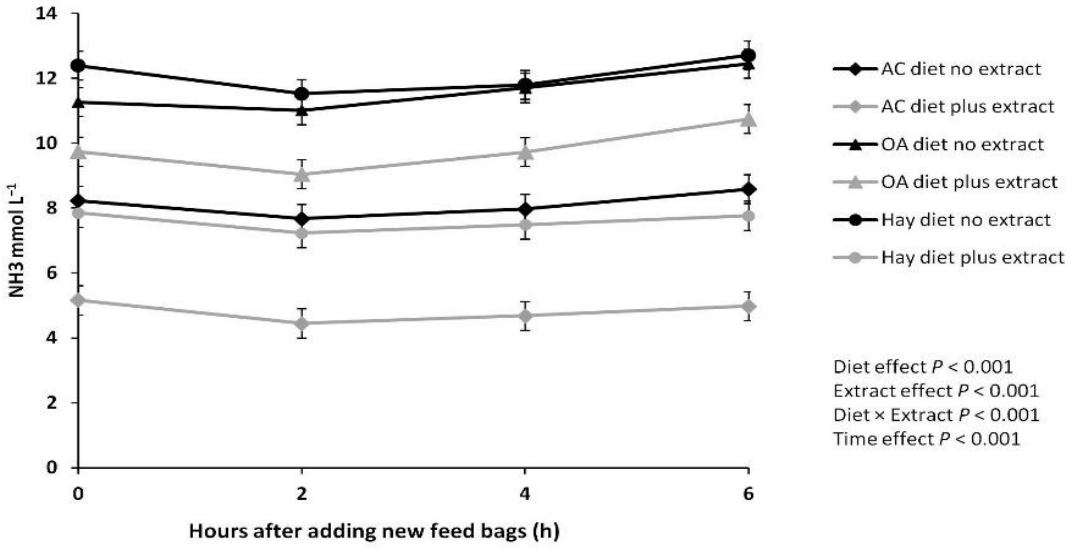


Figure 2. Principal coordinate analysis (PCoA) plots based on Bray Curtis analysis, showing the phylogenetics clustering according to the 6 diets. The 3 axes (PC1, PC2 and PC3) represent the variation among the samples in percent. (□, Hay diet without Scrophularia striata extract; ■ , Hay diet plus S. striata extract; ○ , AC diet without S. striata extract; ● , AC diet plus S. striata extract; ◁ , OA diet no S. striata extract; ▶ , OA diet plus S. striata extract).

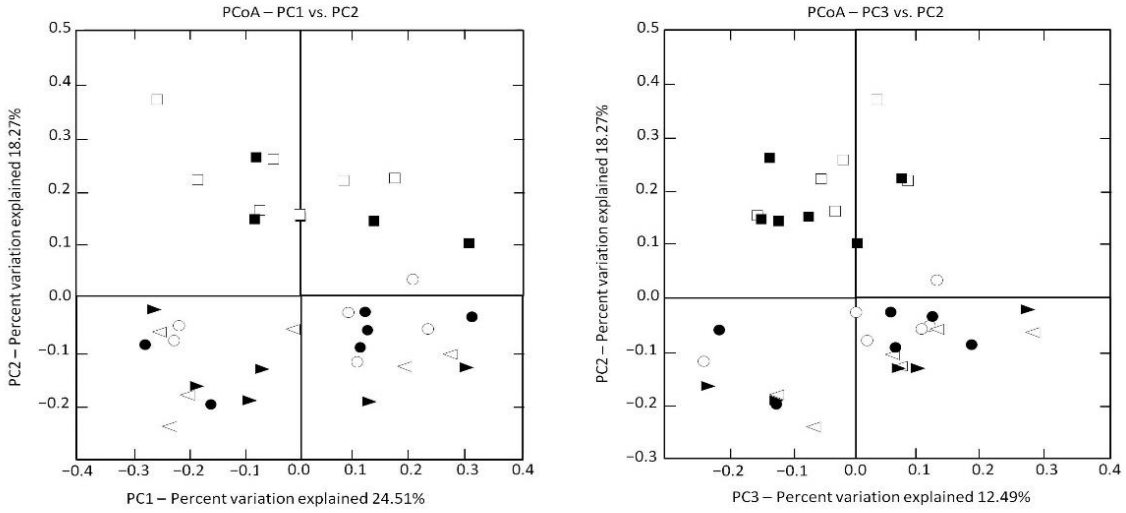
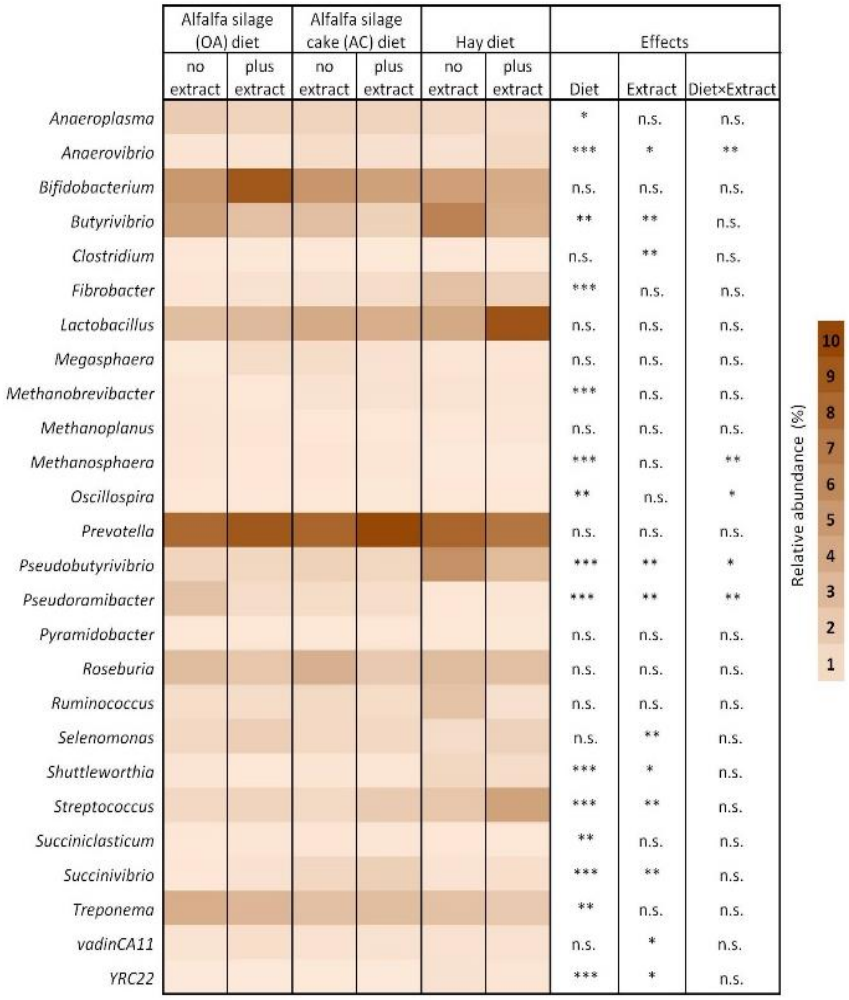


Figure 3. Relative abundance of microbial genera of pooled DNA from liquid and solid fermenter content. Diets contained alfalfa silage cake (AC), or original alfalfa silage (OA) or Hay, either with or without 60 mg g^{-1} DM of *Scrophularia striata* extract. Statistical significance: ***, $P \leq 0.001$; ** $P \leq 0.05$; * $P \leq 0.1$, n.s., non-significant.



Effects of *Scrophularia striata* extract on *in vitro* nutrient degradation and CH_4 formation when added to a diet containing a low-quality fibre source

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Introduction

- *Scrophularia striata* is a herbaceous flowering plant, which has long been used as a medicinal herb in some Asian countries.
- The extract of *S. striata* (SE) is rich in phenolic and flavonoid compounds. It has previously been shown to decrease CH_4 formation, crude protein (CP) degradation and NH_3 concentrations when added to a diet containing high-quality hay in *in vitro* rumen simulation technique (Rusitec) fermenters.
- The biorefinery that uses alfalfa silage juice as valuable amino acid source generates a fibrous by-product, termed alfalfa silage cake (AC).

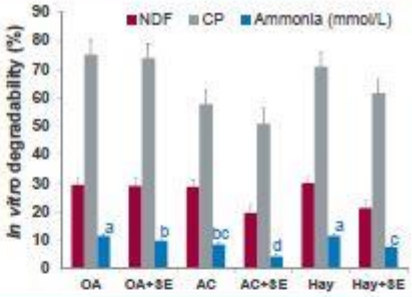
The aim was

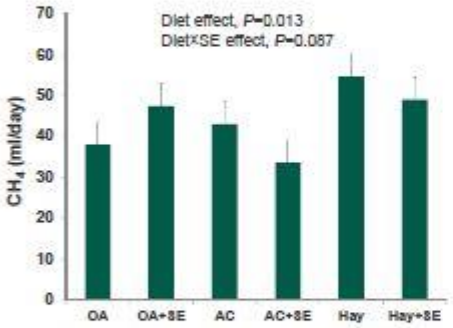
to investigate the effects of *Scrophularia striata* extract (SE) when added to a diet with a low-quality fibre source like the bio-refinery by-product alfalfa silage cake (AC) in the Rusitec system

Materials and Methods

- Three forages, the bio-refinery by-product alfalfa silage cake (AC), the original alfalfa silage (OA) and a fiber-rich hay as control (Hay) were tested as part of a total mixed ration
- Diets were incubated in the Rusitec-system in three experimental runs (each lasting 10 days) either with or without supplementation of freeze dried SE (80 mg/kg DM) resulting in 6 replicates per treatment.
- Parameters measured during the last five days of each run were:
  - Gas production (CH_4 and CO_2)
  - Concentration of short chain fatty acids (SCFA)
  - Concentration of NH_3
  - Nutrient degradation after 48 h of incubation
- Statistics: SAS proc mixed (version 9.4) using a 3 (diets) × 2 (± SE supplementation) factorial design

Dietary components (g/kg DM)	OA	AC	Hay
Grass hay	-	-	600
Alfalfa silage	790	-	-
Alfalfa silage cake	-	600	-
Wheat	200	220	180
Rapeseed meal	-	170	160
Mineral feed	10	10	10
Ca-Caseinate	-	-	40
Chemical composition of diets (g/kg DM)			
CP	17.6	16.3	16.7
NDF	45.4	50.6	49.7
ADF	33.9	38.9	29.4
ADL	5.50	7.07	4.59





	no SE			plus SE				P-value	
Parameter	OA	AC	Hay	OA	AC	Hay	SEM	SE	SE×Diet
pH	6.88	6.89	6.85	6.89	6.85	6.85	0.02	n.s.	n.s.
Redox potential (mV)	-261	-271	-278	-232	-224	-234	10.4	<0.001	n.s.
Total SCFA (mmol/L)	99.8	95.7	93.2	97.0	94.2	87.5	3.65	n.s.	n.s.
Individual SCFA (% total SCFA)									
Acetate	53.9	52.3	51.5	50.6	52.1	52.5	1.86	n.s.	n.s.
Propionate	26.7	25.7	26.7	27.0	27.0	28.2	1.15	n.s.	n.s.
Iso-butyrate	0.81^{ab}	0.83^{ab}	0.84^{ab}	0.93^{a}	0.73^{b}	0.70^{b}	0.04	n.s.	0.001
n-butyrate	11.7	12.1	12.6	12.6	12.0	11.4	0.74	n.s.	n.s.
Iso –valerate	2.50^{b}	3.08^{a}	2.98^{ab}	3.17^{a}	2.94^{ab}	2.53^{b}	0.13	n.s.	0.001
n-valerate	3.21^{b}	4.27^{ab}	3.90^{ab}	4.31^{a}	3.82^{ab}	3.38^{ab}	0.37	n.s.	0.005
Caproate	1.11	1.65	1.47	1.53	1.42	1.23	0.19	n.s.	0.050

Summary and conclusions

Supplementation of SE decreased CP degradability (P=0.002), with effects being strongest with AC and Hay diets, and ammonia concentrations (SE effect, P<0.001; Diet×SE effect, P=0.001).
Supplementation of SE tended to decrease NDF degradability only in the AC and Hay diets (P=0.063).
Likewise inhibitory effects of SE on methane formation were only prominent when SE was supplemented to the diets containing AC and Hay, but not when added to a diet rich in alfalfa silage (Diet×SE effect, P=0.087; Diet effect, P=0.013).

Effects of *Scrophularia striata* extract on *in vitro* nutrient degradation and CH_4 formation when added to a diet containing a low-quality fibre source

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Abstract content

In terms of a sustainable meat and dairy production, by-products of the food production chain are gaining importance as feedstuffs for livestock animals. The bio-refinery that uses alfalfa silage juice as amino acid source generates a fibrous by-product (alfalfa silage cake; AC), which according to our hypothesis can be used as nutritional fibre source for ruminants. However, crude protein (CP) and energy contents are low and additional concentrate is needed to improve dietary nutrients. *Scrophularia striata* is a herbaceous flowering plant, which has long been used as a medicinal herb in some Asian countries. The extract of *S. striata* is rich in phenolic and flavonoid compounds (1) and has previously been shown to have antimicrobial properties, resulting in decreased CH4 formation and decreased CP degradation and ammonia concentrations when added to a diet containing high-quality hay in *in vitro* rumen simulation technique (Rusitec) fermenters (2). Now, we wanted to investigate the effects of *S. striata* extract (SE) when added to a diet with a low-quality fibre source like AC.

Methods: Three forages, the bio-refinery by-product alfalfa silage cake (AC), the original alfalfa silage (OA) and a fiber-rich hay as control (Hay) were tested each as part of a total mixed ration either with or without supplementation of freeze-dried SE (60 mg/g of DM) in a Rusitec-system. The forages differed in CP (g/kg dry matter (DM): AC = 120, OA = 190 and Hay = 70). While NDF content (710 g/kg DM) of AC and Hay was similar, ADL content was considerably higher in AC than in Hay (87 g/kg DM vs. 48 g/kg DM). Concentrates were formulated to balance for similar contents of CP (169 g/kg DM) and non-fibrous carbohydrates (250 g/kg DM) in the unsupplemented diets. The Rusitec experiment consisted of 3 runs, each one lasting 10 days with the last 5 days serving as sampling period. Samples were analyzed for concentrations of ammonia and short chain fatty acids (SCFA) as well as for pH-value and redox potential. In addition, gas production and nutrient degradability of the incubated diets were measured after 24 and 48 hours of incubation, respectively.

Statistical analysis was performed with Proc Mixed of SAS (9.4) using a 3×2 factorial design (three diets and two treatments: no SE or supplementation of 60 mg/g DM SE).

Results: Fermenter pH was in tendency affected by diet (P=0.071), while redox potential increased significantly when SE was included (P<0.001). While NDF degradability of all diets was similar (29.4%), degradability of CP was much lower with the AC diet compared to the others (diet effect P<0.001). Both, degradability of CP and NDF were strongly reduced due to SE supplementation in the AC (-12% and -31%) and Hay (-13% and -29%) diets (effect of SE on CP and NDF degradability, respectively: P=0.002 and P=0.018). Consequently, ammonia concentration was lower when SE was supplemented (P<0.001), whereas concentrations of short chain fatty acids remained unaffected. Unexpectedly, CH4 formation was not inhibited by SE, instead it differed between diets (P=0.013) with a trend for a diet×SE interaction (P=0.087). Supplementation of SE tended to inhibit CH4 formation with the AC (-22%) and Hay (-10%) diets, while CH4 formation was increased when SE was added to the OA diet (+25%).

Conclusions: NDF degradability was similar between diets, indicating AC's suitability as nutrient fiber source for ruminal microbes. Supplementation of SE decreased *in vitro* ruminal NDF and CP degradability and ammonia concentrations. Effects of SE supplementation on ruminal methane formation depended on the dietary composition, as inhibitory effects were only prominent when SE was supplemented to the diets containing AC and Hay but not when added to a diet rich in alfalfa silage. Further research is necessary to elucidate the interaction of SE with dietary components and their effects on the ruminal microorganisms that are involved in ruminal nutrient degradation.

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Original Research Article

In vitro rumen fermentation of feed substrates added with chestnut tannins or an extract from *Stevia rebaudiana* Bertoni

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ABSTRACT

Rumen fermentation parameters and microbiota were evaluated in 3 *in vitro* rumen fermentation experiments after addition of chestnut tannins (CT) or an extract from *Stevia rebaudiana* Bertoni (SB) to substrates. A control (CTR) substrate was fermented alone or added with 1.5% of CT or SB extracts in a batch culture system (Exp. 1, fermentation in 500 mL for 24 h) and in a subsequent continuous culture system (Exp. 2, fermentation in 2 L bottles for 9 d). Experiment 3 used the fermentation system of Exp. 1 and tested 7 doses of each extract added to CTR (additions of 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 1.2% and 1.4% for 48 h). The addition of CT lowered ($P < 0.01$) the *in vitro* rumen ammonia concentration in all experiments and reduced the protozoa counts in Exp. 1 ($P < 0.05$). In contrast, the SB extract did not modify the ammonia concentrations, but significantly lowered the protozoa counts in all 3 experiments (reduction of 47% and 20% in Exp. 1 and 2, $P < 0.05$; and a quadratic reduction in Exp. 3, $R^2 = 0.63$, $P < 0.01$). Neither extract affected the fermentation in terms of gas production (Exp. 1 and 3) nor volatile fatty acids (VFA) yield (Exp. 1 and 2), if we exclude a reduction at the highest CT concentration in Exp. 3. Changes in VFA profile were induced by CT and were limited to reductions in the iso-valerate ($P < 0.01$, in Exp. 2) and iso-butyrate levels ($P < 0.01$, Exp. 2). The CT increased the abundance of *Prevotella ruminicola* and *Selenomonas ruminantium* and decreased that of *Ruminobacter amylophilus* ($P < 0.01$, $P < 0.05$ and $P < 0.05$, respectively). The SB extract increased the relative abundance of *Treponema saccaraphylum* ($P < 0.05$). Both of the studied substances had an impact on rumen metabolism, with SB reducing protozoa counts and CT lowering the rumen ammonia concentration. The effects of both extracts on the rumen were appreciable at low dietary doses, and the negative impacts on fermentation were limited to the reduction in protein degradation with the addition of CT.

1. Introduction

Natural substances extracted from plants can be used as safe dietary additives to favorably modify rumen fermentation and microbiota in terms of pollution mitigation (e.g. methane and ammonia) and improve feed efficiency.

Tannins are known to be bioactive in the rumen, mainly for their capacity to form complexes with proteins and/or to inhibit the enzymatic activity of rumen protease and urease (Patra and Saxena, 2011; Patra and Aschenbach, 2018). However, tannins are a complex family of compounds (e.g. hydrolysable and condensed) with different chemical structures depending on their botanical origin. Moreover, the availability of literature for condensed tannins is extensive, whereas hydrolysable tannins have been less studied in animal nutrition. Chestnut tannins (extracted from chestnut wood, *Castanea sativa* L.; CT) are hydrolysable tannins, that have been studied alone (Hassanat and Benchaar, 2013; Liu et al., 2011; Witzig et al., 2018) or in association with condensed tannins (Aboagye et al., 2018) with positive effects in terms of mitigation of rumen N degradation. Moreover, reductions in methane were measured *in vitro* at high dietary concentrations of CT (Hassanat and Benchaar, 2013; Witzig et al., 2018), and negative impacts on protozoa were found *in vivo* in sheep (Liu et al., 2011).

Stevia (*Stevia rebaudiana* Bertoni, SB) is a perennial shrubby plant belonging to the Asteraceae family known for its steviol glycosides content (mainly, stevioside and rebaudioside A) which have high-intensity sweetness. In addition, Stevia glycosides are thought to have antimicrobial effects and were used to reduce unfavorable bacteria in the gut of young pigs to prevent diarrhea (Munro et al., 2000; Wang et al., 2014). Knowledge on the effects of stevia on the rumen is limited to a recent work (Ramos-Morales et al., 2017) that tested the effects of addition of Stevia leaf extract on rumen fermenters and found a depressive effect on the rumen protozoal population and ammonia and shifts in the bacterial community.

The present research has the aim to study the effects on *in vitro* rumen fermentation of dietary addition of these 2 natural substances

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2405-6545/

Chapter 3. *In vitro* rumen fermentation of feed substrates added with chestnut tannins or an extract from *Stevia rebaudiana* Bertoni

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Abstract

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Keywords: Rumen, ammonia, protozoa, chestnut tannin, *Stevia rebaudiana* Bertoni

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Castanea sativa L.; CT) are hydrolysable tannins, which have been studied alone (Hassanat and Benchaar, 2013; Liu et al., 2011; Witzig et al., 2018) or in association with condensed tannins (Aboagye et al., 2018) with positive effects in terms of mitigation of rumen N degradation. Moreover, reductions in methane were measured *in vitro* at high dietary concentrations of CT (Hassanat and Benchaar, 2013; Witzig et al., 2018) and negative impacts on protozoa were found *in vivo* in sheep (Liu et al., 2011).

Stevia (*Stevia rebaudiana* Bertoni, SB) is a perennial shrubby plant belonging to the Asteraceae family known for its steviol glycosides content (mainly, stevioside and rebaudioside A) which have high-intensive sweetness. In addition, Stevia glycosides are thought to have antimicrobial effects and were used to reduce unfavorable bacteria in gut of young pigs to prevent diarrhea (Munro et al., 2000; Wang et al., 2014). Knowledge on the effect of stevia on rumen is limited to a recent work (Ramos-Morales et al., 2017) that tested the effect of addition of Stevia leaves extract on rumen fermenters and found a depressive effect on the rumen protozoal population and ammonia and shifts in the bacterial community.

The present research has the aim to study the effects on *in vitro* rumen fermentation of dietary addition of these 2 natural substances at low doses, typical of additive supplements. We performed a series of *in vitro* rumen fermentations to evaluate changes in fermentability and the rumen microbiota and to uncover possible dose-dependent effects of the 2 extracts. In our experiments we used a maximum dose of pure tannin (1.1% dry matter DM of tannic acid equivalent) much lower than doses (2.0% DM) assumed to limit intake in ruminants (Jayanegara et al., 2012) and compatible with practical dose of additive supplements in ruminant diets. We adopted the same doses used for CT and SB despite the higher doses already tested *in vitro* (Ramos Morale et al., 2017).

2. Materials and methods

2.1 Diets and plant extracts

SaviotanN Feed supplement, made of hydrolyzable tannins from *Castanea sativa* Miller, was provided by Gruppo Mauro Saviola s.r.l. (Viadana, MN, Italy). SaviotanN Feed supplement was extracted from chestnut wood and used in feed formulations as source of CT (750 g of tannic acid equivalent/kg of DM). The chemical composition and gas chromatographic profile of the CT is available in the study of Campo et al. (2012).

Stevia leaves were obtained by Bio Mondo (Porcia, PN, Italy). Dried and milled leaves were maintained in ethanol solution for 6h (Ramos-Morales et al., 2017) and then filtered, and the liquid

extract was dried by a Univapo 100 ECH vacuum concentrator centrifuge (UniEquip, Planegg, Garmany).

The extracts were added to ground corn meal in Exp. 1 or to a mixture of feeds, which simulated a total mixed ratio for high producing ruminants in Exp. 2 and 3, in amounts ranging from zero to 1.5% of DM. The feed ingredients used as substrate for the 3 experiments were ground through a 1.0-mm screen (Ciclotec Tecator) and were analyzed (AOAC, 2000) for DM, crude protein (CP) and neutral detergent fiber (aNDF, Van Soest et al., 1991) using an Ankom 200 Fiber Analyzer (ANKOM Technology, Macedon, NY).

2.2. In vitro experiments

The study employed 3 different *in vitro* rumen fermentation experiments: a batch system was used to evaluate the fermentation parameters, gas production and protozoa population (Exp. 1), a rumen continuous fermenter to determine the effects of the additives on the microbiome (Exp. 2) and a batch system to study the dose-response effect of the 2 additives (Exp. 3). The rumen fluid for all the fermentation runs of each experiment was collected in the same slaughterhouse in controlled conditions (e.g from culled dairy cows for each rumen collection, animal fed productive total mixed rations base on corn silage, not slaughtered in emergency, in good health status, transported from farms located near the slaughterhouse, rumen fluid sampled within 20 min of slaughter) and was delivered to the laboratory within 0.5 h in airtight glass-bottles refluxed with carbon dioxide and maintained at 39 °C.

2.2.1 Rumen fermentation in a batch system (experiment 1).

Rumen fermentation was conducted in 3 runs using a gas production fermentation system consisting of six 500 mL bottles, closed by a stirring device and connected to a MilliGascounter (measuring range: from 1 mL/h to 1 L/h; measuring accuracy 3%; Dr.Ing. Ritter Apparatebau GmbH & Co. KG). The incubation medium contained 125 mL of the rumen fluid and a double strength buffer (375 mL of bicarbonate-mineral-distilled water mixture, 2:1:3) according to Blümmel and Becker (1997). This adaptation of the original buffer of Menke et al. (1979) permits an increase in amount of the sample for fermentation because the added bicarbonate buffer neutralizes a higher amount of volatile fatty acid (VFA). A larger amount of feed substrate allows to minimize the error in the additives weighing phase. Corn meal (5.5 g of DM) was introduced in each fermentation bottle and was incubated alone as a control and with the addition of one of the 2 extracts, SB and CT (1.5% of corn DM); each treatment was incubated in duplicate bottles per fermentation run. Fermentation bottles were maintained at 39° C for 24 h, and samples for protozoa counts were collected at 4 and 24 h, and samples for ammonia, VFA and pH were collected at 24 h. Gas data were automatically recorded in

a computer connected with the gas counters and equipped with a specific software (Rigamo v3.1,©2012 Dr.Ing. Ritter Apparatebau GmbH & Co. KG) until the end of the fermentation. Fermentation fluid samples at 24 h were taken by completely opening the fermenters at the end of fermentation, the 4-h samples were collected by a syringe through the gas outflow hole. The gas losses during the sample collection were negligible given the small hole with respect to the fermentation bottle volume (10 mm diameter vs. 500 mL), the short sampling duration (approximated 10 s) and the system of gas recording, which is based on a continuous measurement at ultralow flow rates that does not require accumulation of gas under pressure within the bottle.

2.2.2 Rumen fermentation in continuous system (experiment 2).

Two different fermentation runs were performed using six 2-L single-flow continuous fermenters, as described in Mason et al. (2015). A standard diet composed on a DM basis by 15% of hay, 35% of corn silage and 50% of concentrate for dairy cows was used as control (88% DM, 14.3% CP and 42.6% aNDF). The 2 treatments consisted in the addition of 1.5% of DM of SB or CT. Diets were given to each fermenter (2 bottles per treatment) in 2 equal doses (at 09:00 and 17:00) for a total of 18 g/d of DM. Each run lasted 9 d, with 6 of adaptation and 3 of sampling, and the artificial saliva (McDougall, 1948) was continuously infused by a peristaltic pump at 1.3 ml/min. Each day, samples of fermentation fluid were collected for protozoa counts. During the last 3 d before the morning feeding samples for ammonia, pH, and bacterial DNA analysis were collected and stored at - 20° C. For VFA analysis the samples were acidified with H_2SO_4 0.05 mol/L and stored at - 20° C. Ammonia samples were collected just before the morning meal and every hour thereafter for 3 h.

2.2.3 Dose-effect studied by a rumen fermentation batch system (experiment 3)

An independent fermentation experiment was conducted for each extract. Each experiment used 8 fermentation bottles of the same apparatus of Exp. 1 during 3 subsequent fermentation runs. The mixed diet of Exp. 2 was incubated in each bottle alone (5.5 g DM/bottle) or with the following doses of extracts: 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 1.2% and 1.4% of incubated DM. Samples of fermentation fluid were collected at 24 h for protozoa count (SB addition) and ammonia determination (CT addition) as previously described for Exp. 1, and gas production was automatically recorded until 48 h by Rigamo v3.1 software (©2012 Dr.Ing. Ritter Apparatebau GmbH & Co. KG).

2.3 Sample analysis

Protozoa were counted as described by Dehority (2003), and pH was immediately measured after sample collection (GLP 22, Crison Instruments, S.A. Barcelona, Spain).

Samples of fermentation liquid for ammonia analysis were stored at - 20° C. Before the analysis, samples were thawed at room temperature and measured by an ammonia electrode (Ammonia Gas Sensing Combination Electrode, ©Hach Company, 2001). Then the samples were thawed at room temperature, centrifuged at 20,000g for 30 min at 20° C and the supernatant was filtered using polypore 0.45 µm filters (Agilent Technologies, Milano, Italia). The filtrate was injected into a high-performance liquid chromatography instrument (Perkin-Elmer, Norwalk, CN, USA), set to 220 nm according to the method described by Martillotti and Puppo (1985).

2.4 DNA extraction and quantitative PCR

Before DNA extraction fermenter fluid samples of the 3 sampling days were thawed on ice and pooled per bottle. Total DNA was extracted from approximated 800 µl of fermentation fluid using the PowerSoil DNA extraction kit (MoBio Laboratories Inc., Carlsbad, CA, USA) with some modifications. The DNA elution volume was 50µl, and the isolated DNA concentration was determined by NanoDrop One (Thermo Fisher Scientific Inc., Wilmington, DE 19810 U.S.A).

Quantitative PCR (qPCR) was performed using the CFX96 Real Time System (Bio-Rad Technologies Inc, Hercules, California). The total reaction volume was 20 µl and consisted in 0.3 µl of each forward and reverse primer (0.3 µmol/L), 10 µl of iQ SYBR Green Supermix (Bio-Rad Technologies Inc, Hercules, California), 8.4 µl of sterile water and 1 µl of gDNA (appendix Table 1). The amplification program included polymerase activation and denaturation at 98° C for 3 min, 40 cycles of 98° C for 15 s, annealing at 60°C for 30 s and elongation at 72° C for 30 s. A melting curve was established to determine the specificity of the amplification. The amplification efficiency (E) was calculated using the formula: $E = 10^{-1/slope}$. The relative abundance of target bacterial and archaeal groups or species was expressed in proportion of total bacteria 16S rRNA gene and archaeal 16S rRNA gene, respectively. The relative abundance was calculated using the following formula:

Relative abudance $=2^{-\Delta CT}$.

Where CT is cycle threshold, and ΔCT = CT (calibrator) – CT (sample).

2.5 Statistical analyses

In all experiments, the fermentation runs were performed in different periods (weeks) and replicates between runs were the statistical replicates in Exp. 1 and 3. In Exp. 2, continuous fermenters (2 per treatment within each fermentation run) were considered experimental units.

The data from Exp. 1 and 2 (except for protozoa counts of Exp. 1 and ammonia of Exp. 2) were statistically analysed as a factorial randomised complete block (fermentation run) design:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \varepsilon_{ijk},$$

where $y_{ijk,}$ is the experimental data, μ is the overall mean, α_i is the random effect (block) of the fermentation trial (I = 1, 3 in Exp. 1 and I = 1, 2 in Exp. 2); β_j is the fixed effect of the dietary treatment (j = 1, 3); and ε_{ijk} is the random error (k = 1, 2 in Exp. 2).

The protozoa composition of Exp. 1 and 2 and ammonia data of Exp. 2 were analyzed according to a repeated measurement design:

$$Y_{ijkl} = \mu + \alpha_i + \beta_j + \gamma_k + (\beta\gamma)_{jk} + \delta_l + \varepsilon_{ijkl}$$

where y, μ, α, β and ε are as described for previous model; γ_k is the fixed effect of sampling time (for protozoa cell count: 4 and 24 h in Exp. 1 and d 1 to 9 in Exp. 2, k = 1, 2 and 1, 9, respectively; for ammonia of Exp. 2: k = 1, 2) and δ_l is the random effect of fermentation bottle (l = 1, 6).

Volumes of gas from each fermentable bottle were recorded continuously until 48 h in Exp. 3, and cumulative gas values at 2 h intervals of were fitted with the following exponential model without lag phase:

$$y = B \times (1 - \exp^{-k \cdot t}),$$

where y is the cumulative gas volume (mL) produced at time t (h), B (mL) is the asymptotic gas volume (mL) and k is a constant rate (mL/h).

The asymptotic gas volume and the constant rate of gas production of Exp. 3 were statistically analysed as a factorial randomised complete block (fermentation run) design:

$$Y_{ijk} = \mu + \alpha_i + \beta_j + \varepsilon_{ijk}$$

Where y_{ijk} is the experimental data, μ is the overall mean, α_i is the random effect (block) of the fermentation trial (i = 1, 3); β_j is the fixed effect of the dose of each extract (j = 1, 8); and ε_{ijk} is the random error.

The ammonia and protozoal counts measured in fermentation fluids in Exp. 3 were regressed on increasing doses of CT and SB according to the following second order polynomial mixed model:

$$Y_{ij} = \beta_0 + \beta_1 x_{ij} + \beta_2 x^2_{ij} + s_i + e_{ij}$$

where β_0 is the overall intercept across fermentation runs (fixed effect), β_1 and β_2 are the overall regression coefficients for the linear and quadratic effect of x (fixed effects), x_{ij} is the dependent variable for the i^{th} extract doses of the j^{th} fermentation run, ($i = 1, n_j$; $j = 1, 8$), s_i is the random effect of fermentation run i, approximately normal $(0, \sigma^2_s)$, and e_{ij} is the residual error, approximately normal

(0, σ^2_e). Adjusted values for the fermentation run effect, calculated according to St-Pierre (2001), were used to generate 2 dimensional graphs.

For all statistical analyses, probability significance level (*P*) were 0.05 and 0.01 ($P < 0.05$ and < 0.01, respectively).

3. Results

3.1 Experiment 1

The addition of CT and SB to corn meal had no effect on total gas production and total VFA and ammonia was decreased by CT ($P < 0.05$, Table 1). Total protozoa count (Table 2) was significantly affected by treatment ($P < 0.05$) with the lowest value be observed with SB in comparison with that of CT and CTR and there was a significant reduction from 4 to 24 h ($P < 0.05$). The dietary treatment did not modify the protozoa subfamilies proportions, except for a reduction ($P < 0.05$) of Ophryscolecinae due to SB addition compared to CT. Finally, Ophryscolecinae increased their relative abundance from 4 to 24 h ($P < 0.01$).

3.2 Experiment 2

The average ammonia concentration of fermentation liquid in the rumen continuous culture (CC) fermenter was lower with CT addition than with the other treatments ($P < 0.01$, Table 3). Dietary treatments did not modify the total VFA yield and the CT inclusion decreased the proportions of iso-butyrate and iso-valerate ($P < 0.01$) and increased the valerate proportion ($P < 0.01$) compare with the other tretaments. The following rumen microbiota changes were detected: *Prevotella ruminicula group* and *Selenomonas ruminantium* were increased by the CT treatment whereas *Ruminobacter amylphilus* decreased ($P < 0.05$, $P < 0.01$ and $P < 0.01$, respectively). SB positively affected the relative abundance of T*reponema saccarophylum* ($P < 0.01$) compared with CTR and CT.

For protozoa counts the interaction between the sampling day (time) and the dietary treatment was not significant (Fig. 1). Average dietary treatment means of CTR and CT (86.3×10^3 and 76.2×10^3 cell/mL, respectively) differed ($P < 0.05$) from the mean of SB (66.7×10^3 cell/mL). Protozoa counts decreased progressively ($P < 0.05$) with the day of sampling during the first 4 d, and in the following days (from 5 to 9 d) the decrease was less intense (Fig. 1). The percentage of *Entodinium* among the protozoal counts (Fig. 2) increased during the 9 d of sampling and the proportion measured on d 1 was lower ($P < 0.05$) than that on d 9 (45% vs. 78 % of total). In contrast, the relative abundance of Diplodiniinae decreased during this period, with an average lowest value on d 9 ($P < 0.05$).

3.3 Experiment 3

The simple exponential model used to interpolate the gas yields demonstrated satisfactory fitting properties to the experimental data with an average residual mean square errors of + 8.7 and + 6.8 mL, respectively for CT and SB datasets. The calculated kinetic values from the fitting production curve as a result of increasing the dose of CT and SB are shown in Table 4. The maximum cumulative volume of gas production did not differ based on the doses for either additive, and the rate of gas production decreased significantly with increasing dose of CT ($P < 0.05$). Protozoa counts and ammonia concentrations decreased according to a curvilinear trend ($P < 0.05$) with increasing doses of CT and SB, respectively (Fig. 3A and 3B).

4. Discussion

In all the experiments of this research we used as inoculum the rumen fluid collected from cows immediately after slaughter. The collection at slaughter is one of the methods of sampling rumen fluid for microbiota studies (Rumen Microbial Genomics Network, 2019) and is an accepted alternative to sampling through rumen cannula (Yanez-Ruiz et al., 2016). In the present experiments possible differences between rumen fluids collected in different sampling sessions were attenuated by selecting the animal donors (culled dairy cows in good health and fed productive total mixed rations based on corn silage) and by mixing individual rumen fluid of 4 cows for each sampling.

The *in vitro* tests in the present research utilized a rumen CC fermenter designed and tested in our department (Mason et al., 2015) and a batch culture system suitable to evaluate fermentation in terms of continuous gas yield. An initial experiment (Exp. 1) utilized a highly fermentable substrate (e.g. corn meal) to create the conditions for intense bacterial growth (and protein degradation) and to stimulate maximal protozoal growth. In such an environment, we hoped to more effectively demonstrate any effect of the additives (CT and SB) than in less favorable fermentation conditions. Given the quite clear results obtained in Exp. 1, we moved to the CC trial with the objective of evaluating any possible adaptation of the microbiota to the additives and to replicate the effects with a more fibrous substrate, such as a total mixed ration. Finally, in the last experiment, lower doses than those used in previous experiments were studied for each extract in dose-response tests.

4.1 Effects of Stevia extract and Chestnut tannins on rumen fermentation intensity

The extracts studied in this research did not affect the fermentation intensity measured in terms of gas yield (Exp. 1 and 3) and VFA yield (Exp. 1 and 2), if we exclude a modest reduction in the rate of gas production at the highest CT concentration in Exp. 3 (e.g., 1.4% dietary addition). There is no comparable information in the literature, for SB, tannins are known to exert negative effects on

fermentation, although with variable effects according to the family (condensed or hydrolysable), plant origin (Patra and Saxena, 2011) and molecular structure (Mueller-Harvey et al., 2019).

However, Hassanatt and Benchaar (2013) examined increasing doses of CT *in vitro* and obtained a reduction in gas and VFA yield only with additions (e.g. 5%) much higher than the dose levels used in the present trial. Low CT doses (0.15% and 1.5%) were tested in vivo on growing young steers (Aboagye et al., 2018) and did not modify growth performance or rumen VFA concentrations.

4.2 Effect of Chestnut tannins on rumen fermentation and microbiota

The *in vitro* results showed that the addition of CT reduced the yield of ammonia. This result confirms the impact of CT on the overall reduction of proteolytic activity in the rumen, which is well known to be the result of the formation of a reversible complex with proteins and/or inhibition of the enzymatic activity of rumen protease (Patra and Saxena, 2011). The effect was more intense in the CC experiment than in the previous batch fermentation, which could be due to the higher CP content of the substrate used in that experiment. The decrease in rumen ammonia as a consequence of to the CT addition was associated with an increase of concentration of noncellulolytic bacteria, such as *P. ruminicola* and *S. ruminantium*, which are known to use ammonia for amino acid synthesis (Atasoglu et al., 1998), and the concentration of *Ruminococus amilophylus* was reduced.

We were not able to detect a variation in the number of methanogens due to CT addition whereas Witzig et al. (2018) recently measured such a reduction following the addition of a high dose (approximated 10%) of CT.

In both experiments the only significant modification of the VFA profile of the fermentation fluids induced by the CT extract regarded iso-valerate, which was present at levels lower than that in control diet. The iso-valerate comes from the degradation of leucine and its reduction due to CT addition is a well-known effect, demonstrated by the meta-analysis of Jayanegara et al. (2012) and *in vivo* by the work of Liu et al. (2013) and recently by Aboagye et al. (2018). The effect of CT on the protozoa population was different between trials: in the first experiment, there was a reduction in protozoa counts in comparison with the control substrate; in the second experiment this was not confirmed. The effect of CT on the protozoa population is quite controversial in the literature (Patra and Saxena, 2011). Liu et al. (2011) registered a reduction with CT inclusions of 1% and 3% whereas Aboagye et al. (2018) did not find reductions with CT doses of 0.15% and 1.5%. It is known that protozoa growth is favored by diets rich in concentrates (Franzolin and Dehorty, 1996; Hook et al. 2011). Therefore, it could be speculated that a depressive effect of CT on protozoa was detected *in vitro* only in the first experiment, where a starchy substrate was used and not in those case in which a fibrous substrate was used (Exp. 2).

4.3 Effect of Stevia extract on rumen microbiota

In contrast with CT, the SB extract lowered the protozoa counts significantly, and this effect was reproduced in all 3 experiments. In the batch fermentation system (Exp. 1), we measured a reduction in 47% of protozoa (the average between the 4 and 24 h reductions), which is comparable with that reported by Ramos-Morales (2017) after 24 h of incubation (-56%). In the short sampling interval of Exp. 1 (4 and 24 h) the Ophryoscolecinae increased their relative abundance, probably because these organisms have a long generation time (up to 3 d, Sylvester et al., 2009) and could survive more than faster reproducing protozoa strains.

During fermentation in the second experiment, we observed a progressive reduction in the protozoa populations, which are known to be very sensitive to *in vitro* conditions (Cabeza-Luna et al., 2018; Muetzel et al., 2009). The protozoa decline was not attenuated by SB addition and therefore, from the second experiment, we conclude that protozoa were not able to adapt to the SB dietary addition with respect to the control diet. However, the decrease of protozoa induced 307 by SB was lower (approximated -20%, -30%) than that observed in the first experiment and this was probably due to the different conditions of the CC system, which utilizes a continuous flow of liquids and a wash out of the additives.

Additionally, in the third experiment, SB reduced the protozoa counts in decrements proportional to the dose added and Ramos-Morales et al. (2017) speculated that the reduction in the protozoa number was determined by the iminosugar content of SB, even if other unknown phytochemicals may contributing to the effect. Protozoa decrement is generally considered positive in terms of reducing rumen polluting compounds (e.g. ammonia and methane, Newbold et al., 2015), but despite the decrease in the protozoa number, no effects on rumen fermentation parameters were observed in present work. In contrast with the results of Ramos-Morales et al. (2017), SB addition did not result in any reduction in ammonia or any appreciable variation in the patterns of fermentation (total yield and composition of VFA). Moreover, despite the antimicrobial activity claimed for SB extracts (Munro et al., 2000; Wang et al., 2014), we did not observe variation in the relative abundance of bacteria strains, with the only exception of the tripled concentration of *T. saccarophylum*. Therefore, the unaffected composition of the Archaea community and the VFA profile would suggest a nonsignificant variation in methane production, but the gas measure is requested to confirm this indirect suggestion.

5. Conclusion

Both natural substances considered in present paper had an impact on rumen metabolism, but in different ways. For the CT, the lowering rumen ammonia effect was confirmed, however, the assumed action of CT against rumen protozoa was less clear from present results. The SB extract had a relevant negative effect on the rumen protozoa population and no appreciable effects on rumen ammonia levels. While the implication that CT reduce pollution is clear, for SB extract, it will be necessary to better identify the role of protozoa in contributing to the increase in rumen of ammonia and/or methane. However, for both extracts, their effects on the rumen were appreciable at low dietary doses, and negative impacts on fermentation were limited to the reduction in protein with the addition of CT.

Conflicts of interest

None

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Table 1. Effect of addition of chestnut tannin (CT) or *Stevia rebaudiana* Bertoni (SB) extracts to a control (CTR) substrate (corn meal) on the pH, ammonia and VFA of fermentation fluid and gas yield measured at 24 h (Exp. 1).

	Treatment			
	CTR	CT	SB	SEM
24 h gas yield, mL	1621	1584	1648	23.9
pH	7.26	7.25	7.32	0.027
Ammonia, mg/dL	27.9[a]	24.8[b]	27.5[a]	0.58
Total VFA, mmol/L)	115.4	117.7	121.4	1.49
VFA, mol/100 mL				
Acetate	55.46	54.87	54.75	0.299
Propionate	26.16	27.05	27.55	0.427
Iso-butyrate	1.34	1.24	1.26	0.138
Butyrate	12.98	12.89	12.46	0.259
Iso-valerate	2.23[A]	2.08[B]	2.34[A]	0.055
Valerate	1.85	1.88	1.64	0.240
Acetate:Propionate ratio	2.17	2.06	2.01	0.049

SEM = standard error of the means
[a-b]Within rows, means without a common superscript differ ($P < 0.05$).

T**able 2**. Effect of addition of chestnut tannin (T) or Stevia extract (S) to a control (C) substrate (corn meal) on protozoa population in the fermentation fluid measured at 4 and 24 h (Exp. 1).

	Treatment[1]				Sampling time, h		
	CTR	CT	SB	SEM	4	24	SEM
Protozoa, x 10^3 cells/mL	479[A]	334[B]	255[C]	56.6	442[A]	258[B]	25.0
Protozoan subfamilies % of total protozoa							
Entodiniinae	68.4	66.4	71.0	2.25	68.5	67.4	1.84
Diplodiniinae	16.0	17.3	16.3	1.52	18.0	15.0	1.24
Ophryoscolecinae	11.5[ab]	13.7[a]	8.5[b]	1.08	8.4[B]	14.1[A]	0.88
Isotricha	2.2	2.0	1.6	0.43	2.1	1.8	0.35
Dasytricha	1.9	2.4	2.6	0.70	3.0	1.7	0.57

SEM = standard error of the means.
[1] Interaction "treatment × sampling time" not significant.
[a, b] Within rows, means without a common superscript differ ($P < 0.05$).
[A, B] Within rows, means without a common superscript differ ($P < 0.01$).

Table 3. Effect of addition of chestnut tannin (CT) or *Stevia rebaudiana* Bertoni (SB) extracts to a control (CTR) substrate (dry total mixed ration) on the pH, ammonia, VFA and bacterial composition of fermentation fluid in a continuous rumen fermenter (Exp. 2).

	Treatment			
	CTR	CT	SB	SEM
pH	6.05	6.03	6.10	0.065
Ammonia, mg/dL[1]	20.3^{A}	10.5^{B}	19.2^{A}	1.08
Total VFA, mmol/L	47.3	46.1	48.5	3.68
VFA, mol/100 mL				
Acetate	50.3	44.9	47.2	1.55
Propionate	30.2	33.0	32.3	2.24
Iso-butyrate	0.63^{A}	0.25^{B}	0.50^{A}	0.071
Butyrate	9.01	10.3	9.65	0.682
Iso-valerate	4.11^{A}	3.48^{B}	3.93^{A}	0.123
Valerate	5.78^{B}	8.09^{A}	6.45^{B}	0.403
Acetate:Propionate ratio	1.71	1.42	1.48	0.175
Relative abundance, % of total bacteria				
Genus *Prevotella*	35.8	41.8	40.8	2.91
Prevotella ruminicola group	4.66^{b}	5.28^{a}	4.39^{b}	0.928
Fibrobacter succinogenes	0.928	1.02	0.520	0.209
Lactobacillus group	0.145	0.103	0.075	0.024
Ruminococcus albus	0.085	0.075	0.035	0.029
Megasphaera elsdenii	0.318	0.410	0.418	0.133
Selenomonas ruminantium group	0.280^{B}	0.390^{A}	0.243^{B}	0.015
Ruminobacter amylophilus	2.19^{A}	0.08^{B}	2.09^{A}	0.274
Treponema saccarophylum	0.618^{B}	0.552^{B}	1.943^{A}	0.282
Relative abundance, % of Archea				
Methanobrevibacter spp.	79.2	85.4	82.1	4.20
Methanosphaera spp.	1.75	2.42	1.81	0.281

SEM = standard error of the means.
[1] Samples for ammonia analysis were collected just before substrate addition to fermenter in the morning and 3 h after. The data were analyzed considering time effect (not significant) and a repeated measure model (see material and method section).
[A-C] Within rows, means without a common superscript differ ($P < 0.01$).
[a-c] Within rows, means without a common superscript differ ($P < 0.05$).

Table 4. Effect of addition of increasing doses of chestnut tannin (CT) or *Stevia rebaudiana* Bertoni (SB) extracts to a control (CTR) substrate (dry total mixed ration) on kinetic parameters (maximum volume and rate of gas production, V (mL) and k (%/h) measured for 48 h (Exp. 3).

	Treatment			
	CT		SB	
	V	k	V	k
Dose, % of DM				
0	1640	9.07[a]	1522	8.62
0.2	1579	8.95[a]	1494	8.45
0.4	1619	8.93[a]	1518	8.58
0.6	1594	8.87[ab]	1517	8.55
0.8	1551	8.86[ab]	1474	8.71
1.0	1551	8.74[ab]	1497	8.89
1.2	1676	8.71[ab]	1504	8.68
1.4	1629	8.52[b]	1539	8.41
Significance	ns	< 0.05	ns	ns
SEM	26.2	0.087	30.5	0.135

SEM = standard error of the means; ns = not significant.
[a-c] Within columns, means without a common superscript differ ($P < 0.05$).

Fig. 1. Effect of addition of Chestnut tannin (CT) or *Stevia rebaudiana* Bertoni (SB) extracts to a control (CTR) substrate (dry total mixed ration) on total protozoal counts during the days of fermentation in a continuous rumen fermenter (Exp. 2). (Treatment means of CTR and CT (86.3 × 103 and 76.2 × 10^3 cell/mL, respectively) differ ($P < 0.05$) from SB mean (66.7 × 10^3 cell/mL, standard error of the treatment means is 4.65 × 10^3 cell/mL); interaction effect of fermentation day × treatment was not significant; means of days under the horizontal asterisk lines are not significantly different; standard error of the fermentation day means: 8.06 × 10^3 cell/mL).

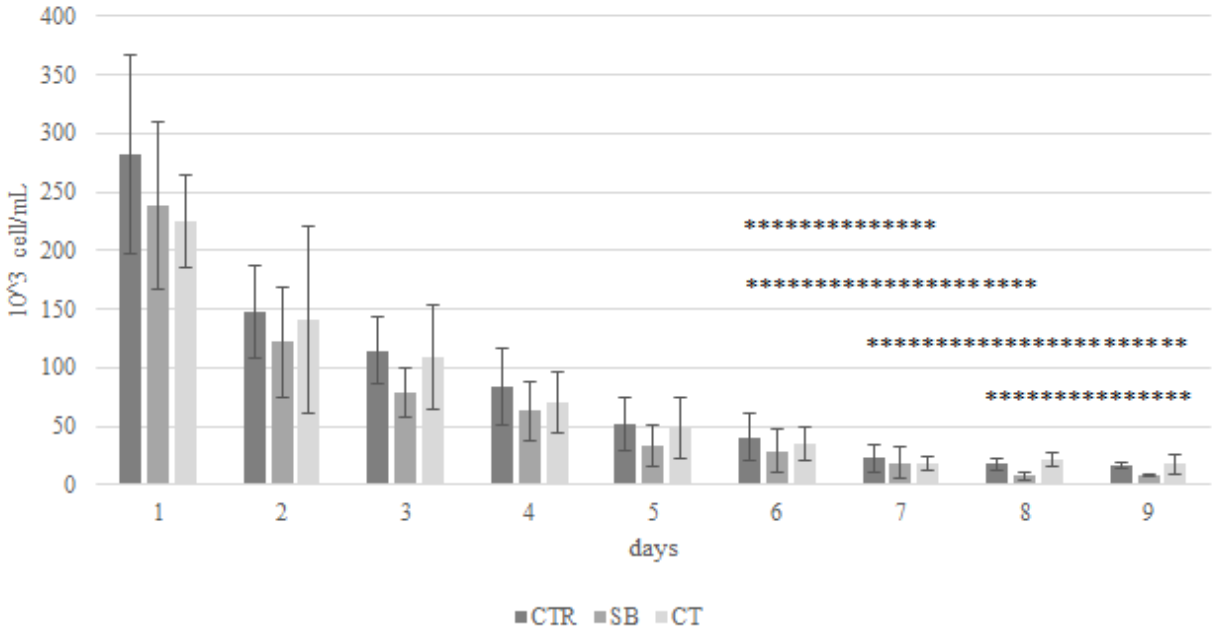


Fig. 2. Percentage of Entodiniinae, Diplodiniinae and remaining other protozoa on total counts over dietary treatments during the days of fermentation in a continuous rumen fermenter (experiment 2). (different letters in the bars denote difference between day averages ($P < 0.05$); the effects of dietary treatment and interaction fermentation day × dietary treatment were not significant; standard error of the fermentation day means is 3.12%, 3.15% and 0.95% for the percentage of Entodiniinae, Diplodiniinae and remaining other protozoa, respectively).

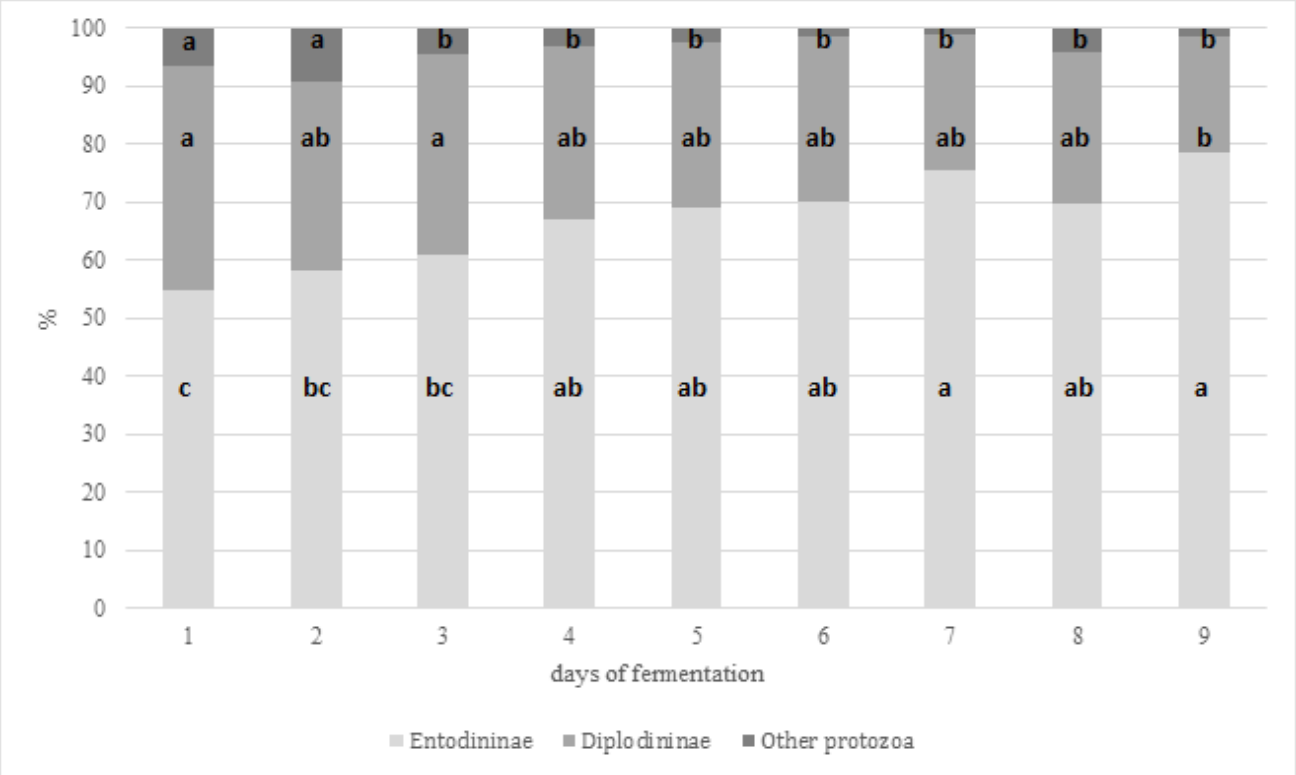
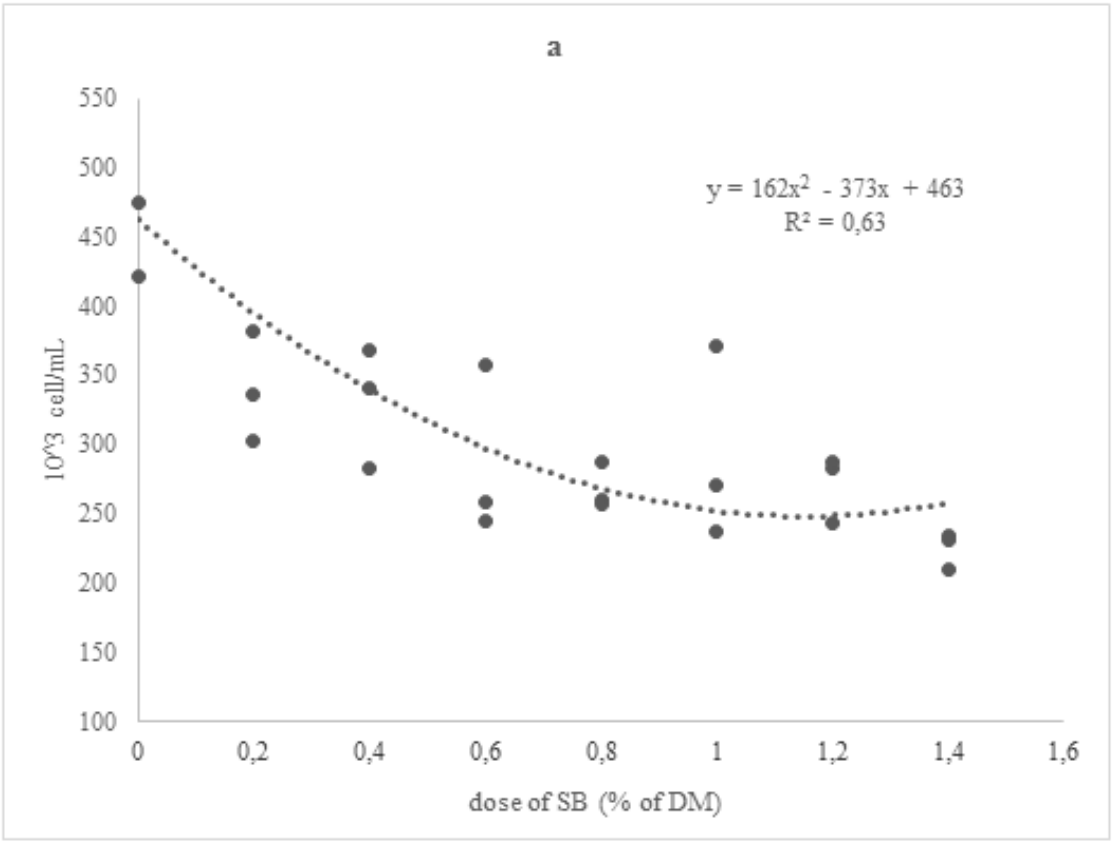
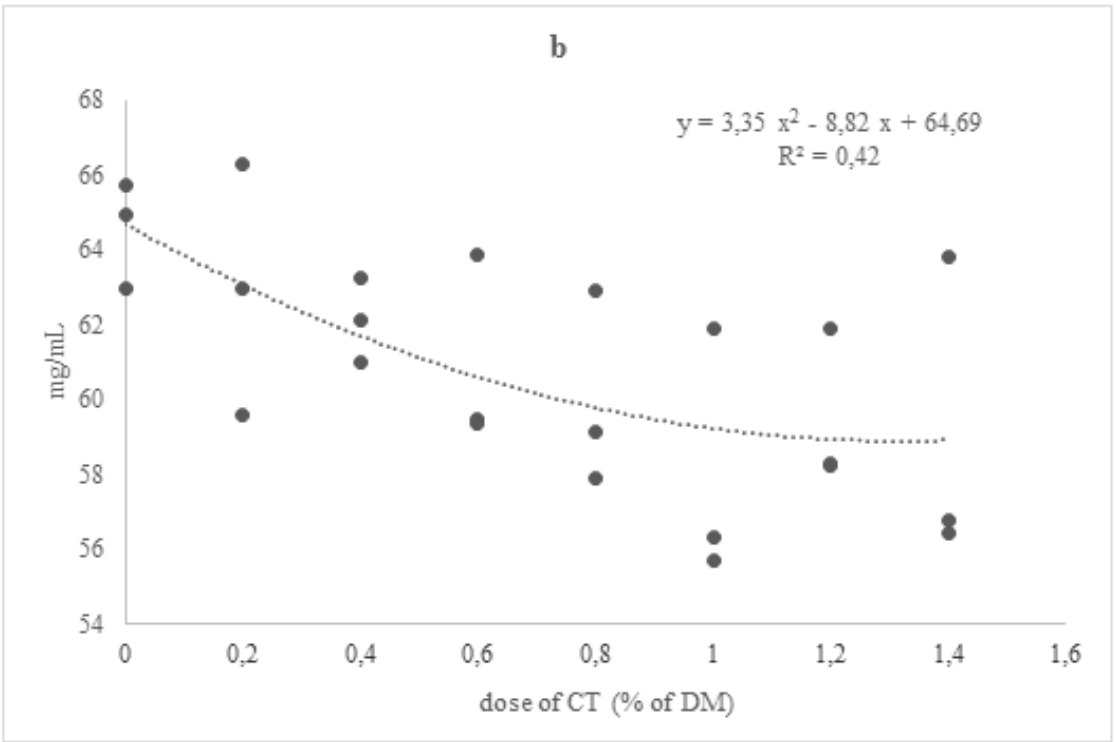


Fig. 3. Polynomial (second-order) regression between total protozoal count and increasing doses of Stevia extract (SB, A), or between ammonia and increasing doses of Chestnut tannin (CT, B), measured after 24 h of fermentation (experiment 3).





Appendix Table 1. Oligonucleotide primers used for quantitative PCR analysis.

Item		Sequence (5'-3')	Product size, bp	Eff., %	R^2	Reference[1]
Universal bacteria	F	CCTACGGGAGGCAGCAG	189	99.3	0.999	Muyzer G et al. (1993)
	R	ATTACCGCGGCTGCTGG				
Archaea	F	CCGGAGATGGAACCTGAGAC	160	99.5	0.997	Chiquette J (2009)
	R	CGGTCTTGCCCAGCTCTTATTC				
Genus *Prevotella*	F	GGTTCTGAGAGGAAGGTCCCC	100	99.5	0.999	Stevenson D M et al. (2007)
	R	TCCTGCACGCTACTTGGCTG				
Prevotella ruminicola group	F	GAAAGTCGGATTAATGCTCTATGTTG	74	98.6	0.998	Stevenson D M et al. (2007)
	R	CATCCTATAGCGGTAAACCTTTGG				
Fibrobacter succinogenes	F	GGTATGGGATGAGCTTGC	446	98.1	0.997	Tajima K et al. (2001)
	R	GCCTGCCCCTGAACTATC				
Lactobacillus group	F	AGCAGTAGGGAATCTTCCA	341	97.1	0.999	Walter J et al. (2001); Heilig H G H J et al. (2002)
	R	CACCGCTACACATGGAG				
Ruminococcus albus	F	TGTTAACAGAGGGAAGCAAAGCA	75	104.0	0.996	Stevenson D M et al. (2007)
	R	TGCAGCCTACAATCCGAACTAA				
Megasphaera elsdenii	F	AGATGGGGACAACAGCTGGA	95	98.3	0.986	Stevenson D M et al. (2007)
	R	CGAAAGCTCCGAAGAGCCT				
Selenomonas ruminantium group	F	GGCGGGAAGGCAAGTCAGTC	83	98.0	0.999	Khafipour E et al. (2009)
	R	CCTCTCCTGCACTCAAGAAAGACAG				
Ruminobacter amylophilus	F	CTGGGGAGCTGCCTGAAT	100	98.0	0.999	Stevenson D M et al. (2007)
	R	CATCTGAATGCGACTGGTTG				
Treponema saccharophylum	F	GGGACAGGGAATGGTCTCGT	466	97.4	0.999	Liu J et al. (2014)
	R	CCGTCAATTTCTTTGAGTTTCAC				
Methanobrevibacter. spp	F	TTTCGCCTAAGGATGGGTCT	171	99.6	0.999	Goberna M et al. (2010)
	R	CGATTTCTCACATTGCGGAG				
Methanosphaera spp.	F	TAAGTCTTTGGTGAAAGCTT	172	99.2	0.996	Goberna M et al. (2010)
	R	GTTACTCACCGTCAAGAT				

F, forward primer; R, reverse primer; Eff., PCR efficiency.

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Effect of chestnut tannins and an extract from *Stevia Rebaudiana* Bertoni on *in vitro* rumen fermentation and microbiota

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ABSTRACT

Plant extracts can be used as safe dietary additives to improve nutritional efficiency and to reduce excretions of animals. The effects of dietary addition of chestnut tannins (T) and an extract from *Stevia Rebaudiana* Bertoni (S) on rumen fermentation parameters and microbiota were evaluated in three *in vitro* rumen fermentation experiments. The Exp. 1 and 3 batch culture systems (fermentation in 500 mL bottles for 24 or 48 h) were used while for Exp. 2 was used a continuous culture system (fermentation in 2 L bottles for 10 d). In Exp. 1 and 2 a control substrate (C) was added with 1.5% of T or S extracts, while Exp. 3 considered 7 doses of each extract added to C (additions of 0.2, 0.4, 0.6, 0.8, 1.0, 1.2 and 1.4 %) in separate runs performed for each extract.

The T addition lowered the *in vitro* rumen ammonia in all experiments: the reduction, in comparison to C, was 12 and 50% in the Exp. 1 and 2 ($P<0.01$) while in Exp 3 the dose effect was linear ($R^2=0.39$; $P<0.01$) and had a negative slope. Tannins effect on protozoa counts depended from the fermentation system, with a reduction in Exp. 1 (334 vs 479 10^3 cell/mL, $P<0.01$) and no effect in Exp. 2. In contrast, the S extract did not modify ammonia concentrations, but significantly lowered the protozoa counts in all three experiments. The reduction was 47 and 23 % in Exp. 1 and 2, respectively, while in Exp. 3 the dose effect was linear ($R^2=0.59$; $P<0.01$) and had a negative slope.

Both the extracts did not affect the fermentation intensity measured as gas (Exp 1 and 3) and VFA yield (Exp 1 and 2). Changes in VFA profile induced by T were limited to reductions of iso-valerate (2.08 vs 2.23 and 3.48 vs 4.11 % total VFA, $P<0.01$, in Exp 1 and 2 respectively) and iso-butyrate (0.25 vs 0.63 % total VFA, $P<0.01$, Exp 1). Tannins increased *Prevotella ruminicula* and *Selenomonas ruminantium* groups and decreased the *Ruminobacter amylophilus* ($P<0.1$, $P<0.05$ and $P<0.05$, respectively). The S extract increased *Treponema saccarophylum* ($P<0.05$).

Both plant extracts did not decrease *in vitro* fermentation but differently affected rumen metabolism (S vs protozoa and T vs ammonia). While the impact of rumen ammonia on pollution is clear the role of rumen protozoa on animal excretions (ammonia and/or methane) requires further research efforts.

Acknowledgements

The chestnut tannins were furnished by the Gruppo Mauro Saviola Srl (Radicofani, Siena, Italy) and stevia leaves by Bio Mondo (Porcia, PN, Italy).

Chapter 4. Diets added with tannins from Chestnut wood or an extract from *Stevia rebaudiana* Bertoni and effects on *in vitro* rumen fermentation, protozoa count and methane production

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Abstract

The dietary use of *Stevia rebaudiana* Bertoni extract (SB) and Chestnut tannin (CT) were investigated to confirm the rumen antiprotozoal action of these plants products and to assess any association with methane yield and rumen fermentation. Both plant products were tested by using *in vitro* rumen batch culture systems at 3 different levels of inclusion (0.75%, 1.5% and 3% of incubated dry matter, DM) in a mixed diet for dairy cows. Total volatile fatty acid concentration, their proportions and gas production were not modified by the plant extracts inclusion, except a significant linear increment of gas production at 24h for SB (Gas24, $p = 0.049$). Ammonia concentration decreased ($p < 0.05$) of about 17% when 1.5 or 3% of CT were included into TMR. Rumen protozoa population was depressed by the SB inclusion ($p = 0.002$) with a maximum reduction of 40% at the highest SB dosage, whereas CT negatively affected total protozoa number (-19%) only at the dose of 3%. *In vitro* DM and NDF degradability were not affected by the supplementation of SB and CT, as well as the methane yield. Thus, the addition of S and CT decrease with different intensity, the *in vitro* protozoa population of the rumen, without effects on fermentation parameters, apart from a reduction of nitrogen fermentability caused CT. Despite the effect on protozoa, no decreasing effect on methane production was detected.

Key words: rumen, protozoa, methane, Chestnut tannin, *Stevia rabaudiana* Bertoni

1. Introduction

In ruminant nutrition research, there is great interest for compounds able to modulate rumen fermentation towards greater efficiency and reduction of pollutants emission (e.g. methane from the rumen and/or ammonia from manure, Mendel et al., 2017). Because of the concern about the utilization of chemicals as additives due to safety issues, natural compounds (such as plant secondary metabolites or plant extracts) are considered an interesting alternative, and current research is focusing on this topic.

Stevia (*Stevia rebaudiana* Bertoni, SB) is a perennial plant native from South America and well known as natural sweetener in human nutrition due the presence of the steviol glycosides (Kohda et al., 1976). Chestnut wood (Castanea sativa L., CT) contain mainly hydrolysable tannins, known in animal nutrition for their effect of reduction of nitrogen degradation and digestion (Patra and Saxena, 2010; Patra and Aschenbach, 2018).

It was recently shown that SB extract reduces the number of protozoa present in the *in vitro* rumen fermentation liquid (Ramos-Morales, 2017, Sarnataro and Spanghero, 2020) and other researches

demonstrated a similar effect also by CT (Lima et al., 2019) or observed only a tendency of reduction (Sarnataro and Spanghero, 2020).

Protozoa contribute up to 50% of the total biomass in the rumen and play a role in fibre, starch and protein digestion (Martin et al., 2010). Moreover, it was demonstrated that protozoa interact with the CH4 production in the rumen (Belanche et al., 2015) and in vivo defaunation experiments had shown a reduction of CH4 of about 12% (Morgavi et al., 2010, Hegarty 1999). Protozoa synthesize fatty acids from fermentation, but also H2, which is used by epi- and endo-symbiotic methanogen attached to protozoa surface to produce methane (Ellis et al., 1994).

This study completes a previous one (Sarnataro and Spanghero, 2020) where SB and CT were used as dietary additives in experiments using batch cultures and continuous culture system to evaluate the effect on fermentation parameters and microbiota. The main results were a consistent reduction of protozoa number with SB and a tendency to decrease with CT without any appreciable effect of adaptation of protozoa to the additives in long-lasting fermentation. The specific aim of present research is to confirm the antiprotozoal action of these plant products also in different experimental conditions (e.g. rumen fluid collected from cannulated sheep and small size batch fermentation system) and to evaluate any associate variation in methane yield and in rumen fermentation.

2. Material and methods

2.1 Diets and plant extracts

As a substrate in all *in vitro* studies a total mixed ration for dairy cows (TMR), calculated for the 30 kg milk production, was used. TMR was composed by (on a DM basis) 495 g maize silage, 123 g grass silage, 63 g wheat straw, 19 g grass hay, 83 g wheat grain, 83 g maize grain, 87 g sunflower meal, 34 g soybean meal and 13 g mineral and vitamin mix (limestone, salt and vitamins and micro minerals) and contained (g kg−1 DM) 158 g CP, 40 g ether extract, 385 g neutral detergent fibre (NDF) and 52 g crude ash. Hydrolyzable tannins from Castanea sativa (CT, Saviotan Feed supplement), was provided by the Gruppo Mauro Saviola Srl (Radicofani, SI, Italy). Chemical and gas chromatographic profile are reported by Campo et al. (2012). Dried and milled *Stevia rebaudiana* leaves were provided by Bio Mondo (Porcia, PN, Italy) and were used to prepare the *Stevia rebaudiana* extract (SB) as described in Ramos-Morales (2017).

2.2 In vitro experiments

An *in vitro* gas production technique and an *in vitro* degradability test were performed to evaluate the effect on rumen fermentation parameters, degradability and methane production at 3 different levels of inclusions (0.75%, 1.5% and 3% of incubated DM). As rumen fluid donors two mature castrated

rams weighing on average 70 kg and fitted with a permanent rumen cannula were used. The rams were fed with medium quality hay ad libitum, 0.25 kg per day of a soybean meal and maize grain mix (1: 1, w/w) to which 25 g of mineral and vitamin were added.

In vitro gas production

In vitro gas production was determined by the procedure of Menke and Steingass (1988) procedure. In each of 36 100 mL glass syringes containing 30 mL mixture of rumen fluid and buffer solution (Menke and Steingass, 1988) 200 mg of the substrate was weighed. SB and CT were added to substrates as aqueous solution immediately before the start of incubation. Gas production was measured manually after 0, 2, 4, 6, 8, 10, 12, 24, 36, 48 and 72 hours. If the volume of produced gas exceeded 80 ml syringes were emptied and volumes recorded. All substrates were incubated in four repetitions. After 24 hours of incubation two among the four syringes of each substrate were withdrawn and the content of syringes divided into three sub-samples for NH3-N, short-chain fatty acid (SCFA) analysis and count of protozoa number. NH3-N and SCFA samples were stored frozen at -20° C until analysis. For preserving the subsamples for protozoa number count an equal volume of 50% formalin solution was added. The experiment was repeated once more in the following week. Altogether, in two runs, eight syringes of each substrate (four for gas production and four for SCFA, NH3-N and number of protozoa) were incubated. In each run, five syringes with blank samples and three syringes with standard hay were also incubated. The coefficient of variation between the batches calculated on the standard hay was always lower than 5%, therefore, no corrections of gas production of substrates between batches were made. Before the analyses, the NH3-N and SCFA subsamples were thawed at room temperature. NH3 was determined by an ammonia electrode (Ammonia Gas Sensing Combination Electrode, ©Hach Company, 2001). The extracts for the SCFA were prepared according to the modified method of Holdeman et al. (1977). SCFAs were analyzed using a gas chromatograph (Hewlett Packard 5890 A; Hewlett Packard, Bellefonte, Pennsylvania, USA) equipped with split/splitless injector and FID detector. For the SCFA separation, a 30 m NUKOL TM, FUSED SILICA capillary column (SUPELCO, Bellefonte, Pennsylvania, USA) was used. The number of protozoa was determined by the method described by Dehority (2003).

In vitro DM and NDF degradability

Eighteen 200 mL glass flasks equipped with a gas-tight rubber stopper and filled with a 40 ml of a mixture of rumen fluid and buffer solution according to a modified procedure of Menke and Steingass (1988) were used. Two hundred seventy milligrams of TMR were weighed into ANKOM F57 filter bags (ANKOM Technology, Macedon, NY, USA) and inserted into flasks. The additives SB and CT were added as the aqueous solutions containing additives at different concentration (0.75%, 1.5% and

3.0% of incubated DM) immediately before the experiment directly into the flasks. Flasks were incubated for 24 h in a water bath at 39±0.5° C together with the standard hay and blank samples. At the end of incubation CH4 concentration was measured by injecting 100 µL of headspace gas into a Shimadzu GC-14A gas chromatograph equipped with a thermal-conductivity detector (TCD). Helium was used as carrier gas. A Porapak-Q stainless steel packed column (4 m length, 0.125 in ID, 2 mm OD) was used. The analysis was run at isothermal conditions (50° C). Injector temperature was 50° C and detector was set to 80° C. The peaks were recorded with Shimadzu Chromatopac C-R6A integrator. The relative percentage of H2, CH4, N2 and CO2 were calculated from peak areas according to the calibration mixture of the same gases. F57 bags with nondegraded substrate were afterwards rinsed with cold tap water until the water was clear and dried at 103° C for 3 h and *in vitro* apparent DM degradability (ivADMD) was calculated. Bags were then treated with boiling neutral detergent solution for 1 h In ANKOM Fiber Analyzer (ANKOM Technology, Macedon, NY, USA), rinsed with cold tap water, dried and the *in vitro* true DM digestibility (ivTDMD) and *in vitro* neutral detergent fibre degradability (ivNDFD) were calculated.

2.3 Calculation and statistical analysis

Gas production kinetic parameters calculation was performed according to Lavrenčič et al. (1997). Briefly, the net volume of gas produced at each time was corrected for blank sample gas production at that time of incubation and adjusted to 1 g of DM substrate. Obtained data were fitted to Gompertz equation (Lavrenčič et al., 1997):

Y_t=〖B×e〗^(-C×e^(-Dt));

where Yt is the gas produced (ml/g DM) at time “t”, “B” the asymptotic volume of produced gas (total potential gas production (ml/g DM)), “C” the specific gas production rate as affected by “t” and governed by constant factor “D”, describing the decay in specific gas production rate and “t” the time in hours.

Parameter values and curve fitting were estimated by the Marquardt compromise for a nonlinear regression method (PROC NLIN; Statistical Analysis Systems Institute, SAS, 2015). Amounts of gas produced in 24 h, times of maximum fermentation rates (TMFRs) and maximum fermentation rates (MFR) were calculated as described by Lavrenčič et al. 2015.

In both experiments the fermentation runs were performed in different periods (weeks) and replicates between runs were the statistical replicates. Each treatment mean was compared against the control diet (named as TMR) using the least squares mean linear hypothesis test (LSMEANS/DIFF) with the Dunnett adjustment. Orthogonal polynomial contrasts were used to determine linear and quadratic

responses to level of additives (0, 0.75, 1.50 and 3.00% DM of each additive) and matrix coefficients were generated by using the proc IML of SAS (SAS, 2015) for unequally spaced contrasts. Statistical significance was declared at $p < 0.05$.

3. Results

Results of gas production are shown in Table 1. The addition of SB and CT at different dosages did not interfere with gas production parameters, except a significant linear increment was detected for SB for gas production at 24h (Gas24, $p = 0.049$). SCFA production was not significant modify and no differences were detected in proportions of acetate, propionate and butyrate (Table 2). Ammonia concentration decreased ($p < 0.001$) with increased amounts of added CT with a reduction of about 17% when 1.5% and 3% of CT were added to TMR (decreased from 21.57 to 18.02 and 17.92 mL/dL, respectively). Large effects of SB and CT additives were detected on the protozoa population. SB decreased the total protozoa count ($p = 0.002$). The reduction was 15% with the SB addition of 0.75%, although no significant differences were detected at this level of inclusion. With an increased amount of SB the reduction of protozoa number became more consistent, reducing their number by around 40% at the 3.0% addition of SB (Table 2), with a linear effect ($p = 0.0003$). CT negatively affected total protozoa number only at the addition of 3%. However, no modification was detected on protozoa proportions.

In vitro DM apparent and true digestibility (ivADMD and ivTDMD) and *in vitro* NDF degradability (ivNDFD) were not affected by the supplementation with both additives (Table 3). In addition, methane calculated as proportion of all fermentation gases was not modified by the additives.

4. Discussion

We aimed to confirm the results obtained in the previous trial despite the adoption of experimental conditions different in terms of fermentation batch systems (syringes vs fermenters of larger dimension) and rumen fluid (collected from cannulated sheep vs slaughtered dairy cows). Moreover, we used doses much higher (1.5-3.0% vs 0.2-1.5% DM) to more effectively demonstrate any effect of the additives on methane production.

In present work we confirm the strong effect of depression of SB on protozoa population (Ramos-Morales, 2017; Sarnataro and Spanghero, 2020) and a reduction due to CT only at high dosages. Total protozoa number significantly decreases (from minus 20 to 40%) using SB at concentration from 0.75 to 3.0%, while CT lowers the protozoa population by about 20% but only when 3.0% of CT were added. The effect of CT on protozoa population is controversial. Aboagye et al. (2018) did not determine any effect when they added 0.15% or 1.5% CT, while Lima et al. (2019) reported that

protozoa number decreased significantly when 3% of CT were added to the diet without any detrimental effects on rumen fermentation and on methane production.

Rumen protozoa population is mainly composed by entodiniomorphs group (Entodioniinae, Diplodiniinae and Ophryscholeciniinae) and the variation of the total protozoa count is usually associated with the variation of this protozoa group. In our experiment changes in protozoal composition was limited without any significant values.

Both additives did not modify the gas production kinetic parameters, the pH values and amounts and proportions of SCFAs released after 24 h of incubation. Morgavi et al. (2012) and Li et al. (2018) associated the reduction of protozoa to a lower production of acetate and butyrate, which was not confirmed in our *in vitro* conditions. Furthermore, tannins are known to form complexes with feed protein or inhibit the protease and urease enzymes in the rumen (Patra and Saxena, 2010, Patra and Aschenbach, 2018) thus lowering the rumen ammonia concentrations. In fact, we observed a 17% reduction in the ammonia concentration when 1.5 and 3.0% of CT were added, although in our previous trial (Sarnataro and Spanghero, 2019) the reduction in ammonia concentration appeared also at much lower CT concentrations.

Despite the observed effects of the SB and CT on protozoa number and rumen ammonia concentration, no effects on rumen degradation of DM and NDF were observed. The reduction of methane concentration is often linked to a general decrease of nutrient digestibility, in particular of fibrous components. It was suggested that the decrease in protozoa population is correlated to lower fibre degradability. Limited fibre degradability is related to lower hydrogen production and thus also methane production (Piluzza et al. 2014). However, in our experiment protozoa number was significantly reduced by supplementation with SB and CT, which did not have any detrimental effect on DM and NDF degradation.

The proportion of methane in the gas production was not affected by supplementation with SB and CT. This was surprising given the assumed association of protozoa with methanogenic microorganisms and therefore a close relationship between protozoa and methane yield. The *in vitro* rumen fermentation systems could be criticized as adequate tools to study the rumen methane emissions, but the recent work of Danielsson et al. (2017) have demonstrated a close relation between methane measures from *in vitro* gas production and in vivo data.

In in vivo experiments of Morgavi et al. (2010) and Hegarty (1999) the defaunation resulted in 12% reduction in methane production. Because in present experiment we reduced the protozoa up to 40%, we expected to decrease methane production of about 4 to 5%.

However, as suggested by Morgavi et al. (2012), the relationship between protozoa and methane production is very complex and "not a simple cause–effect relationship". They were not able to reduce methane production by using the inoculum from defaunated animals in *in vitro* trial, while in in vivo trials the defaunation had only a short-lasting effect. It seems that removal of protozoa from rumen in long-lasting trials was compensated by changes in planktonic microbial community with the increase of the methanogenic bacteria. However, such compensation requires enough time of adaptation and the recent meta-analysis of Li et al. (2018) suggested that after 12 weeks of defaunation the reductions in methane are no longer evident. Moreover, Colombini et al. (2020) were able to find a compound (e.g. eugenol essential oil) which stimulate the *in vitro* rumen protozoal growth, but that increment was not associated to an expected increase in methane yield. Further confirmation of the complexity of the relationship between protozoa number and methane production is given by the meta-analysis of 70 in vivo experiments by Guyader et al. (2014): considering the trials with modifications in methane or protozoa the rumen gas decreased with the number of protozoa only in the 44% of the cases, whereas in the majority of trials (56%) the variations of protozoa and methane were not associated each to the other.

A common interpretation of these controversial results is that decreasing protozoa associated methanogens can be balanced by an increase of rumen fluid methanogenic bacteria and by other modifications of rumen microbial communities, which were not measured in present study.

4. Conclusion

Stevia extract and wood tannin reduce, with different intensity, the *in vitro* protozoa population, without chancing rumen fermentability, apart from a reduction of nitrogen fermentability caused by tannins. Despite the change in protozoa number the methane yield was not affected, and this requires further research efforts on the complex rumen microbial adaptations following the protozoa changes.

Conflict of interest

None

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Table 1. *In vitro* gas production parameters of the different levels of inclusion of Stevia extract (SB) and chestnut tannin (CT) compared to the control diet (TMR).

	Treatment								P-value[1]				
		SB			CT					SB		CT	
	TMR	0.75%	1.5%	3%	0.75%	1.5%	3%	RMSE	Trt	L	Q	L	Q
B (mL g^{-1} DM)	299	324	306	316	309	306	305	7.392	0.147	0.233	0.306	0.614	0.384
C	2.74	2.72	2.68	2.66	2.76	2.79	2.84	0.027	0.004	0.014	0.512	0.006	0.963
D	0.117	0.107	0.118	0.114	0.120	0.117	0.111	0.006	0.406	0.887	0.695	0.258	0.493
TMFR (h)	8.789	9.535	8.429	8.744	8.746	8.972	9.579	0.432	0.212	0.438	0.935	0.086	0.509
MFR (mL/h)	12.7	12.6	13.2	13.1	13.4	13.0	12.4	0.406	0.302	0.225	0.776	0.236	0.129
Gas_{24h} (ml g^{-1} DM)	249	258	259	261	258	254	247	4.430	0.107	0.049	0.216	0.302	0.083

B: total potential gas production; C: the specific gas production rate as affected by time of incubation and governed by a constant D, describing the decay in specific gas production rate, TMFR: time of maximum fermentation rate; MFR: maximum fermentation rate, Gas_{24h} = gas produced in 24 hours of incubation

[1]P-values for Trt = treatment; orthogonal contrasts for L = linear and Q = quadratic effects

Table 2. The total amount of short-chain fatty acids (SCFAs), molar proportions of acetate, propionate and butyrate, pH, concentration of NH_3 and protozoa count in Stevia extract (SB) and chestnut tannin (CT) supplemented diets after 24h of incubation.

	Treatment								P-value[1]				
		SB			CT					SB		CT	
	TMR	0.75%	1.5%	3%	0.75%	1.5%	3%	RMSE	Trt	L	Q	L	Q
SCFA (mmol g^{-1} DM)	9.80	9.57	8.81	9.26	9.86	9.87	9.34	0.592	0.559	0.338	0.277	0.424	0.576
Acetate (g g^{-1} SCFA)	0.804	0.803	0.796	0.799	0.799	0.794	0.797	0.005	0.343	0.180	0.266	0.142	0.108
Propionate (g g^{-1} SCFA)	0.100	0.102	0.106	0.104	0.109	0.108	0.104	0.005	0.704	0.451	0.520	0.768	0.163
Butyrate (g g^{-1} SCFA)	0.094	0.095	0.098	0.097	0.092	0.099	0.099	0.003	0.408	0.387	0.505	0.098	0.809
pH	7.40	7.36	7.26	7.24	7.24	7.35	7.40	0.118	0.623	0.197	0.663	0.660	0.367
NH_3 (mL/dL^{-1})	21.57	21.58	21.38	21.39	19.98	18.02*	17.92*	0.474	0.0006	0.648	0.900	0.0002	0.007
Total protozoa (10^3 cell/mL^{-1})	436	371	310*	275*	424	432	354*	22.2	0.002	0.0003	0.054	0.008	0.238
Entodiniinae, %	90.3	91.9	93.3	93.2	92.8	94.8	93.7	1.59	0.289	0.118	0.311	0.083	0.084
Diplodiniinae, %	4.47	3.80	2.82	3.50	4.78	3.46	3.64	1.02	0.590	0.363	0.267	0.314	0.775
Ophryscolecinae, %	2.87	2.58	2.24	1.82	1.37	1.16	1.24	0.828	0.361	0.233	0.907	0.139	0.163
Isotricha, %	1.62	1.30	1.28	1.33	0.930	0.482	1.38	0.636	0.670	0.720	0.663	0.823	0.104
Dasytricha, %	0.727	0.428	0.381	0.140	0.089	0.091	0.000	0.247	0.182	0.062	0.650	0.044	0.114

[1]P-values for Trt = treatment; orthogonal contrasts for L = linear and Q = quadratic effects

*Means within a row difference from the TMR ($P<0.05$).

Table 3. *In vitro* dry matter apparent digestibility (*iv*ADMD), true digestibility (*iv*TDMD), *in vitro* NDF degradability (*iv*NDFD), CH_4 concentration in diets supplemented with Stevia extract (SB) and chestnut tannin (CT) at 24h of incubation.

	Treatment								P-value[1]				
		SB			CT					SB		CT	
	TMR	0.75%	1.5%	3%	0.75%	1.5%	3%	RMSE	Trt	L	Q	L	Q
*iv*ADMD (g kg^{-1})	616	614	581	582	589	603	579	29.7	0.742	0.223	0.656	0.328	0.908
*iv*TDMD (g kg^{-1})	748	757	740	743	738	749	730	24.1	0.939	0.707	0.990	0.546	0.790
*iv*NDFD (g kg^{-1})	345	368	324	332	320	348	299	62.6	0.939	0.707	0.990	0.546	0.790
CH_4 %	4.29	4.45	4.93	5.13	4.88	5.48	4.70	0.499	0.374	0.113	0.747	0.489	0.066

[1]P-values for Trt = treatment; orthogonal contrasts for L = linear and Q = quadratic effects

Chapter 5. Dietary addition of two essential oils from *Achillea moschata* or their main pure components (camphor, eucalyptol and bornyl acetate) on *in vitro* rumen fermentation

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Abstract

Aim of the study was to investigate the effects of *Achillea moschata* essential oils and the oil main pure compounds - camphor (CAM), eucalyptol (EUCA) and bornly-acetate (BORN) – on rumen fermentation and microbiota. *In vitro* batch fermentation systems were used in a first experiment on TMR samples to measure gas production (GP) and CH_4 whereas a continuous culture system was used to evaluate potential adaptation of microbiota to pure oil compounds in a second experiment. There was an effect of the additive on CH_4 production (% total GP) at 48 h (P=0.004) with lower values (P<0.05) for EUCA (22.5%) and CAM (22.4%) compared to the Control (i.e. no additive; CTR; 24.2%). All the compounds increased the total number of protozoa. In the first experiment, all the treatments increased the genus Entodinium (% total protozoa) (on average 91.8 for additives vs 83.0 for CTR; P<0.001) whereas the genus Diplodinium was decreased (on average 6.60 for additives vs 13.6 for CTR; P=0.001). Bacteroidetes and Firimicutes relative abundances were decreased by all treatments whereas Proteobacteria 23 increased as compared to CTR.

The complexity of bacterial population (number of different species) was decreased by the additives ranging from a minimum of 48 (EUCA and CAM) to a maximum of 76 (CTR). Results of Exp. 2 (i.e. continuous culture system) showed a decrease in total VFA of about 20% for EUCA as compared to CTR (P=0.029) and an increase in valerate proportion (P=0.001). BOR and CAM increased the acetate proportion compared to the CTR (61.26, 58.44 vs 54.34 for BOR, CAM and CTR, respectively). EUCA increased the presence of Ruminocuccus as compared to CTR (P=0.0018). Stechiometric calculated CH_4 (mmol/L) was lower (P=0.0245) for EUCA (7.40) than the other treatments (on average 8.87). The results of the present study showed that all the additives modulated rumen fermentation pattern although with different results among the 2 experiments and EUCA seems the most promising compound.

Keywords: methane, rumen fermentation, achillea moschata, protozoa, microbiome

1. Introduction

Anthropic activities are an important source of greenhouse gases emission in the atmosphere with the consequent increase in global warming. Among anthropic emissions, livestock represents about 14-15 % of total emissions with CH_4 being the major greenhouse gas (Gerber, 2013). The evaluation and development of dietary strategies that can decrease enteric CH_4 production are beneficial both for climate change mitigation, but also for animal production because CH_4 is an energy loss for the animals (Johnson and Johnson, 1995). The use of rumen modulators, such as plant secondary metabolites, seems to be promising because of their direct effect on methanogens, protozoa and on

fermentation process (Cobellis et al., 2016). Plants produce a wide variety of secondary metabolites against 46 pest, disease and predator attacks (Jouany and Morgavi, 2007). Among secondary metabolites, essential oils have been widely evaluated as feed additives for improving microbial metabolism in the rumen and inhibition of methanogenesis (Patra and Yu, 2012). Essential oils are obtained from steam distillation of plants and they include several compounds (Aziz et al., 2018) but only a few give to each essential oil its properties (Bakkali et al., 2008).

Most of the active compounds in the essential oils have antimicrobial activity which should be evaluated in order to select additives that can decrease CH_4 production without compromising overall gas and volatile fatty acids (VFA) production in the rumen. With this regard, only a few individual active compounds of essential oils have been tested for their effects on rumen fermentation and CH4 production (Joch et al., 2018). Recent studies (Joch et al., 2016; Joch et al., 2018) showed interesting results for some compounds such as camphor (CAM) and bornyl-acetate (BOR) in decrease CH_4 production without negative effects on VFA production at short time of *in vitro* incubation. Together with eucalyptol (EUCA), CAM and BOR represent the main components of *Achillea moschata* essential oil (Vitalini et al., 2016). *A. moschata* is a plant showing antimicrobial activity (Vitalini et al., 2016), traditionally used to treat human disorders, but also in the veterinary field, especially as a digestive for cows (Vitalini et al., 2015).

The antibacterial properties of achillea ssp. essential oil are probably due to the high content of CAM (about 27.0 % of oil) and EUCA (about 11.0% of oil), whose noticeable antimicrobial potential is known (Si et al., 2006). Bornyl acetate seems to contribute to the antimicrobial activity of the essential oils when it is among the main constituents (Fidan et al., 2019; Zerkani et al., 2019, Baali et al., 2019). Another issue in the search for feed additive-based mitigation strategies is that the rumen microbial ecosystem may adapt to the use of feed additives. In that case, only a transient reduction of CH_4 emissions can be achieved 69 (Klop, 2016). Cardozo et al. (2004) reported a transient effect of plant extracts on fermentation characteristics that disappeared after six days indicating that microbial adaptation can occur after short term exposure. Hence, the evaluation of additive by a rumen continuous fermenter should be another step which should be performed prior to in vivo study.

The current study aims to investigate the effect of *Achillea moschata* essential oils collected from two sites and of their main pure compounds (CAM, EUCA and BOR) in terms of modifications of rumen fermentation and microbiota. *In vitro* batch fermentation systems were used for an overall screening of essential oil extracts and pure compounds whereas the continuous culture system was used to evaluate a potential adaptation of microbiota to pure oil components.

2. Materials and Methods

2.1 Plant material and essential oil isolation

A. moschata was collected at the blossom period in July 2017 in two different valleys of the Rhaetian Alps (Sondrio Province, Northern Italy). Specifically, the samples were harvested at 2400 m a.s.l. in Valle dei Forni and at 2000 m a.s.l. in Val San Giacomo, respectively. Two voucher specimens (No. AMVF 104 and No. AMVC105, respectively) were deposited at the Department of Agricultural and Environmental Sciences, Milan State University (Milan, Italy), after its identification according to morphological traits described in Flora d'Italia (Pignatti, 1982). The air-dried aerial parts (50 g) of *A. moschata* were subjected to steam-distillation for 1 h in a Clevenger-type apparatus. The obtained distillates were dried over anhydrous sodium sulfate and concentrated with a rotary evaporator at 30 °C to give a pale-blue yellow oils.

2.2 Pure compounds

The three main components of *A. moschata* essential oils, namely BOR (6.21% of essential oil), CAM (27.6%) and EUCA (10.7%) (Vitalini et al., 2016; Tava et al., 2019), were purchased from Sigma-Aldrich (Milan, Italy).

2.3 Experiment 1: in vitro incubation and analysis

Ruminal fluid was collected from 2 fistulated dry Italian Friesian dairy cows fed a diet composed of (g/kg DM): corn silage (434), grass hay (323), corn meal (105) and soybean meal (136) (forage:concentrate ratio of 75:25 on a DM basis). Through-out the experimental period, the animals were handled as outlined by the Directive 2010/63/EU on animal welfare for experimental animals. Rumen liquor was strained through four layers of cheesecloth and poured into a flask pre-warmed at 39°C purged with CO2. Buffer solution was prepared according to Menke and Steingass (1988). Each compound was dissolved in ethanol and the concentration used was 200 mg compound/L of inoculum. Two commercial TMR samples were used as substrates. Diets were characterized by corn silage (352 g/kg DM on average), corn meal (172 g/kg DM on average), solvent soybean meal (170 g/kg DM on average), alfalfa hay (145 g/kg DM on average). Diet chemical composition (g/kg DM) was, on average: Crude protein 155, aNDF 348 and starch 243. Approximately 200 mg of each TMR was weighed in duplicate in serum bottle (120 mL) and each bottle was inoculated with 30 mL of inoculum. For each additive (2 *A. moschata* essential oils, BOR, CAM and EUCA), a corresponding blank (inoculum+ethanol+additive) was incubated and each additive was tested against a negative control (TMR+inoculum+ethanol). Three incubation runs were conducted in a shaking water bath at 39°C, for 48 h. At 24 and 48 h of incubation, headspace pressure was recorded in each serum bottle, using a digital manometer (model 840082, Sper Scientific, Scottsdale, AZ, USA), and a sample of air was collected from bottle headspace using a gas tight syringe (Hamilton, USA) and stored in gas tight

vials (Labco Exetainer Vials, UK). At the end of incubation, pH was recorded, and three samples of liquor were collected: 10 mL for rumen microbiota characterization, 5 mL plus 1 mL of 50% formalin for protozoa count and 5 mL plus 5 mL of 25% metaphosphoric acid for volatile fatty acids (VFA) determination. The samples for microbiota and VFA were immediately frozen. Air samples were analyzed for CH_4 concentration using an Agilent 3000A micro GC gas chromatograph (Agilent Technologies, Santa Clara, CA, USA). Volatile fatty acid concentrations were determined using a Varian 3800 gas chromatograph (Varian Chromatography Systems, Walnut Creek, CA, USA) according to Pirondini et al. (2012).

2.4 Experiment 2: continuous rumen fermenter

Eight 2L single-flow continuous fermenters were used as described in Mason et al. (2015) to perform two fermentation runs which lasted each 9 d with 6 days of adaptation and 3 days of sampling. For the fermentation runs a standard dairy cow TMR (90% DM, 16.0 % CP and 40.2 % aNDF) was used. The treatments consisted in the addition of 100 mg/L of inoculum per day of the three pure compounds BOR, CAM, EUCA, using two fermenters per treatment per each run. Two control fermenters per run were added only with ethanol. Essential oils and the standard diet were given twice a day in equal doses (at 09:00 and at 17:00) for a total of 18 g/d of DM. Artificial saliva (Slyter et al., 1966) was continuously infused using a peristaltic pump at 1.3 mL/min. During the last 3 days before morning feeding pH was directly measured (GLP 22, Crison Instruments, S.A. Barcelona, Spain), whereas samples for ammonia-nitrogen NH_3-N, VFA, protozoa and bacterial DNA analyses were collected. Samples for NH_3-N were directly stored at -20°C, thawed at room temperature and measured using an ammonia electrode (Ammonia Gas Sensing Combination Electrode, ©Hach Company, 2001) whereas VFA samples were stored at -20°C adding H_2SO_4 0.1N; after thawing were centrifuge at 20.000 g for 30 min at 20°C, filtered by a polypore filter (0.45 µm, Agilent Technologies, Milano, Italia). The VFA concentration was measured as described by Martillotti and Puppo (1985). The CH_4 yield was estimated by the equation of Moss et al. (2000), considering the hydrogen recovery of 90% (default):

CH_4 (mL/L) = 0.45 (acetic, mmol/l) – 0.275 (propionic, mmol/L) + 0.40 (butirric, mmol/L)

Protozoa and microbiome analysis

Protozoa were counted as described by Dehority (2003). For the extraction of DNA from the rumen microbiota 350 µl of rumen fluid was stored at -20°C pending extraction. Particular attention was devoted to this operation: the 350 µl were taken immediately after shaking as the rumen fluid has a rapid precipitation, in this way all the analyzed samples had the same characteristics. DNA from rumen fluid has been extracted using the NucleoSpin Soil kit (Macherey-Nagel); the first steps of

sample preparation and adjust lysis condition were performed with SL1 and enhancer SX solutions. The DNA was finally recovered in 70 µl of SE buffer as suggested by the kit manufacturer.

PCR amplification of 16S gene

For the identification of the bacterial community present in the rumen fluid a portion of the 16S gene was used, as described by Takahashi et al. (2014). For the amplification the following primers were used: Pro341F: 5′-CCTACGGGNBGCASCAG -3′ and Pro805R: Rev 5′-GACTACNVGGGTATCTAATCC -3′. The amplifications were performed using 5 µL of the extracted DNA in a final reaction volume of 25 µl using Platinum Taq DNA polymerase high fidelity (Thermofisher) following the manufacturer instructions. The amplifications were performed for 27 cycles using 55 °C as annealing temperature.

2.5 PCR products sequencing and Bioinformatics analyses

For the identification of the bacterial community present in the rumen fluid a portion of the 16S gene was used, as described by Takahashi et al. (2014). For the amplification the following primers were used: Pro341F: 5′-CCTACGGGNBGCASCAG -3′ and Pro805R: Rev 5′-GACTACNVGGGTATCTAATCC -3′. The amplifications were performed using 5 µL of the extracted DNA in a final reaction volume of 25 µl using Platinum Taq DNA polymerase high fidelity (Thermofisher) following the manufacturer instructions. The amplifications were performed for 27 cycles using 55 °C as annealing temperature. The libraries were purified with Beads Amplure XP 0.8X, amplified with Indexes Nextera XT Illumina, normalized, mixed and loaded on Miseq with 2x300bp (paired-end) approach to generate a minimum of 50,000 sequences (± 20%). The raw sequences R1 and R2 (raw reads) were verified and filtered by quality, trimmed by the primers and fused by the Qiime2 v8 software. The DADA2 (Qiime2) software isolates the ASVs (formerly OTUs) whose sequences were compared against the Greengenes v13-8 to obtain the taxonomic assignment.

2.6 Statistical analyses

The data from Exp. 1 and 2 were statistically analysed as a factorial randomised completely block (fermentation run) design by proc mixed procedure of SAS 9.4:

$Y_{ijk} = \mu + \alpha_i + \beta_j + \varepsilon_{ijk}$

where yijk, is the experimental data, µ is the overall mean, αi is the random effect (block) of the fermentation trial (i=1,3 in Exp. 1 and i=1,2 in Exp. 2); β j is the fixed effect of the dietary treatment (j =1,6 in Exp.1 and j=1,3 in Exp. 2); and εijk is the random error. Least squares means estimates are reported. For all statistical analyses, significance was declared at $P \leq 0.05$ and trends at $P \leq 0.10$.

3. Results

3.1 First experiment. In batch gas and CH_4 production and rumen fermentation parameters

The results of GP are in Table 1. Gas production was not affected by additive. There was an effect of the additive on CH_4 production as percentage on total GP at 48 h (P=0.044) with lower values for EUCA and CAM compared to the CTR (22.5% and 22.4% respectively, versus 24.2% of the CTR, P<0.05). Among the other parameters, tendencies (P<0.10) were found for CH_4 production (mL/200 mg DM) at 24 and 48 h and for CH4 percentage on total GP at 24 h with EUCA and CAM being the most promising compounds. The two essential oils did not affect any of the parameters evaluated. Regarding the rumen fermentation parameters (Table 2), pH and total VFA were not affected by treatment. The CTR had highest values (% total VFA) for Iso-butyrate (1.74) (P=0.003) and Iso-valerate (3.11) (P<0.001) than the other treatments (on average: 1.62 and 2.79 respectively for Iso-butyrate and Iso-valerate). Acetate to propionate ratio (A:P) was not affected by treatment

(P=0.299).

3.2 Protoza count and relative abundance of main bacteria Phylum and Euryarchaeota

Results on protozoa and relative abundance of main bacteria Phylum and Euryarchaeota are in Table 3. All the experimental compounds increased the total number of protozoa (P=0.006) as compared with the CTR. All the treatments increased the genus *Entodinium* as percentage of total protozoa (on average 91.8 for additives vs 83.0 for CTR; P<0.001) while the genus *Diplodinium* (% total protozoa) was decreased by all the experimental treatments (on average 6.60 for additives vs 13.6 for CTR; P=0.001). The percentage of other protozoa (Ophryscolecinae, Isotricha and Dasytricha) was lowest in BOR, CAM and EUCA (on average 0.906) than CTR (3.38) (P=0.018). There was an inversely proportional relationship between total protozoa and CH_4 production; however, other protozoa (Ophryscolecinae, Isotricha and Dasytricha) were positively correlated to CH_4 production (Figure 1). Additives affected *Bacteroidetes* (P=0.0031), *Proteobacteria* (P=0.014) and *Firmicutes* (P=0.014) relative abundance. *Bacteroidetes* and *Firimicutes* were decreased by all treatments as compared with CTR while *Proteobacteria* abundance increased with all the compounds. No effect related to additive was observed for total *Euryarchaeota* (P=0.189) although at genus level VadinCA11 was higher (P<0.05) for CTR (0.625% relative abundance) than for the other treatments (0.328%). Overall, relating the CH_4 as percentage of total 48h GP, it is possible to observe a positive relationship with *Euryarchaeota* (*Euryarchaeota* (%) = 0.0582* CH_4 production (% total GP) -0.658; r2 = 0.3129). The correlations among Protozoa and main Bacteria Phylum are reported in table 4. *Euryarchaeota* was positively correlated with *Ophryscolecinae* (r=0.697) and negatively correlated with *Entodinium* (r=-0.584). On the other hand, Proteobacteria was positively correlated with *Entodinium* (r=0.658).

Bacteroidetes and *Firimicutes* were negatively correlated with *Entodinium* (r=-0.630 and – 0.732, respectively for *Bacteroidetes* and *Firimicutes*). The regressions among main Bacteria Phylum and CH_4 emission (% total GP) are in Figure 2. *Firimicutes* and *Bacteroidetes* were positively related to CH_4 emission whilst *Proteobacteria* and *Spirochaetes* were negatively correlated.

3.3 Complexity of bacterial population

The total number of bacterial species identified in the different samples treated with the different additives were determined and this analysis showed a wide variability depending by the additive; the value varies from a minimum of 27 to a maximum of 82 (Table 5). In order to verify the bacterial complexity, the number of species needed to reach 90% of the total bacterial population was also calculated. In all the samples inoculated with the additives a reduction is observed for both evaluations. This reduction is much more pronounced in the treatments EUC, CAM and BOR. On the basis of these results, the relationship between CH_4 (ml/200 mg DM) and bacterial species found was verified. This analysis was made considering only the species that were present at least for a 4% in at least on 1 sample for each treatment. Therefore, 7 species have been identified that cover a variable percentage of the entire bacterial population: from 72 to 84%. The multilinear regression is in table 6. Three unclassified species (codified as A, B and D) show a significance effect ($P<0.10$) on CH_4 production. The species A belongs to the order of *Bacteroidales*. The bacteria of the families belonging to this order are colonizers of the gastro-intestinal tract of many animals. The B species, order *Pseudomonadales*, belongs to the *Proteobacteria* phylum, one of the most prominent bacterial groups in the rumen. The third species that has significance (D) is represented by a bacterium belonging to the family of the *Ruminococcaceae*. This last species had the coefficient of regression (0.596), whilst the species B had the lowest (0.27).

3.4 second experiment. Continuous rumen fermenter system

Results of continuous rumen fermenter are shown in Table 7. The inclusion of BOR and EUCA increased the pH in the fermentation liquid ($P<0.001$). Total VFA shown a decrease of about 20% when EUCA was added compared to the CTR ($P=0.029$), while proportions were not modified by this oil except for an increase in Valerate ($P=0.001$). BOR and CAM affected the proportion of acetate in the fermentation liquid whit an increase of about 10% compared to the CTR (61.26, 58.44 vs 54.34 for BOR, CAM and CTR, respectively), while Valerate was modified by BOR inclusion resulting in a lower value ($P<0.05$) than CTR and EUCA. Protozoa were highly affected by the addition of the three oils, whit an increase of the number of about 50% ($P<0.001$). No modification for the protozoa groups were detected instead. Relative abundance of Phylum and Genera were affected only considering EUCA addition that increment the presence of *Anaerovibrio* and *Ruminocuccus* (Table

8). Moreover, the addition of EUCA tended to increase Firmicutes while lower *Bacteriodetes* percentage (P=0.090 and P=0.084, respectively). Stochiometric calculated CH_4 (mmol/L) was lower (P=0.0245) for EUCA (7.40) than the other treatments (on average 8.87).

Discussion

The aim of the present study was to evaluate the possible effects of the main pure compounds of *A. moschata* essential oil and the essential oil on rumen fermentation and microbiome both at short and long incubations. *A. moschata* is a medicinal plant which has been traditionally used in ethnomedicine to treat various digestive disorders in human but also in animal health. For this plant, Vitalini et al. (2016) showed a broad spectrum of antimicrobial activity against some food pathogen bacteria such as *B. cereus*, *S. aereus*, *E. Coli*, *P. Mirabilis* and *P. aeruginosa*. However, to the best of our knowledge, the role of this essential oil to possibly modify rumen metabolism to enhance nutrient utilization by animals was never investigated. The main compounds of *A. moschata* essential oil are BOR, CAM and EUCA.

Interestingly, these compounds gave promising results in terms of lower enteric CH_4 production in short time *in vitro* incubations without decreasing VFA production (Joch et al., 2016, 2018). In the research of additives, molecules that decrease enteric CH4 without compromising overall gas and VFA production should be identified. In the first experiment 271 (48 h), the GP and VFA production was not decreased by the additives whilst in the second Experiment (9 d), the addition of EUCA determined a lower VFA production which is possibly related to a lower digestibility of the diet. The difference between the experiments may be due to several reasons such as: different dose used, different donor animals and possible adaptation of rumen bacteria to the additives.

The selected compounds are oxygenated monoterpenes which are not very degraded in the rumen; for example, Maleky et al. (2011) showed that after 24 h of incubation with caprine rumen, oxygen containing terpenes were less degraded than linear and monocyclic terpenes. Hence, it can be speculated that in the short trial the additives were slowly degraded however, in the second Experiment the rumen microbiome was probably more adapted to the additives. In agreement with this result, Cardozo et al. (2004) observed in continuous fermenter a different VFA profile due to essential oil supplementation between 2 and 6 d of fermentation, although the difference disappeared after d 6 suggesting that ruminal microbes were adapted to additives after 6 d of incubation.
The VFA profile was affected by the additive; particularly, in the long-term experiment, EUCA increased valerate proportion. Ungerfeld (2015) suggested that an increase in ruminal hydrogen availability following methanogenesis inhibition enhances fermentation pathways that consume

hydrogen, such as formate, valerate and caproate which is positively correlated to a lower CH_4 emission. The estimated CH_4 production underlined a lower value for EUCA than other treatments. On the other hand, CAM and BOR increased acetate proportion (without affecting A:P ratio) as compared to CTR which should be related to a better fiber digestibility. In according with this hypothesis, the pH value of BOR was higher than CTR. All the selected additives and essential oils modulated the rumen microbiome although with different results between the 2 experiments. In both experiments, the 3 dominant phyla were Bacteroidetes, Firmicutes and Proteobacteria.

However, the CTR samples (i.e. ethanol) had a higher abundance of Proteobacteria and a lower abundance of Firmicutes in 48 h Experiment than in the 9 d Experiment using rumen fluid collected from different animals. Other studies (among these Jami et al., 2014; Li et al., 2009) reported a large variation between animals for the abundance of the main Bacteria phyla. In the present study, the rumen inoculum was collected from different animals fed different diets which probably caused the observed difference. In the 48 h Experiment, all the treatments increased the relative abundance of Proteobacteria and decreases that of Firmicutes and Bacteroides. Wallace et al. (2015) found out that in beef cattle Proteobacteria were 4-fold more abundant in animals with lower CH_4 emissions than in animals with higher emissions; similarly, Danielsson et al. (2017) reported a higher abundance of Proteobacteria in low emitting cows than in high emitting cows. These results seem in agreement with the results for EUCA and CAM treatments in Exp. 1. Overall, the results of the *in vitro* trial showed a negative correlation between Proteobacteria and CH_4 production.

Moreover, there was a reduction of bacterial diversity due to additive. This reduction is probably due to a selective action of the additives used which can act in two ways: a) eliminating some bacterial species (with the consequent increase in % of the other species) or b) favoring some bacterial species (which therefore increase their percentage presence). This is an interesting result, since a comparison of microbiome richness across animals revealed significantly lower richness in efficient cows' microbiomes (Shabat et al. 2016). Despite the effects were less marked, in the 9 d Experiment there was a tendency for a higher relative abundance of Firmicutes and for a lower Bacteoroides abundance due to EUCA addition in comparison with CTR. Overall this determined a higher Firmicutes-to-Bacteroidetes ratio 316 for EUCA; this ratio was recently found to be positively correlated with daily milk-fat yield (Jami et al. 2014). Delgado et al. (2019) found that more efficient cows presented a larger relative abundance of Bacteroidetes, and a lower, but not significant, relative abundance of Firmicutes. A decreased amount of Bacteroidetes in the digestive microbiota has been also associated with impaired feed conversion rate and residual feed intake (Jami et al. 2014) and with an increased fat deposition in mice (Turnbaugh et al. 2006). Overall, a lower amount of Bacteroidetes in the rumen

might redirect energy intake to an increased fat deposition at the expense of lowering milk production per unit of feed intake (Delgado et al. 2019). Another effect observed in the second experiment, was the increase in Ruminococci relative abundance due to EUCA treatment. Ruminococci play a fundamental role in cellulose degradation (Christopherson et al., 2014) producing succinic acid as a major fermentation product together with acetic and formic acids, H2 and CO2. In agreement with this finding, previous data (Colombini, unpublished) reported a positive effect of EUCA on Italian ryegrass NDF *in vitro* digestibility. Moreover, in the second experiment, the rumen pH value of EUCA was higher than CTR which is in agreement with a positive effect on fiber digestion. Although not significant, Patra and Yu (2012) reported on a TMR sample a value of NDFD (%) of 36.7 with the addition (250 mg/l) of eucalyptus oil versus a value of 33.9 for control. Sallam et al. (2009) showed that the addition of eucalyptus oil (25 ul/75 inoculum) decreased CH_4 without a decrease in total GP.

No effects of additives on Euryarchaeota relative abundance was underlined in the present study. Shi et al. (2014) underlined that the number of archaea is not essential for the level of CH_4 production, but rather the metabolic activity of individual methanogenic species is important. However, it has to be noticed that in the present study there was a positive correlation between Euryarchaeota and CH_4 production expressed as percentage of total gas and that there was an effect in decreasing the abundance of vadinCa11 due to the experimental treatments as compared with CTR.

In both experiments, the number of Protoza increased due to the additive supplementation. Effects of essential oils on protozoal population vary in the literature. Some studies report a lack of effect on protozoal (Benchaar et al., 2007, Newbold et al., 2004) whilst others found a stimulatory effect of essential oils on protozoa (Patra et al. 2006), although the mechanism has not been elucidated (Patra and Saxena, 2010). The relationship between CH_4 and protozoa was unexpected. A meta-analysis (Guyader et al. 2014) summarized that in the analyzed database, 31% of the *in vivo* experiments reported a concomitant reduction of both protozoa concentration and CH_4 emission. By contrast, 21% of *in vivo* studies reported a variation in CH_4 emission without changes in protozoa numbers, indicating that methanogenesis is also regulated by other mechanisms. Belanche et al. (2014) demonstrated that holotrich protozoa have a different endosymbiotic correlation with methanogens than entodiniomorphids which is possible due to more hydrogenosomes than entodiniomorphids (Paul et al., 1990) or the synthesis of glycogen by holotrich protozoa that generates more hydrogen (Hall, 2011; Denton et al., 2015).

These differences may explain the greater impact of holotrich protozoa on rumen methanogenesis compared to entodiniomorphids (Belanche et al. 2015). The results of the present experiment showed

a quadratic relationship between CH_4 and Ophryscolecinae which were also negatively correlated to *Proteobacteria*. To the best of our knowledge, this result in the first screening trial, was never reported in the literature and deserves further studies. In agreement with the results of the present study, the numbers of large protozoa were increased by *Achillea millefolium* (Broudiscou et al. 2000). An increase in the number of rumen protozoa can have some beneficial effects for the animal. A recent study (Newton et al., 2015) showed that the elimination of rumen protozoa significantly decreased NDF (–20%) and ADF digestibility (–16%), probably as a result of the loss of protozoal fibrolytic activity.

Conclusions

The results of the present study showed that all the additives modulated rumen fermentation pattern although with different results among the 2 experiments. Although the effect of the additive was less strong in the second experiment, EUCA treatment determined some positive effects, such as an increase of Ruminococci abundance, which may be positively related to a higher fiber digestion by cows and which can compensate for the lower VFA production due to this treatment. The essential oils had different effects than the pure compounds and this suggests the importance of also evaluate pure compounds in the research of feed additives to decrease CH_4 production. Although the first experiment was mainly a screening trial, some important interactions were observed about rumen Protozoa, Bacteria and CH_4 emission. These observed results should be confirmed by other studies in order to better understand the role of rumen microbiome and the relationship with important aspects of dairy cow nutrition such as CH_4 production, fiber digestibility and protozoa role in the rumen ecosystem.

Authors contributions:

SC writing original draft, review and editing, conceptualization, data elaboration, funding acquisition

SV selection of plant material and oil extraction and processing

PP microbiome analysis, data elaboration, writing review and editing

ARG 48 h *in vitro* analysis, writing review and editing, data elaboration

CS continuos fermenter analysis, writing review and editing, data elaboration

MS conceptualization, data elaboration, writing review and editing,

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Figure 1. Linear and quadratic regressions between Protozoa and and CH_4 emission

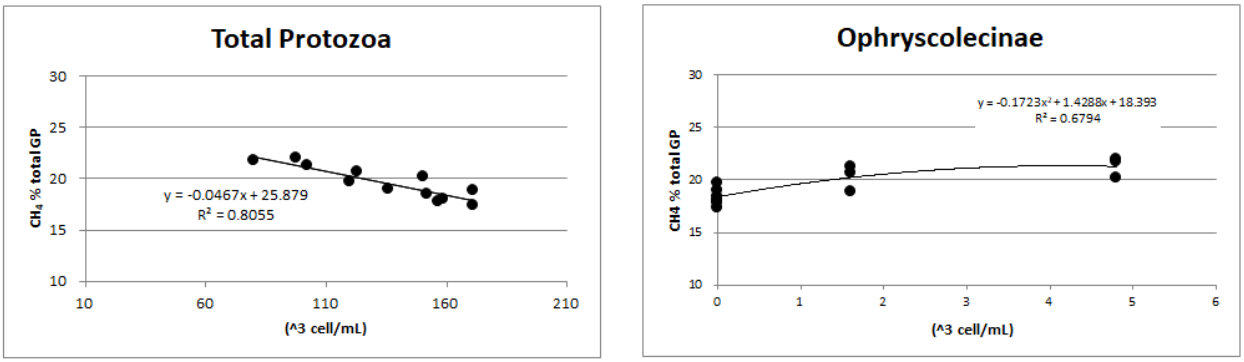


Figure 2. Linear regression between main bacteria Phylum and CH_4 emission

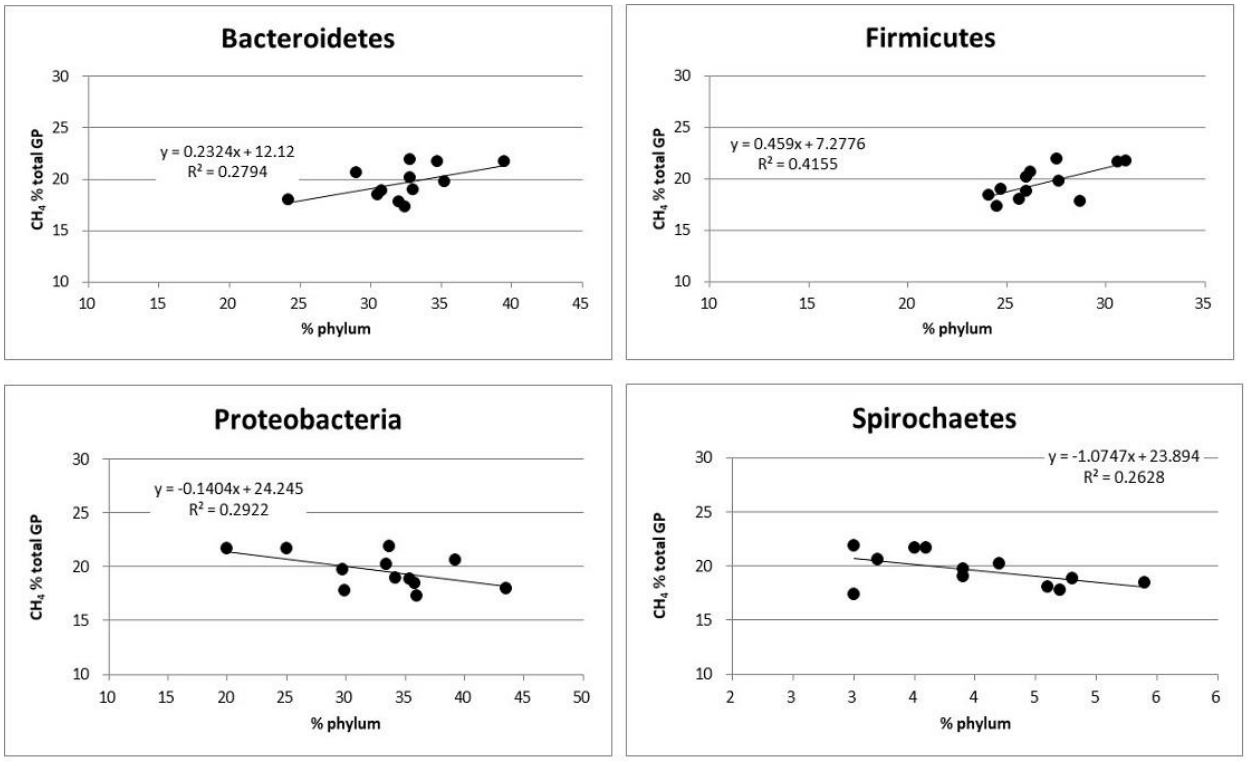


Table 1. Gas production (GP) and CH_4 production of diets with the different additives. Data are expressed as ml/200 mg DM unless otherwise stated.

		Pure compound[1]			Essential oils[2]		RMSE	P
	CTR	BOR	CAM	EUCA	OIL 1	OIL 2		
GP 24h	31.1	30.2	30.3	30.3	30.5	30.0	2.90	0.772
GP 48h	46.3	45.2	44.7	44.7	45.2	44.8	2.97	0.545
GP 24-48h	15.2	15.0	14.4	14.4	14.6	14.8	1.05	0.334
CH_4 24h	7.84	7.44	7.02	7.06	7.97	7.52	1.18	0.063
CH_4 48h	11.3	10.9	10.2	10.2	11.1	10.5	1.54	0.075
CH_4 24-48h	3.59	3.68	3.35	3.35	3.32	3.31	0.747	0.744
CH_4 %GP 24h	22.9	22.1	20.9	21.0	23.6	22.5	1.03	0.082
CH_4 %GP 48h	24.2^{a}	23.8^{ab}	22.4^{b}	22.5^{b}	24.4^{a}	23.2^{ab}	2.06	0.044

[1] CTR: control; BOR: bornyl-acetate; CAM: camphor, EUCA: eucalyptol

[2] OIL1 *Achillea moschata* essential oil derived from sample 1; OIL2 *Achillea moschata* essential oil derived from sample 2

ab: means in the same row with different superscripts are statistically different for P<0.05

Table 2. Rumen fermentation parameters of diets with the experimental additives

		Pure compound[1]			Essential oils[2]		RMSE	P
	CTR	BOR	CAM	EUCA	OIL 1	OIL 2		
pH	6.74	6.68	6.70	6.73	6.72	6.77	0.096	0.207
Total VFA (mMol)	63.8	62.4	60.6	59.3	59.2	57.7	5.29	0.189
% total VFA								
Acetate (A)	63.1	64.5	64.2	63.3	63.5	63.4	1.61	0.286
Propionate (P)	17.2	16.7	17	17.3	17.2	17.3	0.870	0.594
Iso-butyrate	1.74^{a}	1.58^{c}	1.59^{bc}	1.65^{b}	1.65^{b}	1.64^{bc}	0.060	0.003
Butyrate	13.0	12.7	12.8	13.1	13.1	13.0	0.480	0.481
Iso-valerate	3.11^{a}	2.83^{b}	2.72^{b}	2.79^{b}	2.83^{b}	2.80^{b}	0.045	<0.001
Valerate	1.81^{a}	1.69^{b}	1.69^{b}	1.75^{ab}	1.80^{a}	1.78^{ab}	0.187	0.040
A:P	3.89	3.81	3.67	3.71	3.68	3.68	0.287	0.299

[1] CTR: control; BOR: bornyl-acetate; CAM: camphor, EUCA: eucalyptol

[2] OIL1 *Achillea moschata* essential oil derived from sample 1; OIL2 *Achillea moschata* essential oil derived from sample 2

abc: means in the same row with different superscripts are statistically different for P<0.05

Table 3. Rumen protozoa count and relative abundance (%) of main bacterial Phylum

		Pure compound[1]			Essential oils[2]		RMSE	P
	CTR	BOR	CAM	EUCA	OIL 1	OIL 2		
Total protozoa (^3 cell/mL)	68.0^{b}	92.0^{a}	104.0^{a}	92.3^{a}	89.5^{a}	86.9^{a}	26.3	0.006
% total protozoa								
Entodinium	83.0^{b}	93.6^{a}	92.4^{a}	93.8^{a}	89.8^{a}	89.4^{a}	1.81	<0.001
Diplodinium	13.6^{a}	5.85^{b}	6.69^{b}	4.92^{b}	7.30^{b}	8.24^{b}	1.31	0.001
Other protozoa	3.38^{a}	0.577^{c}	0.900^{c}	1.24^{bc}	2.88^{ab}	2.35^{ab}	0.953	0.018
Phyla (% total observations)								
Firmicutes	30.7^{a}	25.8^{b}	27.8^{b}	25.1^{b}	26.9^{b}	26.0^{b}	0.802	0.031
Bacteroidetes	37.1^{a}	32.8^{b}	27.7^{d}	28.3^{cd}	30.9^{bcd}	31.8^{bc}	1.11	0.014
Proteobacteria	22.5^{b}	32.8^{a}	36.7^{a}	39.7^{a}	36.5^{a}	34.4^{a}	1.95	0.014
Spirochaetes	3.59	4.67	4.61	3.77	2.98	4.52	0.375	0.107
Euryarchaeota	0.71	0.385	0.41	0.45	0.46	0.505	0.077	0.189
Others	5.31	3.52	2.82	2.71	2.35	2.80	1.01	0.445

[1] CTR: control; BOR: bornyl-acetate; CAM: camphor, EUCA: eucalyptol

[2] OIL1 *Achillea moschata* essential oil derived from sample 1; OIL2 *Achillea moschata* essential oil derived from sample 2

abcd: means in the same row with different superscripts are statistically different for P<0.05

Table 4. Correlations among Protoza and main Bacteria Phyla

	Archea	Bacteroidetes	Proteobacteria	Firmicutes	Spirochaetes
Total Protozoa	-0.526	-0.627	0.646	-0.709	0.538
Entodinium	-0.584	-0.630	0.658	-0.732	0.530
Diplodinium	0.304	-0.105	-0.039	0.157	0.210
Ophryscolecinae	0.697	0.442	-0.398	0.432	-0.377

Table 5. Complexity of bacteria communities due to additive supplement

Pure compound[1]	Total[2]	90%[3]
CTR	76	21
BOR	64	18
CAM	48	15
EUCA	48	15
OIL 1(c)	62	17
OIL 2(v)	73	19

[1]CTR: control; BOR: bornyl-acetate; CAM: camphor, EUCA: eucalyptol.

[2]Number of bacterial species identified in the samples analyzed; [3]Number of species needed to reach 90% of the total bacterial community contents.

Table 6. Multiple regressions between main Bacteria species and CH_4 production (ml/200 mg DM)

	Regression Coefficient	SE	P
Intercept	-18.73	10.5	0.148
species A[1]	0.312	0.131	0.076
species B[2]	0.27	0.099	0.053
species C[3]	0.397	0.234	0.165
species D[4]	0.596	0.185	0.032
species E[5]	0.872	0.567	0.199
species F[6]	-0.212	0.161	0.259
species G[7]	-0.116	0.175	0.543

R^2 0.824; F=8.38; P=0.029

[1] Bacteria/Bacteroidetes/Bacteroidia/Bacteroidales/unclassified/unclassified/unclassified

[2] Bacteria/Proteobacteria/Gammaproteobacteria/Pseudomonadales/Moraxellaceae/Acinetobacter/unclassified

[3] Bacteria/Firmicutes/Clostridia/Clostridiales/Lachnospiraceae/unclassified/unclassified

[4] Bacteria/Firmicutes/Clostridia/Clostridiales/Ruminococcaceae/unclassified/unclassified

[5] Bacteria/Bacteroidetes/Bacteroidia/Bacteroidales/Prevotellaceae/Prevotella/unclassified

[6] Bacteria/Spirochaetes/Spirochaetes/Spirochaetales/Spirochaetaceae/Treponema/unclassified

[7]Bacteria/Proteobacteria/Gammaproteobacteria/Aeromonadales/Succinivibrionaceae/Ruminobacter/unclassified

Table 7. Fermentation parameters and protozoa count

		Pure compound[1]				
	CTR	BOR	CAM	EUCA	RMSE	P
pH	5.98[b]	6.15[a]	6.06[b]	6.20[a]	0.032	<0.001
NH_3 (mg/dL)	13.33	13.86	13.85	12.60	0.543	0.356
Total VFA (mMol)	33.28[a]	30.98[ab]	32.29[a]	26.80[b]	1.358	0.029
% total VFA						
Acetate	54.34[b]	61.26[a]	58.44[a]	51.49[b]	1.287	0.001
Propionate	15.39[ab]	15.94[ab]	17.26[a]	12.52[b]	1.044	0.048
Iso-butyrate	0.441	0.255	0.312	0.159	0.071	0.092
Butyrate	17.16	13.76	13.85	19.75	1.554	0.055
Iso-valerate	0.917	0.802	0.917	0.905	0.110	0.853
Valerate	11.74[b]	7.99[c]	9.21[bc]	15.17[a]	0.874	0.001
A:P	3.55	4.04	3.42	4.15	0.285	0.250
Calculated CH_4 mmol/L	9.02[a]	8.75[a]	8.85[a]	7.40[b]	0.344	0.0245
Total protozoa (^3 cell/mL)	54.9[b]	84.1[a]	80.7[a]	79.6[a]	2766	<.0001
% total protozoa						
Entodinium	95.3	95.5	95.1	95.4	0.911	0.990
Diplodinium	3.52	3.10	3.93	3.08	0.883	0.889
Other protozoa	1.20	1.38	1.00	1.52	0.599	0.934

[1]CTR: control; BOR: bornyl-acetate; CAM: camphor, EUCA: eucalyptol.
ABCD: means in the same row with different superscripts are statistically different for P<0.05

Table 8. Relative abundance of main bacterial Phylum and Genera

		Pure compound[1]				
	CTR	BOR	CAM	EUCA	RMSE	P
OTUs	403.8	322.5	398.0	351.0	65.44	0.788
Phyla (% total observations)						
Firmicutes	55.10	54.20	54.75	62.78	2.428	0.090
Bacteroidetes	26.55	30.80	31.00	22.08	2.484	0.084
Proteobacteria	10.80	9.275	7.825	6.475	1.109	0.088
Spirochaetes	2.925	1.975	2.175	3.050	0.410	0.224
Actinobacteria	2.700	2.425	2.650	2.775	0.381	0.923
Euryarchaeota	0.985	0.680	0.743	0.885	0.222	0.765
Others / Unclassified	1.925^{ab}	1.325^{b}	1.600^{ab}	2.850^{a}	0.251	0.040
Genera (% total observations)						
Anaeroplasma	0.110	0.162	0.310	0.143	0.064	0.113
Anaerovibrio	0.520^{ab}	0.333^{b}	0.290^{b}	0.575^{a}	0.737	0.028
Bifidobacterium	2.08	1.602	2.193	2.075	0.377	0.675
Butyrivibrio	4.87	3.387	5.933	4.525	0.714	0.118
Clostridium	0.885	0.489	1.093	1.440	0.423	0.419
Fibrobacter	0.288	1.336	0.043	0.233	0.555	0.361
Megasphaera	7.21	5.550	8.550	6.085	1.361	0.366
Methanobrevibacter	0.585	3.301	0.550	0.830	1.203	0.325
Oscillospira	1.28	0.880	1.610	2.290	0.617	0.378
Prevotella	20.2	15.72	22.37	15.75	3.792	0.441
Pseudobutyrivibrio	0.758	10.70	1.978	1.930	4.581	0.399
Ruminococcus	1.18^{b}	0.813^{b}	1.370^{b}	2.595^{a}	3.720	0.0118
Shuttleworthia	5.11	3.518	3.320	9.185	2.332	0.214
Succiniclasticum	4.10	4.498	5.028	4.670	1.186	0.993
Succinivibrio	0.623	1.948	2.368	1.173	0.972	0.496
Treponema	2.96	1.753	1.770	3.093	0.829	0.437
YRC22	0.613	0.510	0.808	0.53	0.224	0.708

[1]CTR: control; BOR: bornyl-acetate; CAM: camphor, EUCA: eucalyptol

ABCD: means in the same row with different superscripts are statistically different for P<0.05

Chapter 6. Comments and implications

The following discussion points consider some experimental aspects which are based on the results obtained across several experiments of the thesis.

In vitro rumen fermentation systems

In present thesis several different rumen vitro technologies were used, as can be seen from Table 2.

Table 2. *In vitro* techniques and type of rumen liquid collection used for the experiments in the thesis.

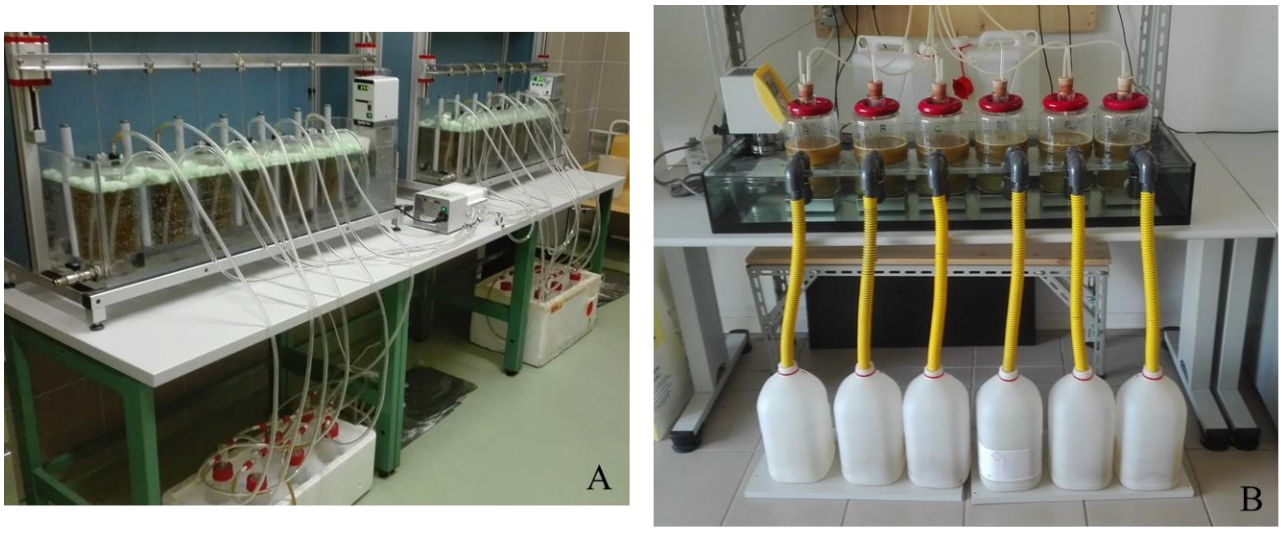
	In vitro technique	Rumen liquid collection
Paper 1	Rumen simulation technique (Rusitec)	cannulated cows
Paper 2	Gas production system (batch)	slaughterhouse
	Continuous culture system	slaughterhouse
	Gas production system (batch)	slaughterhouse
Paper 3	Gas production system (batch)	cannulated sheep
	In vitro DM and NDF degradability (batch)	cannulated sheep
Paper 4	*In vitro* gas production (batch)	cannulated cows
	Continuous culture system	slaughterhouse

A first difference between techniques is the origin of rumen fluid, since it can be taken directly from the animal via cannula or esophageal probe or by sampling from the rumen at the slaughterhouse. The use of rumen liquid from slaughtered animals is often criticized, although no specific comparison between sampling methods were published (Spanghero et al., 2019). In fact, in literature only some contrasts between the collection from cannulated animals and esophageal probe are reported (van Gastelen et al., 2019, Shen et al., 2012, Duffield et al., 2004), but none compared these two methods with sampling at slaughterhouse. This lack of comparison is probably due to the high cost to organize experiments where animals with cannula are slaughtered to obtain the two measures in each animal. In the fermentations of present thesis rumen liquid from fistulated animals and from slaughterhouse was used and we did not directly compare the two sampling methods. However, we were able to replicate the same results in experiments differing for type of collection of rumen fluid. A first confirm is given by the study of the effects of *Stevia rebaudiana* extract and chestnut tannin. The dietary additives were analyzed both at University of Udine with liquid from slaughtered animals and

at the University of Ljubljana where liquid from fistulated sheep was used. The inhibition effect of *Stevia rebaudiana* extract on protozoa population was confirmed in both experimentations as well the effect of reduction of ammonia was detected in both cases with tannin supplementation. In addition, in the paper in collaboration with the research group from Milan the addition of essential oils (camphor, bornyl acetate and eucalyptol) to the substrate determined an increase in protozoa population and this was observed both in batch cultures with rumen fluid coming from fistulated cows and in continuous culture system with fluid collected at the slaughterhouse. The above discussed agreement of results between experiments using different rumen fluids is a gross and indirect support to the assumption that rumen fluid collected from slaughterhouse has the same fermentative properties of that from cannulated animals. However direct comparisons between the two types of liquids are needed to validate these hypotheses.

In vitro ruminal fermentation systems can be divided into two categories: closed systems (“batch”) and continuous systems. In this thesis both systems were used: two types of continuous systems (Rusitec and a fermenter developed at the University of Udine, Figure 7) and two types of batch systems for the study of gas production and rumen degradation.

Figure 7. Rumen simulation technique (Rusitec, A) and the fermenter developed at the University of Udine (Mason et al., 2015, B)



In a meta-analysis from Hristov et al. (2012) different continuous culture system were compared with Rusitec, with concordant results in terms on fermentation parameters and digestibility. Rusitec system was developed by Czerkawski and Breckenridge (1977) in a period when the measures of degradability based on incubation of feeds in nylon bags were spreading a lot (Orskov e and Mc Donald, 1979). Perhaps this is the reason why in this system the substrate is administered to

each vessel by inserting nylon bags and the measure of degradability in porous bags is a peculiarity of this system.

However, I directly verified that this fermentation system is more complex to use and need an intense maintenance. On the contrary, the system set up by Mason et al. (2015) at the University of Udine is simpler and even construction and maintenance cost are limited. The disadvantage is the impossibility to perform degradation experiments, due to the feeding system used, and to measure or collect fermentation gases. Nevertheless, experiments have shown that the system from Udine is able to maintain the protozoa population due to a gentle agitation system and the maintenance of suitable ecological conditions by the formation of fibrous matter. If we compare the mean protozoa counts in diets without *Scrophularia striata* extract supplementation in Rusitec and the means of control diets in the system from Udine (used in Chapter 3 and 5) we could observe a relevant difference between the two fermentation systems: Rusitec shows a substantial reduction of protozoa that result to be 85% less than the other system ($10*10^3$ cells/mL vs $80*10^3$ cells/mL, for Rusitec and Udine's system, respectively).

Batch systems are generally simple apparatus, consisting of a single fermentation flask, containing less fermentative liquid than continuous systems, utilise small amount of substrate and the duration of fermentation is generally around 24-48h. However, thanks to their simplicity, they allow to screen many diets and additives simultaneously, reducing time and costs of experimentation. For the thesis different batch cultures were used: Menke and Steingass gas production test, Ritter technology and degradability NDF test. Interesting is the comparison between the two gas production tests. Menke and Steingass (1988) technique is an historical method that allows the determination of nutritive value of the substrate. It is simple and fast and allow the study of many samples at the same time. Ritter technology (Dr.-Ing. Ritter Apparatebau GmbH & Co. KG) is an innovative method for the determination of gas production, which is composed by large volume bottles (500mL) and allows the continuous measurement of the gas production and the possibility to collect gases for analysis. This system, due to its volume capacity, allows the use of more quantity of substrate (3-3.5 g instead of 0.2 g for Menke and Steingass (1988) technique). The use of a large quantity of feed is an advantage because the possibility to study the inclusion of additives in very low percentage of inclusion without weighing problems. Therefore, this system allows the sampling during all the fermentation trial due to the gas outlet that allows the use of a syringe for the sampling. In contrast to the fixed pressure systems like ANKOM gas production system (Ankom^{RF} Gas Production System, Ankom Technology, NY, US), Ritter technology enables gas exit, avoiding the pressurization. It has been

shown that in fixed pressurized systems, there is a partial dissolution of CO_2 in the fermentation liquid and a consequent underestimation of total gas production (Tagliapietra et al., 2010).

Fermentation metabolites and methane

The main rumen metabolites originating from fermentation are VFA and NH_3 and their concentrations in *in vitro* fermentation liquid are used to evaluate the effects of different dietary treatments. Modification of these components are usually induced by a shift of microbiota, that is involved in the digestion the substrate. Several authors (Patra and Saxena, 2010, Arowolo and He, 2018, Cobellis et al., 2016, Vasta et al., 2019, Olagaray and Bradford, 2019) found that the use of secondary plant compounds in general did not modify total VFA production and profiles, as already described in the introduction section (Figure 3). In present thesis for *Stevia rebaudiana* extract, chesnut tannin and *Scrophularia striata* extract the effect on total VFA and VFA profile was negligible. On the contrary, eucalyptol reduced VFA yield, especially in the continuous culture system experiment, showing a detrimental of fermentation.

Ammonia concentration in the rumen fermentation fluid is related to the degradation of proteins and our investigations showed an important effect of tannin on this metabolite. The involvement of tannin in rumen protein metabolism is already demonstrated, because they act complexing proteins and/or inhibiting protease and urease (Patra and Saxena, 2010, Patra and Aschenbach, 2018) and cause a reduction of ammonia release in the rumen. The use of chestnut tannin in Udine experiments and in the collaboration with Ljubljana confirm this trend. Also, *Scrophularia striata* extract significantly decreases ammonia concentration in rumen fluid and this effect seems to be correlated to flavonoid compounds present in *Scrophularia striata* extract. In fact, flavonoids and tannins are both polyphenolic compounds. The other extracts and oils tested in this thesis did not affect ammonia production, even if an effect on *Stevia rebaudiana* extract was suggested by Ramos-Morales et al. (2017).

Methane is one of the major greenhouse gases and the reduction of its production in the rumen by using secondary plant compounds has been widely investigated. The most accurate method for CH_4 determination is the respiration chamber technique, where methane and CO_2 concentration are directly measured in the air flux (Johnson and Johnson, 1995). However only few research centers in the world have the availability of respiration chambers, which require elevated equipment investments and maintenance costs. A recent study from Danielsson et al. (2017) has verified an excellent correspondence (R^2 = 0.96) between the *in vivo* measures of methane yield with that obtained by *in vitro* rumen gas production system. Moreover, the yield obtained *in vitro* were quite accurate compared with that observed *in vivo* (e.g. 399 L/d compared with 418 L/d for a total of 49

diets for dairy cows). In present thesis methane concentration was examined in the collaborations with Vienna, Ljubljana and Milan using *in vitro* techniques. Despite the encouraging results on methane mitigation for *Scrophularia striata* extract, tannin and *Stevia rebaudiana* Bertoni extract (Bagheri Varzaneh et al., 2018, Witzig et al., 2018 and Ramos-Morales et al., 2017), no significant reduction has been found in our experiments. In general methane production is related to fiber degradation and no detrimental on this parameter has been detected. Essential oils studied in the collaboration with Milan group determined a decrease of methane concentration in the fermentation gas at 48h for camphor and eucalyptol in batch culture. On the contrary, in continuous culture system the effect was found only for eucalyptol, by predicting methane yield by using VFA concentration. In that case, eucalyptol had lower amount of VFA leading a significant reduction of calculated methane.

Microbiota

In the last decades the study of rumen microbiota rapidly increased with the development of molecular biology methods such as DNA sequencing and the more advanced metagenomic analysis (McCann et al., 2014). Results obtained by sequencing analysis of bacterial DNA samples extracted by fermentation fluids after the use of *Scrophularia striata* extract in Austria and the fluids from fermenters added with essential oils in the collaboration with Milan were not in agreement. There were relevant differences for several important Phylum in terms of the relative abundance, such as for Bacteroidetes and Proteobacteria (8.91 vs 27.60 and 0.97 vs 8.59, respectively). These discrepancies can be explained firstly by the difference in the diet used as substrate and in the type of continuous fermentation system used (Rusitec vs Udine fermenter). Finally, the different sample preparation (e.g. extraction DNA kits) and conservation can interfere with the analysis (Fligerova et al., 2014). A further situation of lack of agreement was found in the trial with researchers from Milan. In fact, while batch cultures underlined a modification of Phylum, results of sequencing for continuous culture system were not affected. In this case a probable responsible of differences, in addition to type of substrate, was the adaptation of microbiota to *in vitro* conditions, which is assured by the continuous culture system and not by the batch system.

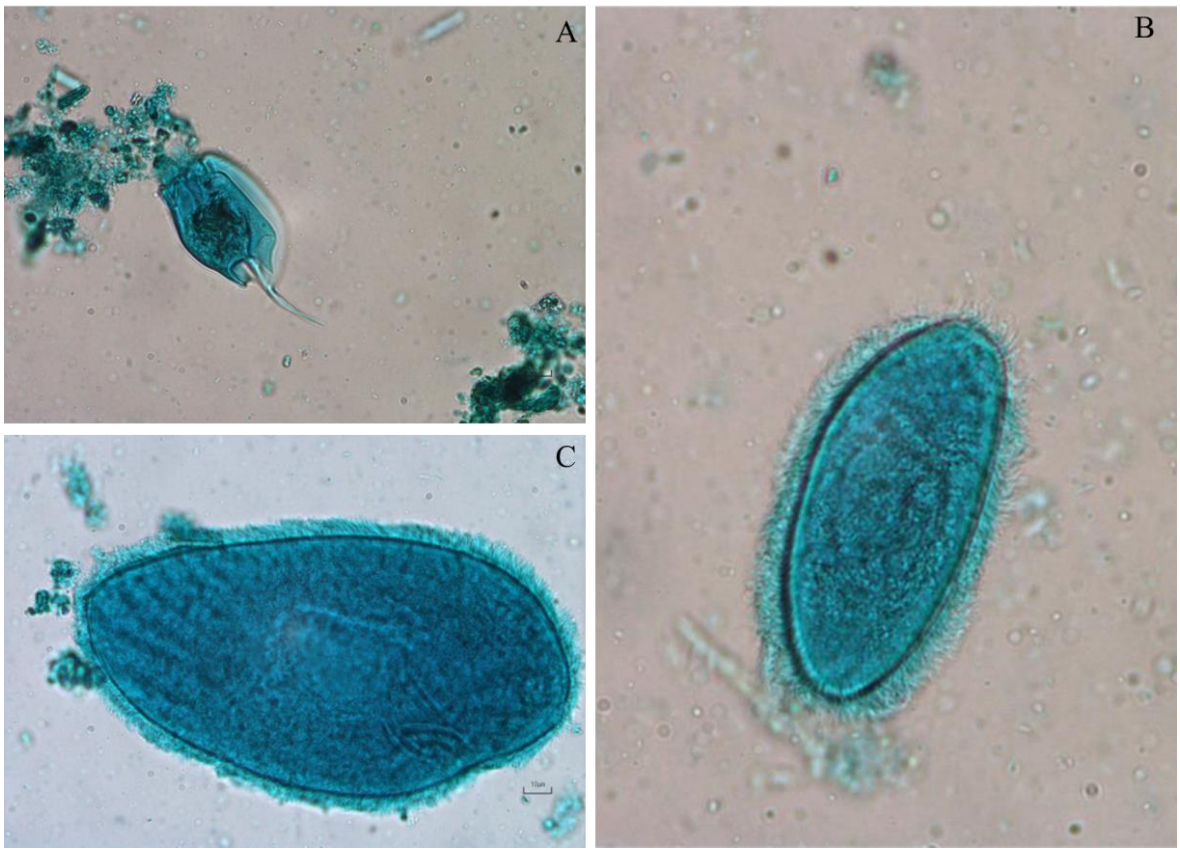
Another point of discussion regarding the DNA sequencing data is that found for the *Scrophularia striata* extract. Plant extracts have often antimicrobial properties at rumen level (Cobellis et al., 2016) and for *Scrophularia striata* extract this effect was reported by Dixon et al. (2004) and Bagheri Varzaneh et al. (2018). In our experiments we partially confirmed such properties because there was a clear decrease of richness and diversity of microbes (Chao 1 and Shannon index) but this did not cause a reduction in parameters of fermentability. Therefore, the only reasonable interpretation is that

there was a compensative phenomenon within the bacterial community: however, there are some difficulties to adapt a pure quantitative approach to molecular biology results.

For the second experiment, in which chestnut tannin and *Stevia rebaudiana* Bertoni were analyzed, bacterial composition was studied using a qPCR technique, that allowed to check the relative abundance on target bacteria. QPCR is a method widely used for specific genes, or microbial groups analysis in rumen environment (Belanche et al., 2012; Buccioni et al., 2015). Whereas for Stevia no significant effects were detected, tannins determined an increase of *Prevotella ruminicola* group and *Selenomonas ruminantium* relative abundance, which are known to be involved in ammonia use and amino acids synthesis (Atasoglu et al., 1998). For this reason, the microbial change is in accordance with the decrease of ammonia concentration.

While for bacterial composition biomolecular tools were used, protozoa were analyzed using a simpler technique set up by Dehority (2003) that includes the use of microscope, with a direct count and classification of protozoa cells. This analysis was performed in all the experiment reported in the thesis and some imagines of the most present protozoa species are shown in figure 8.

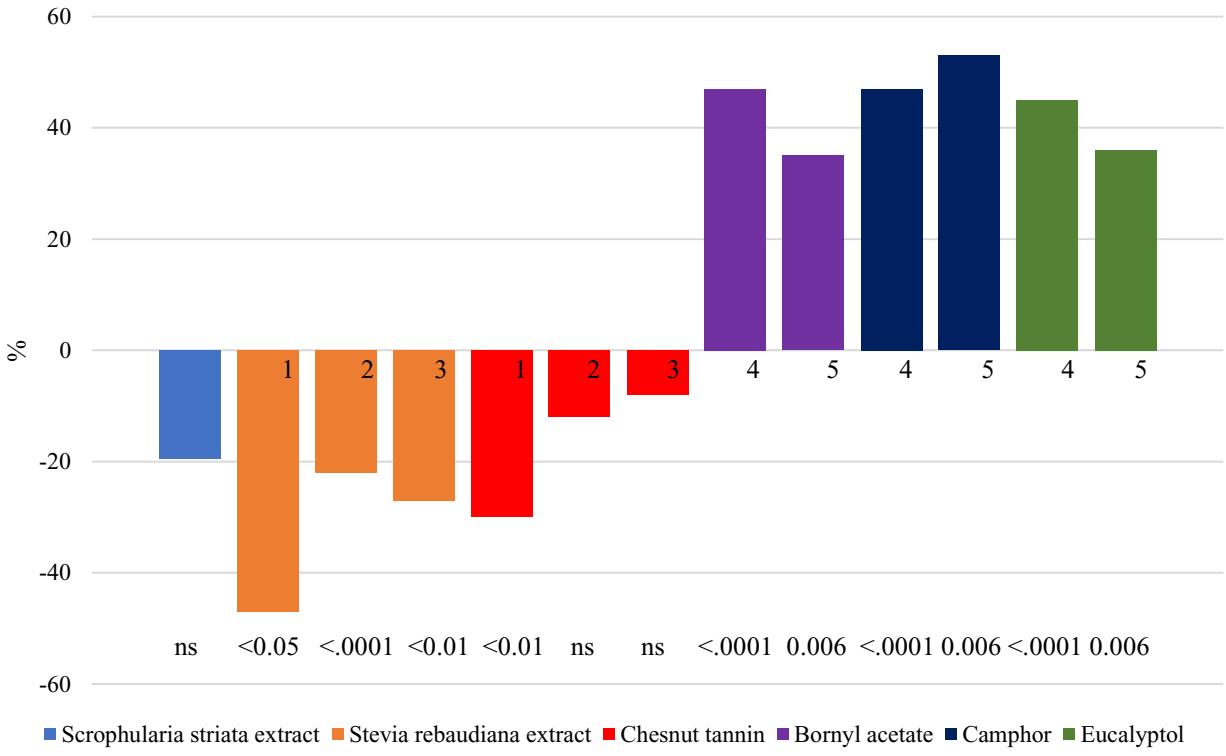
Figure 8. Examples of protozoa cells from different ruminal species in fermentation fluids.



Entodinium caudatum (A), *Dasytricha ruminantium* (B)*, Isotricha intestinalis* (C)

In general, we were able to obtain with protozoa more clear and reproducible results than those obtained with the bacterial populations, where lack of agreement between trials and high variability were found, as previously discussed. The inclusion of different additives has led to a significant variation of protozoa counts in fermentation liquid, as shown in figure 9.

Figure 9. Percentage of variation of protozoa counts in fermentation fluids after different plant extract additions with respect to the control fermenters (data from trial described in Chapter 2, 3, 4, 5)



[1]Experiment 1 in Chapter 3

[2]Experiment 2 in Chapter 3

[3]Gas production test in Chapter 4

[4]Continuous culture system in Chapter 5

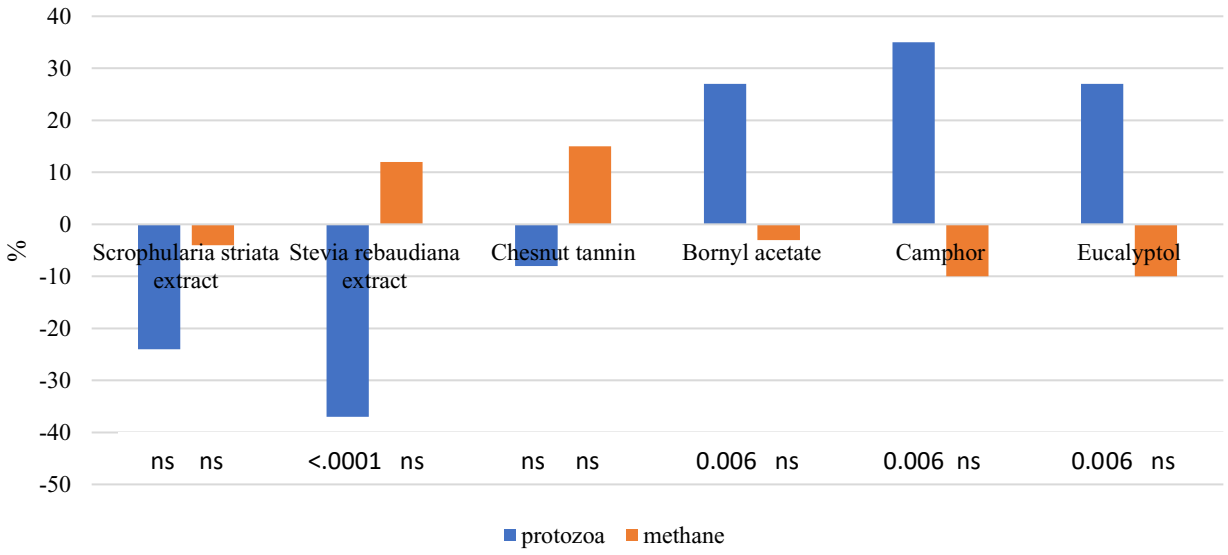
[5]Batch culture in Chapter 5

Stevia rebaudiana extract decreased protozoa population in all the tests performed and confirm the previous results obtained by Ramos-Morales (2017). It is worth noting that the different fermentation systems (batch and continuous fermenter) and rumen inocula (collected from cows or sheep, from slaughtered or cannulated animals) did not influenced this effect, which was replicates in all the experiments. The effect of tannin addition was analyzed in the same trials as Stevia, but results were less in agreement. In fact, only in one experiment (Exp. 1 in Udine trial) the reduction was statistically significant, while continuous system trials and fermentations performed in Ljubljana revealed only a

not significant reduction. Finally, the addition of camphor, bornil acetate and eucalyptol oils showed a significant increase of protozoa counts, which was confirmed in two experiments performed in two labs with different *in vitro* systems, substrate of fermentation and rumen inoculum. While the inclusion of *Scophularia striata* extract influenced bacteria richness and diversity, protozoa number was reduced but did not reach the statistical significance. As commented in a previous point, the scarce aptitude of Rusitec to contrast the protozoa decline is probably also responsible of a high variability of protozoa counts.

Even if the thesis was not focused on methane detection, results of this gas were reported for all the experimental additives. In our experiments there were no significant effects of additives on methane yield, even if the production of this gas varied from a +15% for tannin inclusion (from Chapter 4) to -4% for *Scrophularia striata* extract (from Chapter 1). The symbiosis between protozoa and methanogens suggests a role of protozoa in methane production and in literature there are some evidence of methane reduction with the decrease of protozoa counts or defaunation (Morgavi et al., 2010, Hegarty, 1999). In figure 10 results of methane and protozoa number were reported: despite no significant effects on methane yield, the trend in comparison with protozoa counts seems to be opposite to what expected.

Figure 10. Percentage of variations of protozoa counts (blu) and methane yield (orange) in fermentation fluids after different plant extract additions with respect to the control fermenters.



However, the role of protozoa is still controversial and recent work from authors (Morgavi et al., 2012) who have long research experience in the field of rumen methane yield indicate that the cause-effect relationship between methanogens and protozoa is quite complex. They conclude that "…the absence of protozoa from the rumen microbiota did not systematically reduce methane production in

rumens (...). Some of the reasons could be explained by changes in the community of methanogens, which following defaunation seem to develop slowly as evidenced by the differences between recent and long-term defaunated animals." Moreover, other authors recently stated (Ye et al., 2018) that "Methane production can be uncoupled from methanogen abundance (Mosoni et al., 2011) and is not necessarily correlated with protozoal counts (Morgavi et al., 2012). Thus, inhibition strategies probably reduce cellular metabolic capacity of protozoa (not necessarily cell counts)."

Final conclusion and implications

In present thesis we confirmed the role of tannins in reducing the rumen ammonia and their potential role as additives to mitigate N excretions by the ruminants. However, the most relevant and new result is the depression of rumen protozoa caused by the use of *Stevia rebaudiana* Bertoni extract, which was demonstrated in several independent experiments. On the contrary, in two experiments, protozoa were favorite by camphor, bornyl acetate and eucalyptol essential oil. Unfortunately, we were not able to find a close relationship between *in vitro* variations of protozoa population and methane yield, as suggested by most part of the literature. However, the important result is that we found some plant compounds able to modulate the presence of protozoa in the rumen. This can have some important implications because it is possible that rumen protozoa have an ideal dimension in the rumen microbiota to assure an optimal ruminal function. In conditions of excess of protozoa the efficiency of rumen can be reduced by increment of excretions (ammonia and methane), whereas in situations of reduced presence (e.g. high concentrate diets) it has to be considered the absence of some positive effects of protozoa (engulfment of starch and mitigation of rumen fermentation and reduced biohydrogenation of unsaturated dietary fatty acids as recently demonstrated by Francisco et al., 2019).

In conclusion, further research efforts could be addressed to utilize such compounds as dietary additive suitable to adjust the development of the rumen protozoa population, according to the different dietary conditions.

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