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Ciclo XXXV

"**Rumen batch fermentation systems to measure methane yield**"

Dottorando

Dott. Matteo Braidot

Supervisore

Prof. Mauro Spanghero

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Abstract

Ruminants constitute an important element of the food supply chain representing approximately 30% of total global protein intake. Their breeding negatively impacts the environment and special attention is given to methane derived from the enteric fermentation process, which accounts for 17% of global methane generated by the agricultural sector and so accounts for about 29% of the world's methane emissions. Considering the rise of food demand in the next future, the study of strategies to reduce the global impact of the livestock sector is fundamental to improving its sustainability.

Since *in vitro* techniques represent a helpful approach in this sense, the main aim of this thesis is the study of new *in vitro* equipment for methane detection based on infrared technology. The equipment allows the continuous measurements of methane during the whole fermentation promoting an accurate study of methane kinetics and a precis evaluation of the total methane produced. Furthermore, the system can be used to test different factors that can affect total methane production representing a useful tool to meet the needs of the next future. Overall, the thesis is composed of three experiments that evaluate different aspects that can affect methane production.

The first experiment investigates the application of the *in vitro* equipment to the study of gas production (GP) and methane production (MP) kinetics obtain from different feeds. Barley meal (BM), alfalfa hay (AH), corn silage (CS), and soya bean hulls (SH) were used as substrates in four consecutive fermentation runs. Cumulative volumes of GP and MP and the percentage of methane on total GP were recorded continuously until 48 h. The average values at 1 h intervals were fitted with an exponential model with a lag phase. GP and MP reached the highest plateau levels for SH (1836 and 370 mL, respectively; $P < 0.01$) and the lowest for AH (1000 and 233 mL, respectively). The remaining substrates showed intermediate values. MP kinetics showed a discrete lag phase (from 0.09 to 1.12 h) whereas it was equal to zero for the total GP (except for SH). The methane concentration in gas flowing increased rapidly at the beginning of fermentation (from 0.35 to 0.95 /h) and reached a plateau after approximately 8-12 h. Overall, the rumen fermentation system generates methane

values comparable to those reported in the literature and has demonstrated that the GP and MP kinetics differ.

In the second experiment, the system was used to evaluate the efficacy of nitrate (NO_3) addition at different inclusion levels. The work's main topic is to study the effect of progressive inclusion levels of sodium nitrate ($NaNO_3$) on methane production. Four different NO_3 inclusion levels (2.5, 5, 7.5, and 10 % of DM) were used to assess the relationship between dosage and methane reduction over time. NO_3 addition reduced CH_4 yield in a dose-dependent response. Compared to the control, the 10% and 7.5% inclusion levels demonstrated a significant impact up to 48 h of fermentation (149 vs 276 mL and 217 vs 273 mL, $P<0.01$). The inclusion level of 5% showed a significant decrement in CH_4 production (195 vs 222 mL, $P<0.05$) until 24 h while the lowest dosage demonstrate a significant effect only in the first 12 h (158 vs 173 mL, $P<0.05$). Considering the total gas, the high inclusion levels (10% and 7.5%) showed a significant reduction to relative control (1118 vs 1381 mL and 1264 vs 1387 mL respectively, $P<0.01$), while the lower levels of additive (2.5% and 5%) not affected the total production after 48 h of incubation. At the end of fermentation, the measure of pH, ammonia, and protozoa counts in the fermentation fluid did not show differences between treatments. The treatment does not affect the production of VFA at the end of fermentation but alters their composition. The experiment confirmed the potential of $NaNO_3$ to mitigate rumen methane yield. However, the dose-effect results are dependent on the fermentation time. The impact of low doses is appreciable only at the beginning of the fermentation, while high doses can demonstrate a complete effect only at the end of the fermentation process. Assuming a duration length of 24 h, in our conditions, it can be estimated that a safe dose (1% DM) could reduce 5-10% of methane.

The last experiment is a meta-analysis that evaluates how protozoa number is relating to methane yield and rumen fermentation parameters. The study considers recent *in vitro* rumen batch experiments, where a significant change in the protozoa population was measured after the addition of different substances. Approximately 80% of the 46 trials from the selected 27 papers (for a total

of 201 dietary treatments) tested plant-derived substances, either alone or in mixtures, and 24 trials used rumen fluid from cattle and 22 from sheep. *In vitro* fermentations with sheep fluid used slightly larger bottles (113 *vs* 94 mL, $P<0.05$), but the inoculum volume, substrate amount, and fermentation duration (38.2 mL, 328.9 mg, and 32.3 hours on average, respectively) did not differ ($P > 0.05$). Only ammonia concentration in cattle fluids was higher compared to sheep (274.2 *vs* 137.3 mg/L, $P<0.01$). Within each trial, we calculated the percentage variation of protozoa compared to control bottles and the majority of the experimental treatments reduced the number of protozoa, by an average of -27.5%. The relationship between fermentation parameters and variations of protozoa was studied by linear regressions, adjusted for the trial effect. In general, protozoa variation in fermentation liquids did not affect total GP or total volatile fatty acids. Contrarily, a decrease in protozoa numbers was associated with a significant decrease in MP, both in absolute terms ($R^2 = 0.604$) and as a proportion of total gas ($R^2 = 0.528$). In terms of individual volatile fatty acids, there was a slight decrease in acetate ($R^2 = 0.298$), an increase in propionate ($R^2 = 0.388$), resulting in a decrease in the acetate to propionate ratio ($R^2 = 0.379$), and no effect of the protozoa on butyrate. Finally, there appeared to be a positive relationship ($R^2 = 0.580$) between protozoa counts and NH_3 concentration. In conclusion, rumen protozoa counts of *in vitro* batch systems are positively associated with methane and ammonia yields confirming the *in vivo* observations. This regression study predicts that the complete removal of protozoa involves a reduction of about 25 and 33% of total ammonia and methane yields, respectively. Batch fermentation systems appear to be appropriate for testing substances that can modulate protozoa counts and related fermentative characteristics including methane.

Summary

Introduction

The human population will increase from 8 billion to slightly less than 10 billion by 2050 with a consequent rise in the demand for resources, particularly food. The livestock sector is critical to the food supply chain; consider that ruminant breeding accounts for more than 30% of total global protein intake (FAO, 2009). These animals are widely farmed for their capacity to transform low-quality feeds into products with high biological value such as meat and milk. This capability is derived from the presence of the rumen apparatus where a complex microbial ecosystem converts the fibrous substrate and the low-quality protein into a suitable nutritional source for the animal. The ability to use feed sources with low biological values and not suitable for human consumption promotes ruminants as a possible candidate to respond to the increasing demand for food in the next decades.

On the other hand, the increment in the ruminants number reared worldwide has been demonstrated to harm the environment due to their emissions, particularly greenhouse gas (GHG) (Gerber, 2013). Special consideration is given to methane derived from the enteric fermentation process, which accounts for 17% of global CH_4 generated by the agricultural sector and so accounts for about 29% of world methane emissions (Lynch et al., 2021).

Methanogenesis is the biological process that leads to methane formation and takes place in the rumen to maintain a constant redox potential ensuring optimal conditions for the catabolic reaction that occurs during the fermentative process. The hydrogen generated during the degradation of carbohydrates is used by the methanogenic *Archaea* to reduce the carbon dioxide in methane that is released in the environment by eructation. This process is conditioned by several factors such as type and feed intake, genetics, and environmental conditions and represents a dietary energy loss that up to 12% that decreases the feed efficiency (Johnson et al., 1995).

Despite improvements in animal efficiency and the resulting reduction in methane generated per product unit, the overall absolute amount of methane produced is expected to rise. In light of climate change and future environmental sustainability goals, measures that allow for a reduction in

total CH_4 generated by ruminants are required. Given this objective, different strategies have been proposed and three different macro-areas could be identified: animal and feed management, diet formulation, and rumen manipulation (Arndt et al 2022). All of these areas share the requirements of technology for methane measurements and predictions.

Different methods have been proposed to assess methane production *in vivo* however these techniques present some disadvantages such as complex equipment, high cost, difficulty in results standardization, and the need of considering animal behavior and welfare. A valid alternative could be represented by the adoption of *in vitro* techniques that are widely diffused to assess the nutritive values of different feeds but could be also used for the methane assessment. These techniques ensure precise evaluation of the total methane, less labor and cost in virtue of a large number of treatments that can be tested simultaneously, and a reduction in the time needed for analysis. As a result, *in vitro* approach used for measuring methane produced during fermentative processes may be a useful tool for future research on this topic (Yáñez-Ruiz et al., 2016).

To summarize, the challenges associated with the environmental impact of the livestock sector, as well as the need for new tools to investigate potential solutions in this regard, have led to the current work. The main aim is to develop and evaluate the efficacy of a new *in vitro* batch system that allows a kinetic study of methane production through the continuous measurement of its production during the whole fermentation process. Secondarily, thanks to the application of *in vitro* techniques, the effectiveness of different solutions proposed to reduce methane output has been evaluated.

Thesis outline

The main topic of the thesis is to study some factors affecting methanogenesis in ruminants by the use of rumen batch fermentation systems. Investigations were performed in the laboratory of animal nutrition at the Department of Agricultural, Food, Environmental, and Animal Sciences of the University of Udine. This thesis is the result of three different research projects, with the main theme of methane production brought together through the application of a new *in vitro* batch system for the CH_4 assessment. Before the experimental part is reported, an introduction regarding the application of rumen batch fermentation systems is followed by a general biological concept about methanogenesis and a short description of dietary factors and strategies that might influence methane production in ruminants.

The initial experiment represents the first application of our *in vitro* infrared system for the continuous measurement of methane. The total gas and methane production of four different feeds was tested, and the kinetics obtained from the data were evaluated. The results obtained were presented orally at the 24th Congress of the Animal Science and Production Association (ASPA) in Padova. Following the presentation, a scientific paper was published in the Journal of Animal Physiology and Animal Nutrition.

The second experiment considers the application of the continuous measurement system to the study of anti-methanogenic additives efficacy. Several dosages of the same additive (sodium nitrate) were tested and the efficacy was evaluated over time. Moreover, a linear regression was performed at different time points between the CH_4 production and progressive $NaNO_3$ dosages to assess the methane reduction over time at other inclusion levels. Results obtained from this research will be presented in a scientific paper prepared for submission to a scientific journal.

The third study focuses its attention on the correlation between protozoa and methane. The recent scientific publications were analyzed and data collected were used in a meta-regression to evaluate the correlation between the main fermentative traits, including methane, and the number of

protozoa. The results achieved were published in the journal of Animal Feed Science and Technologies. The correlation founds shows a possible future application of the *in vitro* system to confirm the outcome of this study.

The appendix included some additional research and technical papers related to the thesis's overall topic.

The thesis concludes with a general comment on the results obtained and some thoughts on the possibility of a practical application of the scientific contribution of the works proposed in the previous chapters.

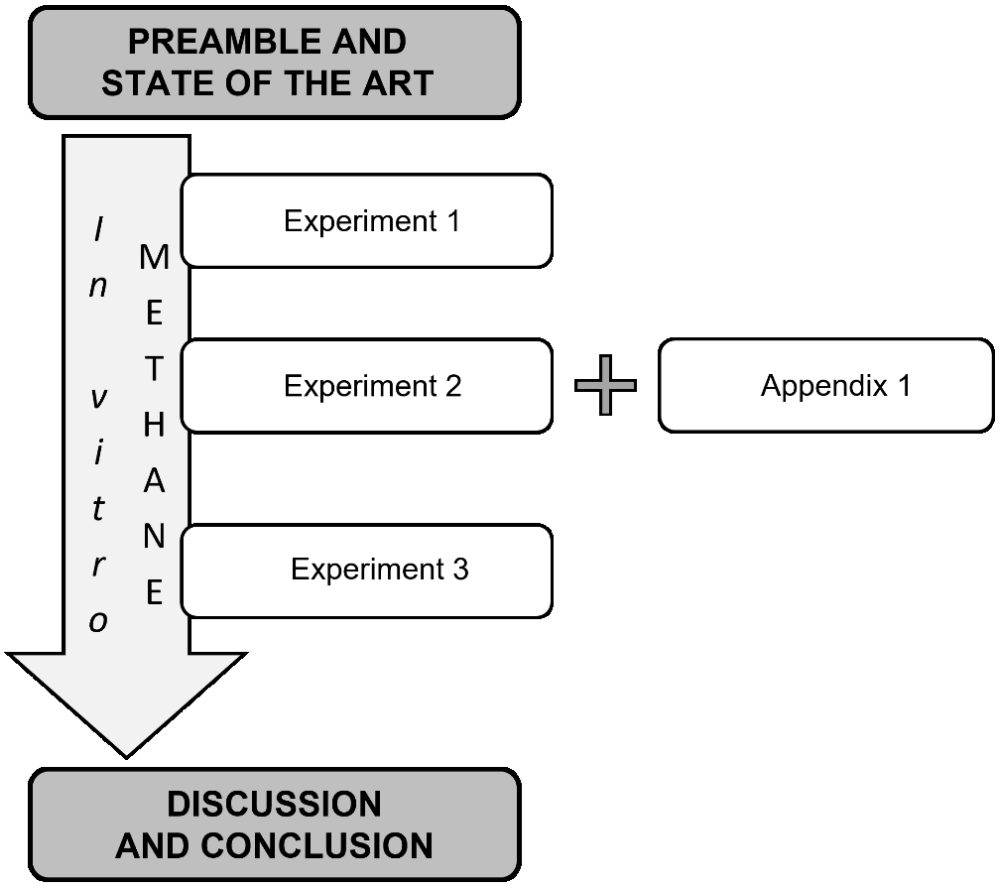

Figure 1 Schematic representation of thesis structure.

Chapter 1. State of the Art

1.1 Rumen methanogenesis: general concepts

The primary goal of the fermentative activity that takes place in the rumen environment is the generation of the energy required for the upkeep of the animal metabolic functions, as well as the preservation and growth of the microbial population. To achieve these objectives, several chemical processes must occur to convert the main macromolecules of the substrate into an available source of energy. Complex carbohydrates are degraded and utilized by rumen microorganisms for fermentative processes while feed proteins are brake down to amino acids and NH_3. At the same time, microbial proteins are synthesized and are available for digestion in the subsequent digestive tracts. The principal end products of fermentative processes in the rumen environment are volatile fatty acids (VFAs), CO_2, H_2 NH_3, and microbial biomass. In addition to the main reactions, other secondary processes related to the microbiological activity occur in the rumen (e.g. bio-hydrogenation of lipids, vitamin synthesis, and detoxification). Among these, methanogenesis follows the enteric fermentation process and reduces the available carbon dioxide into methane. This gas is eructated by the ruminants and is a relevant part of the GHG release in the atmosphere.

Throughout the digestive process, the carbohydrates from plants' origin are hydrolyzed in simple molecules that are used as a substrate by the microorganisms present in the rumen environment. During fermentation cofactors (NADH, NADPH, and FADH) are re-oxidized (NAD^+, $NADP^+$, FAD^+) producing hydrogen, which tends to accumulate in the rumen environment. The hydrogen concentration increment has an inhibitory effect on the metabolic pathway with a consequent reduction in microbial activity and to prevent this phenomenon, different hydrogen-consuming reactions take place. The main reaction that occurs is methanogenesis, which adsorbs approximately 80% of the total hydrogen produced during fermentation. This process plays a key role to maintain an optimal redox potential in the rumen ensuring optimal conditions for the reaction involved in metabolism.

Methane production can follow three different pathways: hydrogenotrophic, acetoclastic, and methylotrophic. These reactions differ in the source of methyl group used for the conversion to methane. Hydrogenotropic reaction uses carbon dioxide, acetoclastic utilizes acetate while the methylotrophic path employs methylated compounds. All these possible pathways for methane formation share several steps: demethylation of methyl-coenzyme M and the reduction of the heterodisulfide of coenzyme B and coenzyme M. Among all possible paths for methane formation, only hydrogenotrophic and acetoclastic might occur in the rumen environment. This second way appears to have a marginal role since in normal conditions the passage rate of the rumen is too high to allow the conversion of acetate to methane. (Jansen, 2010).

The major part of ruminants' methane is produced using the hydrogenotrophic pathway and Figure2 reports the complete set of reaction that occurs during the reduction of CO_2 to CH_4

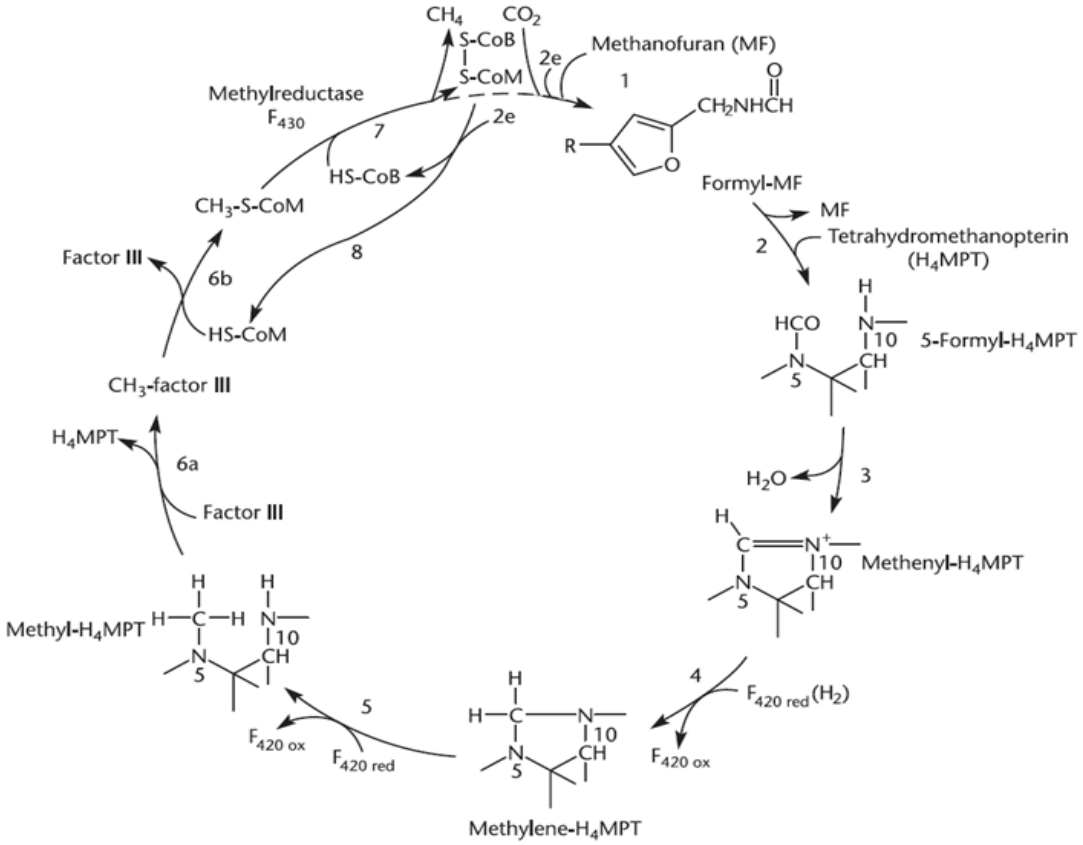

Figure 2 Reactions that occour during the carbon dioxide reduction to methane in the hydrogenotropic pathway of methanogenesis (Lessner 2009).

This process is mainly mediated by methanobacteria that belong to the *Archaea* family and to the genus *Methanobrevibacter* which utilizes this reaction to produce energy for their growth. As previously reported this process is necessary to guarantee optimal conditions for fermentative processes but it also generates an energy loss of around 2-12% with a consequent reduction in the efficiency of the feed conversion rate. Moreover, methane production represents an important point for the livestock sector given its environmental impact.

1.2 Rumen methanogenesis: strategies for its mitigation

The objective of reducing the global warming caused by GHG build-up in the atmosphere is a pressing global issue. Considering the contribution of farm ruminants to total anthropogenic CH_4 emissions there is a need to adopt breeding strategies suitable to attenuate the gaseous emissions from livestock units. In a recent meta-analysis, Arnd et al. (2022) analyze different mitigation solutions described and tested in 430 experimental reports. It results that methane reduction strategies can generate an absolute decrement in total production or a relative reduction expressed on the product base. Overall, approaches to reduce methane production can be classified into three main categories that are reported in Figure 3.

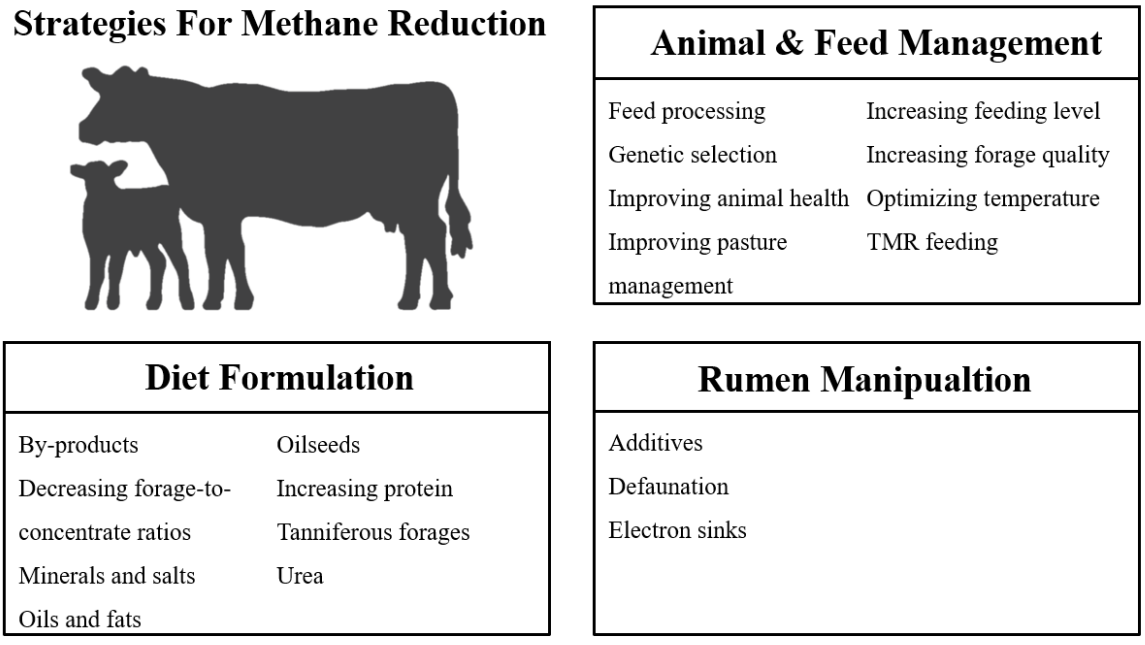

Figure 3 Principal categories of methane reduction strategies (Arndt et al., 2022).

The first group is represented by animal and feed management strategies that include solutions concerning the general management of livestock. The second category includes options that consider diet formulation, from general consideration about the raw materials and their balance in diets to the application of other substances. It was demonstrated that diet composition influences the fermentation activity in the rumen environment (Morgavi et al 2010, Knapp et al., 2014) and consequently affects also methane production as demonstrated *in vivo* (Gislon et al.,2020). Some examples of actions are decreasing forage/concentrate ratio or grass maturity, increasing dietary oils, fats, and oilseeds, etc. The last class consists in strategies of rumen manipulation, which are approaches with a direct effect on the rumen environment modifying specific microorganisms or metabolic pathways. Recently Tricarico et al. (2022) have reported that the majority of the feeding strategies (approximately 64%) failed to reduce enteric methane emissions. Only five choices between those proposed to reduce enteric methane production were effective without reducing milk output, and only three options have reduced emissions intensity while boosting animal productivity. This shows that identifying, developing, and validating efficient enteric methane mitigation methods that yield net emissions reductions for milk and beef production while also meeting farmers' and broader socioeconomic needs remains a major problem.

In the present thesis, attention is given to strategies that can be classified into the rumen manipulation category. These solutions reduce absolute methane production by modifying the rumen environment through the utilization of substances (e.g. chemical or natural additives) that influence directly the rumen microbiome (e.g. protozoa population) or that affect the metabolic pathway (e.g. alternative hydrogen acceptors). Since they generate an absolute reduction in methane production, the application of the *in vitro* techniques results be an appropriate methodology for their study.
Different substances realize mitigation through two principal modes of action. In the first one, the additive target directly the microbial population reducing the methanogenic *Archaea* abundance. In the second, additives alter the methanogenesis reaction affecting the enzymes involve or redirecting

the electron flow (Honan et al., 2021). Table 1 reports the most common substances that have been studied for their antimethanogenic capacity, classified according to their mode of action.

Table 1 General classification of antimethanogenic additives based on their principal mode of action (Honan et al., 2021)

Antimethanogenic Additives		
Effect on rumen microbiota	Effect on methanogenesis reaction	
Effect on methanogenic *Archaea*	Electron sink	Enzymatic inhibitor
• Plant metabolites • Antimicrobial agents	• Lipids • Nitrates • Organic Acids • Plant compounds	• Nitro-compounds • Organic acids • Plant metabolites • Halogens

Focusing the attention on additives that affect directly methanogenesis, a large number of substances that influence different steps of the reaction can be found and two main subcategories can be identified: enzymatic inhibitors and alternative electron sinks. To this last category belong substances such as malate and fumarate that can promote a metabolic shift, promoting reactions like propionate formation that compete with methanogenesis for hydrogen consumption. Other molecules that can work as alternative electron acceptors are lipids (Samal and Dash, 2021) or nitrate (van Zvijderveld et al., 2011). Since nitrate inclusion has been demonstrated to generate possible side effects at high inclusion level a more accurate investigation is needed. A more in-depth discussion will follow in chapter 3.

1.2.1 Nitrous dietary additives

In this category can be included nitroderivates, which are characterized by the presence of one or more nitro-functional groups, and nitrates. These molecules have demonstrated their ability to reduce methanogenesis through different mechanisms of action: altering electron transfer, influencing the methanogen population, and modifying enzyme activity.

Several nitrocompounds have been studied for their antimethanogenic capacity and particular attention is given to 3-Nitrooxipropanol (3-NOP). The principal effect of this compound is the modification of the mcrA gene expression. This gene encodes the coenzyme M reductase that catalyzes the last step of methanogenesis, the conversion of methyl coenzyme M to methane (Anderson et al 2003; Zhang et al., 2020; Yu e al., 2021).

Recently, Kebreab et al. (2023) have studied the overall effect of 3-NOP and its relationship with the nutrient profile of diet in dairy cattle considering the results from 25 *in vivo* studies. In synthesis, the metanalysis demonstrates that the inclusion of 70.5 mg/kg DM of 3-NOP generates a reduction of 32.7% of methane. Moreover, a negative correlation between its efficacy with NDF and crude fat content is observed while the starch content in the diet demonstrates a positive effect on the 3-nop antimethanogenic capacity. In addition to 3-NOP, other nitrocompounds like 2-Nitroethanol, Nitroethane, and 2-Nitroalcohol have demonstrated an impact on methanogenesis and they have been tested as a possible solution for methane abatement Zhang et al. (2018). Among them, 2-Nitrothetanol has a detrimental effect on methane production (-90%) suggesting an effect on several aspects of methanogenesis. Three main effects have been proposed: direct inhibition of methanogens, reduction of formate dehydrogenase activity, and possible alternative electron acceptor (Teng and Kim, 2021).

Another important group of nitro dietary additives is represented by nitrate (NO_3) which is used as an alternative electron acceptor redirecting the hydrogen flow. Nitrates supplementation represents a useful mitigation option in particular in the case of low-protein diets since nitrate can be used as a nitrogen source for nitrate-reducing bacteria (Fouts et al., 2022). Assuming a diet for

growing bulls or heifers contains 13% DM of crude protein, this corresponds to a nitrogen concentration of 2.08% DM. If the addition of NO_3 is 1.5%, it translates to a nitrogen supply of 0.33% which accounts for around 16–17% of the dietary crude protein content. If slow-release NO_3 is used, the amount of nitrate could be increased even further. The NO_3 can be provided by different sources from plants that contain it to salts (e.g. $NaNO_3$, $Ca(NO_3)_2$, KNO_3), and its origin affects its efficacy against methanogenesis (Latham et al., 2016).

The reaction of nitrate reduction to ammonium has a lower Gibbs free energy (ΔG^0) and consequently is thermodynamically favored over methane production (-600Kj/mol *vs* 136Kj/mol). During the reduction process, nitrate is converted to nitrite and then to ammonia as reported in the following reactions:

$$NO_3^- + H_2 \rightarrow NO_2^- + H_2O \qquad \Delta G^0 = -163.2\text{Kj/mol}$$

$$NO_2^- + 3H_2 \rightarrow NH_4^+ + 2H_2O \qquad \Delta G^0 = -436.4\text{Kj/mol}$$

A fundamental aspect to consider is the complete conversion of NO_3 to ammonium to prevent nitrite (NO_2) accumulation because this intermediate results to be toxic and this phenomenon decreases the nitrate efficacy against methane production. Nitrate supplementation has shown good results in methane reduction but the application of high nitrate levels in diets presents possible side effects on animal health. Nitrite can interact with hemoglobin causing the oxidation of the Fe atom of the eme prosthetic group from ferric (Fe^{2+}) to ferrous (Fe^{3+}) producing methemoglobin that is not capable to transport oxygen. The consequence of this phenomenon varies according to poisoning level but can range from feed intake depression and weight gain reduction to more serious symptoms like cyanosis or respiratory disease. To mitigate this adverse effect it is important an adaptation period to nitrate supplementation, this precaution reduces significantly the phenomenon (Lee and Beauchemin 2014). Considering the possible side effects, became important to investigate the relationship between the inclusion level of NO_3 and methane reduction. This was one of the arguments evaluated in this thesis

and will be presented later. Table 2 reports a list of recent papers that evaluate *in vitro* the antimethanogenic efficacy of NO_3.

Table 2 List of the article considered to evaluate the effect of nitrate supplementation, the nitrate source used in different trials, and their in-vitro conditions.

Author	Year	Nitrate source	Substrate (mg)	Volume incubated (mL)	Time (hour)
Alzarez-Hess et al.	2019	$Ca(NO_3)_2*4H_2O$	1000	100	24
Bozic et al.	2009	$NaNO_3$	200	10	24
Capelari et al.	2017	$5Ca(NO_3)_2*NH_4NO_3*10H_20$	500	50	48
Correa et al.	2017	$NaNO_3$	200	10	24
Guo et al.	2009	$NaNO_3$	200	40	72
Henry et al.	2021	$5Ca(NO_3)_2*NH_4NO_3*10H_20$	700	50	48
Lee et al.	2017	$5Ca(NO_3)_2*NH_4NO_3*10H_20$	500	60	24
Lin et al.	2011	$NaNO_3$	200	50	24
Lund et al.	2014	$5Ca(NO_3)_2*NH_4NO_3*10H_20$	500	75	48
Nguyen et al.	2016	$NaNO_3$	200	10	23
Patra et al.	2013	$NaNO_3$	400	40	24
Wu et al.	2019	$NaNO_3$	400	40	48
Yang et al.	2019	$NaNO_2$	200	50	24

Only recent papers where trials are conducted *in vitro* and that report methane, total gas, and other fermentative parameters have been considered. As can be noted, the trials utilize different NO_3 sources and the most common is $NaNO_3$. The data show that incubation conditions differ across studies and in general the duration of the fermentation process ranges from 24 to 72 hours.

Also, the amount of substrate incubated varied from 0.2g to 1g while the volume used for incubation is between 10 to 100mL. To perform an accurate evaluation of the results from different works, the standardized mean difference (SMD) between control and treatment is considered to compare the outcomes (Figure 4).

Each SMD is related to a specific confidence interval (CI) of 95% calculated using the standard error of a single trial. The same additive tested in two different experiments in the same scientific paper is considered as two different trials even if the same concentration has been used. The bubbles dimension represents the dimension of CI, large dimension depends on a greater CI. The trend obtained is a general reduction in methane production as expected but the efficacy varies significantly across studies. This result can depend on several factors such as fermentation conditions and the nitrate source. In addition, applied dosages vary in a wide range affecting the outcome. Moreover, it should be underlined that some of them are not suitable for their application *in vivo*. This result clearly suggests that nitrate addition causes a methane reduction but considering the dosages tested *in vitro* and the variability of the outcome further studies are needed to better understand the implications of its application.

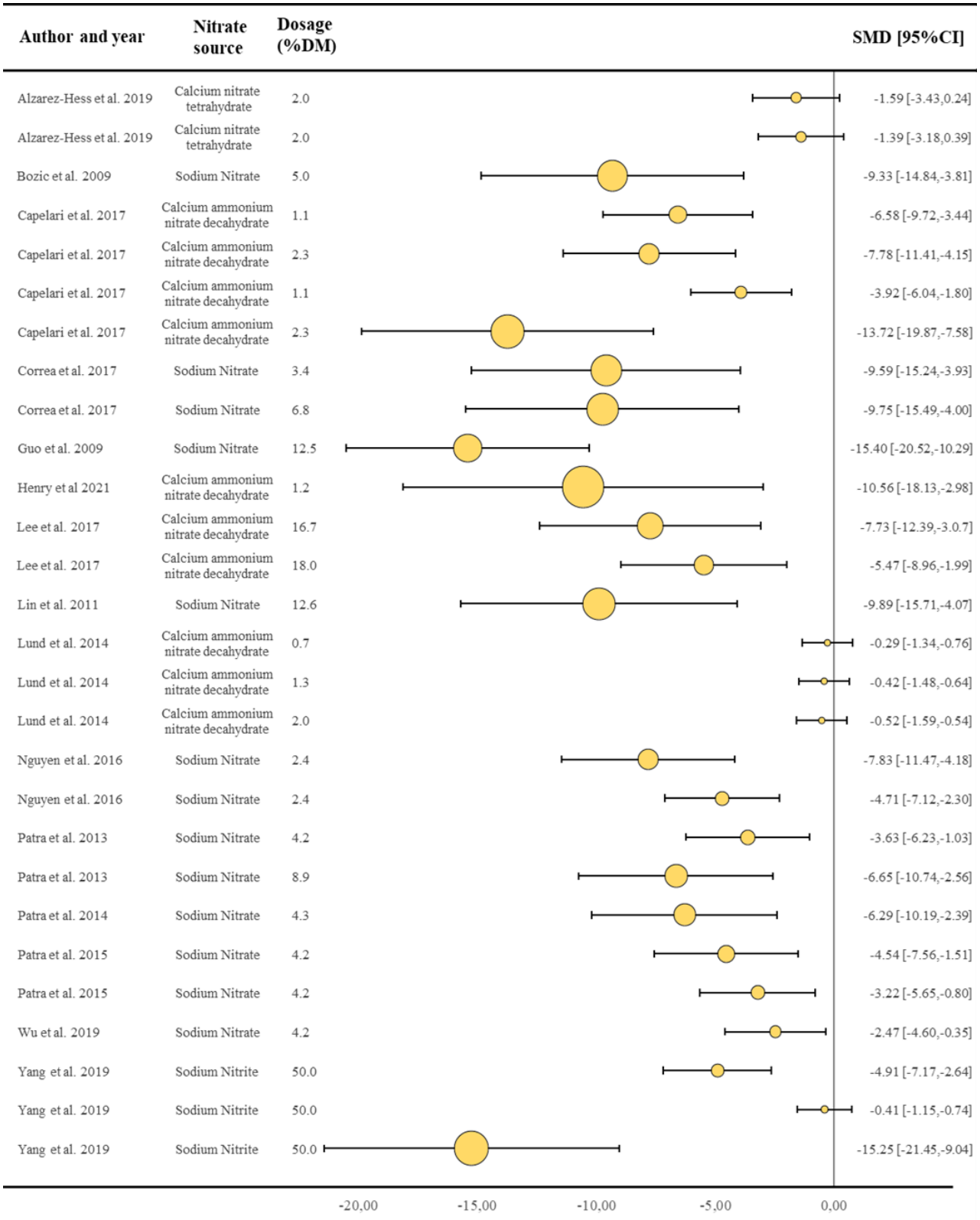

Figure 4 Forest plot of standardized means difference (SMD) and their 95% confidential interval (CI) obtained from the outcome of different trials that indicate the effect on methane production of nitrate addition. Bubble dimension is proportional to the inverse of CI. The zero value represents the control treatment in each trial.

1.2.2 Protozoal population control

The protozoa population is an important part of the rumen microbiota that represents 50% of the total rumen microbial mass (Newbold et al., 2015). Ciliates are the most abundant protozoa found in the rumen, ranging from 10^4 to 10^6 cells/mL of rumen contents.

They take part in fiber's brake down, engulfing starch granules, and act as predators toward other microorganisms playing an important role in the microbiome (Wright 2015). Protozoa are classified based on their morphological characteristics and two principal groups can be individuated: isotrichids and entodinomorphs (Patel 2018). In Figure 5 are reported images obtained with optical microscopy of common protozoa cells from different ruminal species in fermentation fluids.

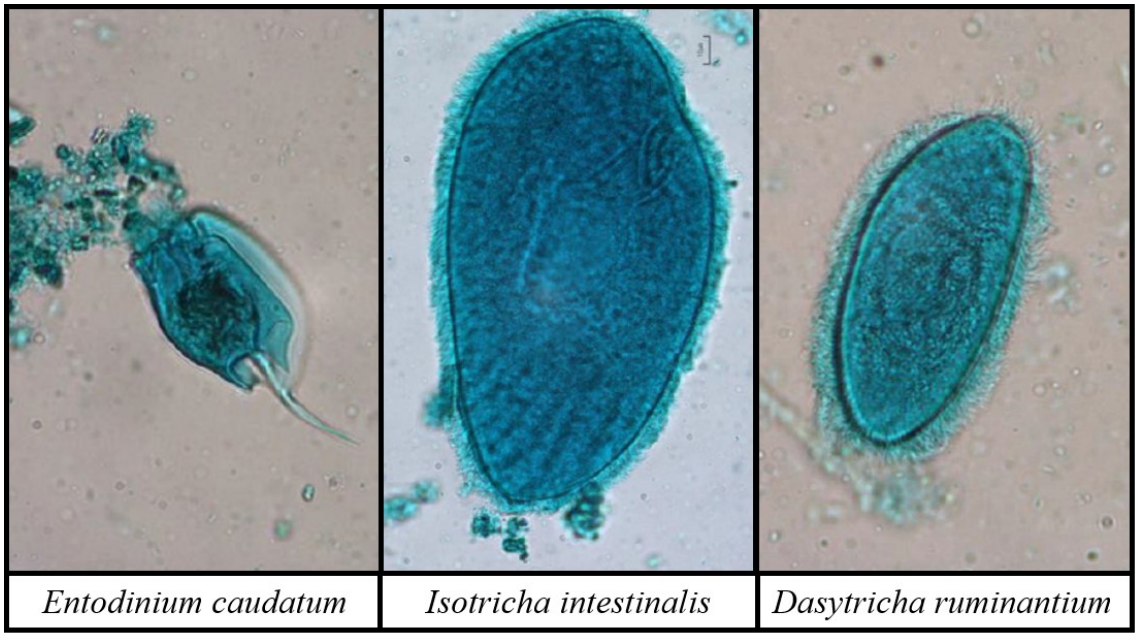

Figure 5 Different species of protozoa cell derived from different fermentation liquids.

Protozoa are responsible for the production of a high amount of H_2 since these microorganisms contain specialized organelles called hydrogenosomes that transform pyruvate into acetate, H_2, and CO_2. (Guyader et al., 2014) The acetate generated is used as an energy and carbon source while the H_2 and CO_2 are used by *Archaea* for methanogenesis (Holmes et al., 2014). Given this complex symbiosis between protozoa and methanogenic *Archaea,* the role of protozoa in methane production has been evaluated (Morgavi et al., 2011). The two main protozoa groups are differently correlated to total methane production, isotrichids have a more significant role since they have more

active hydrogenosomes compared to entodinomorphs and consequently generate more methane (Belanche et al., 2015). This is confirmed in a recent meta-analysis (Dai et al., 2022) that considers the role of the two main rumen protozoa groups on methane emissions in *in vivo* trials. The outcomes confirm the correlation between protozoa and methane and demonstrate that the isotrichids population is better correlated with methane production while the entodinomorphs play a marginal role.

Tannins, flavonoids, saponins, and essential oils are proposed to modify the rumen protozoa population since they have demonstrated antimicrobial activity (Ku-Vera et al., 2020,) but also lipids supplementation results as a possible solution to alter the protozoa population. Dai and Faciola (2019) evaluate the linkage between complete or partial protozoa defaunation on ruminal fermentation and animal performance. The results of 76 *in vivo* trials have been evaluated for this purpose. The outcomes show that ruminal protozoa play a fundamental role in the rumen affecting several aspects of the rumen environment as the ruminal ammonia concentration and fiber digestion. As regards the production of methane emerges a significant correlation with the protozoa population and a complete defaunation decreases the methane yield by around 18%. All these considerations underline the importance of these microorganisms in the rumen environment but even though their correlation with ruminal fermentation has been widely studied *in vivo* there is a lack of studies that evaluate this correlation *in vitro.* Further studies are needed to explore this association and with this aim, a meta-analysis based on recent publications that report a modification of the protozoa population *in vitro* has been realized. The results obtained are presented in an appropriate section (Chapter 4).

1.3. *In vitro* batch fermentation systems to measure rumen methane yield

The investigation of rumen metabolic processes and its microbiome plays an important role to understand better this complex environment and to develop practical feeding strategies to improve feed efficiency or to reduce emissions and excretions. Studies of rumen metabolism in *in vivo* animals have several constraints (e.g. costs, equipment, animal behaviors, and welfare concerns) while there is a growing interest to develop alternative methods. *In vitro* rumen techniques have been developed to overcome these problems indeed they are more rapid and simpler, less expensive, and give the possibility to test several samples using similar experimental conditions.

Feedstuffs are incubated with a microbial starter (rumen fluid) and a buffer solution to simulate the process that occurs *in vivo*. Microorganisms degrade and utilize the substrate for their metabolic processes with consequent development of microbial mass, gas, and fermentation acids. All the *in vitro* systems use a buffer solution and rumen inoculum and therefore these two main components influence the outcoming results. The incubation medium is crucial to maintain optimal environmental conditions for the fermentative process. The main purpose is to provide the correct amount of macro and micronutrients for the correct evolution of the fermentative process and to maintain pH in an acceptable range that does not compromise substrate degradation (Mould et al., 2005a). The ratio between the rumen inoculum and the buffer solution is important to prevent the accumulation of intermediates or end-reaction products that can have a feedback effect on the fermentative process. Moreover, the medium must promote the growth of ruminal microorganisms in the same way without promoting a particular group.

The other fundamental component of *in vitro* incubation is the microbial inoculum that is necessary to simulate the fermentative environment. This component represents the major source of variation in this technique since several factors can affect microbial activity (Mould et al., 2005b). First of all, the microorganism's abundance and activity depend on donor species and donor diets. It

is well known that diet influences the microbial population in the microbiome present in the rumen environment and consequently, it has a significant impact on fermentative performances. Secondarily, the origin of the rumen inoculum can alter the results that are obtained (Yang 2017). Finally, factor as sampling, inoculant manipulation, and storage has been demonstrated to impact the efficacy of rumen inoculum. Taking into account these factors plays a key role to standardize the conditions adopted for inoculum collection to minimize the variation across trials.

Batch culture systems represent a subclass of the *in vitro* rumen fermentation techniques. The main characteristic of these types of cultures is that all nutrients or additives are added at the beginning of the fermentation and during the whole process, there are no further additions. For this reason, usually, these cultures are commonly called “closed systems”. Given this characteristic, the fermentative process will be particularly efficient at the beginning and will tend to run out gradually since the reduction of substrate availability and the waste product accumulations. *In vitro* batch systems are largely applied to evaluate the total gas generates during fermentation. Since this parameter produced is directly correlated to the anaerobic digestion of feeds, this approach allows us to assess the nutritive value of feeds and classify them. The first *in vitro* system for gas production has been proposed by Czerkwski and Breckenridge (1975) and later developed by Menke et al. (1979). In the following years, different adaptations of the *in vitro* system have been proposed and Table 3 reports a summary of the most common.

Table 3 Summary of common methods used in the in vitro gas production technique and the general fermentation condition utilized (adapted from Rymer et al., 2005)

	Menke et al. 1979	**Blummel & Orskov 1993**	**Pell & Schoffiel 1993**	**Theodorou et al. 1994**	**Cone et al. 1996**	**Davies et al. 2000**	**Mauricio et al. 1999**
Fermenter	Glass Syringes	Glass Syringes	Bottle	Bottle	Bottle	Bottle	Bottle
Inoculum	Liquid phase	Liquid and solid phase	Liquid phase	Liquid and solid phase	Liquid phase	Liquid and solid phase	Liquid phase
Incubation volume(mL)	30	30	10	100	100	100	100
Inoculum volume(mL)	10	20	2	10	33	10	10
Container Volume(mL)	100	100	50	125	250	125	125
Buffer solution	Menke et al. (1979)	Menke et al. (1979) added with trypticase	Goering and van Soest (1970)	Theodorou et al. (1993)	Steingass (1983)	Theodorou et al. (1993)	Theodorou et al. (1993)
Sample (mg)	200-300	200	100	500-1000	400-500	1000	1000

An aspect that has been considered in batch culture is represented by gas accumulation. A lack of an adequate system for managing the gas produced during substrate degradation can generate a reduction in the rate of the fermentative process and a modification in the release of gas from the liquid fraction. To avoid this possibility the gas is periodically released (manually or automatically) from fermenters at a pre-determinate time point or if a pressure detector is available at pre-determinate pressure levels. This solution guarantees a more accurate determination of the fermentative potential of feeds (Yang 2017).

As can be observed in the table above, to simulate the rumen fermentative processes small quantities of buffer and substrate are commonly employed. The buffered rumen fluid ranges from 30 to 100mL while the substrate varies from 200 to 1000mg. The Ritter Company (Dr.-Ing. Ritter Apparatebau GmbH & Co. KG) proposes a fermentation system where higher volumes (500mL) can

be applied ensuring the possibility to test additives at low inclusion levels and facilitating the manual procedure. Furthermore, the apparatus is equipped with an automated mixing system that can be preset and with a gas counter (Ritter MGC-1, Ritter Apparatebau GmbH & Co. KG) that consents to continuous measurements of the outflow gas.

As previously described batch rumen systems are commonly applied to assess the feed quality and to test feed additives for ruminants. In the last few years, the application of these techniques to study methane production has been deepened. Since this approach presents different advantages when compared to *in vivo* methods and guarantees precise evaluation of methane yield, *in vitro* techniques are a useful tool for future research on this topic (Yáñez-Ruiz et al., 2016). Hatew et al (2015) have compared the methane production obtained from *in vitro* and *in vivo* trials. They evaluate the methane production of four dietary treatments *in vivo* and subsequently they use the rumen liquor of the animal employed in the *in vivo* trial to test the methane produces with the *in vitro* technique using the same diets. The methane estimated from the *in vitro* techniques is slightly different from that obtained with *in vivo* but a good correlation between them is founded. Recently, Danielson et al (2017) have confirmed this correlation. Using the *in vitro* technique, the methane produced for a total of 49 diets was assessed and compared with the methane produced from the same diets *in vivo*. The results show a very high correspondence (R^2=0.96) between *in vivo* and *in vitro* outcomes. These results demonstrate that *in vitro* techniques are suitable to study methane production in the rumen.

Usually in the *in vitro* systems, methane is quantified at the end of the fermentative process from a sample of total gas produced using gas chromatographic analysis. In this condition, the pressure could increase inside the fermenter causing a modification in gas solubility and representing a possible disruptor for the fermentative process. Moreover, the CO_2 dissolution causes an additional problem in the assessment of total gas produced and consequently also in methane estimation (Cattani et al., 2014; Alvarez Hess et al., 2018). Some authors hypothesize that *in vitro* CH_4 kinetics differs from that of total gas and underline the need to improve overall accuracy (Pellikan et al., 2011; Ramin and Huhtanen, 2012). These authors tested batch fermentation systems where gas was manually

sampled at different time points, more frequently at the beginning of fermentation, reducing also the pressure increment due to the gas accumulation. This approach allows taking into account the kinetics of methane production instead of end-point measurements. The automated batch fermentation system for gas and CH_4 measurement proposed by Muetzel et al. (2014) represents a further improvement of this type of approach. The apparatus is composed of small fermentation bottles equipped with a mechanized system of valves, pressure detectors, and devices for the management of outflow gas. The methane is detected automatically in the total gas with gas chromatographic analysis.

As can be observed throughout these different approaches, automated systems are becoming more used for methane quantification in *in vitro* systems since they allow several sampling points during fermentation.

The system studied in this thesis meets this need with the further improvement of a simplification of the outflow gas management and the application of an infrared sensor instead of a gas chromatographic. The Ritter gas analyzer (RI. sens mono IR1, Ritter Apparatebau GmbH & Co. KG) is a non-destructive analyzing system based on high-performance light-emitting diodes. It can be used to detect several gases, either individually or simultaneously. The device applied in our experiment is calibrated to detect only CH_4 in a concentration that ranges from 0 to 30% of the total gas and specific software (MarsTool version 0.0.127, Wi.Tec-Sensorik GmbH) permits data collection at different sampling times.

The system was previously tested with a standard gas sample to guarantee the accuracy of the measurements. This solution guarantees an accurate, rapid, and continuous assessment of methane during the whole fermentative process to ensure a precise kinetic study. A schematic representation of the equipment layout is reported in Figure 6.

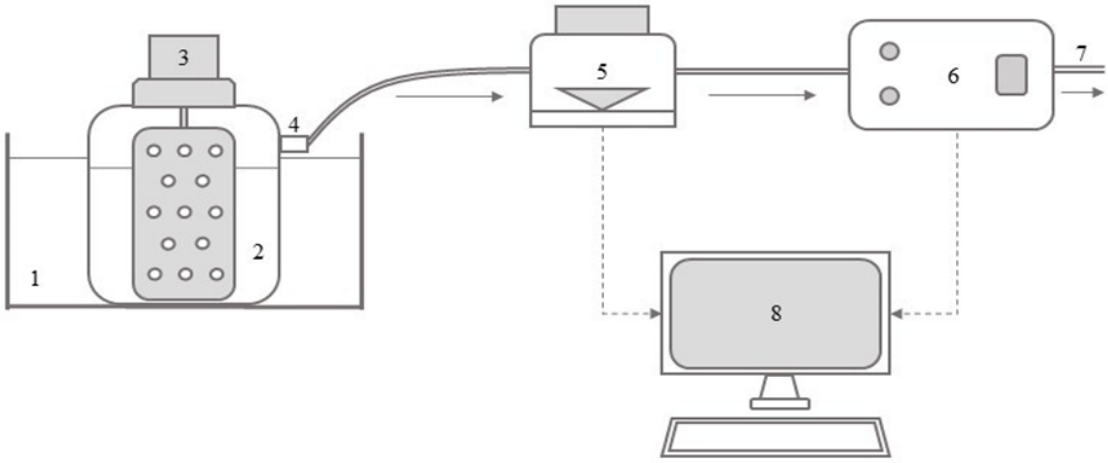

Figure 6 Schematic representation of the in vitro system proposed by Braidot et al., 2022. The layout of the fermentation system unit: 1, water bath; 2, fermentation bottle containing rumen fluid and buffer solution; 3, stirring device; 4, gas outlet hole; 5, gas flow counter; 6, methane infrared analyzer; 7, gas discharge; 8, Computer connected for the data collection. The arrows indicate the gas flow while the dashed arrows indicate the line of data acquisition.

Thesis aim

The thesis is composed of three distinct experimental contributions that apply *in vitro* methodologies to assess the effects of various conditions on total methane production. Following are reported the objectives of the various trials:

- The first trial aims to assess total methane generated from different substrates throughout the application of new equipment based on infrared (IR) technology to *in vitro* batch systems. The work wants to describe the batch fermentation system adopted and explain the relationship between the kinetics of methane production and that of total gas production.
- The second experiment aims to study the anti-methanogenic effect of nitrate ($NaNO_3$ used as a source) with the utilization of the *in vitro* batch system described and tested in the first experiment. The final goal is to consider progressive doses of nitrates and use a regression approach to predict the impact of nitrates at dosages low enough to be considered compatible with *in vivo* health conditions (e.g., around 1% of the substrate).
- The third experimental contribution is a meta-analysis that collects and analyzes recent *in vitro* rumen batch experiments where a significant change in the protozoa population was measured after the additions of different substances intended to modify *in vitro* rumen metabolism. The purpose is to determine how the protozoa number is related to methane yield and rumen fermentation parameters.

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SHORT COMMUNICATION

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A new equipment for continuous measurement of methane production in a batch in vitro rumen system

Matteo Braidot | Chiara Sarnataro | Alberto Romanzin | Mauro Spanghero

Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, Udine, Italy

Correspondence
Matteo Braidot, Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, Via Sondrio, 2/A, 33100 Udine, Italy.
Email: braidot.matteo@spes.uniud.it

Abstract

A new rumen batch fermentation system that allows continuous measures of total gas (GP) and methane production (MP) was tested. The fermentation system is composed of glass bottles connected to gas counters (Ritter Apparatebau GmbH & Co. KG) and an infrared gas analyser that measures the methane concentration. The system allows direct and continuous measurement of GP and MP for accurate kinetic studies. The aim of the work was to test the rumen fermentation system and compare the GP and MP kinetics obtained. Barley meal (BM), alfalfa hay (AH), corn silage (CS), and soya bean hulls (SH) were used as substrates in four consecutive fermentation runs. Cumulative volumes of GP and MP and the percentage of methane on total GP were recorded continuously until 48 h and average values at 1 h intervals were fitted with an exponential model with a lag phase reaching a good fit ($R^2 > 0.992$). GP and MP reached the highest plateau levels for SH (1836 and 370 ml, respectively; $p < 0.01$) and the lowest for AH (1000 and 233 ml, respectively). The remaining substrates showed intermediate values. MP kinetics showed a discrete lag phase (from 0.09 to 1.12 h), whereas it was equal to zero for the total GP (except for SH). The methane concentration in gas flowing increased rapidly at the beginning of fermentation (from 0.35 to 0.95 h^{-1}) and reached a plateau after approximately 8–12 h. In conclusion, the rumen fermentation system evaluated generates methane data comparable to those reported in the literature and allows simple continuous measurement of methane release throughout fermentation.

KEYWORDS

Gas production, In vitro fermentation, Methane, Rumen batch systems

1 | INTRODUCTION

Emissions of greenhouse gases, such as carbon dioxide and methane, into the atmosphere, cause global warming and serious damage to the planet's ecosystem. The livestock sector is responsible for 14.5% of anthropogenic greenhouse gas emissions, according to the Food and Agriculture Organization of the United Nation. Of these, ruminant enteric methane production account for 40% (Gerber et al., 2013). The EU recently agreed to reduce 36% of methane emissions by 2030 compared to 2005 levels (Commission of the European Community, 2020), and as a result, new feeding strategies to

A new equipment for continuous measurement of methane production in a batch *in vitro* rumen system

Matteo Braidot, Chiara Sarnataro, Alberto Romanzin, Mauro Spanghero

Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, Italy

Via Sondrio, 2/A, 33100 Udine, Italy

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Abstract

A new batch rumen fermentation system that allows continuous measures of total gas (GP) and methane production (MP) was tested. The fermentation system is composed of glass bottles connected to gas counters (Ritter Apparatebau GmbH & Co. KG) and an infrared gas analyzer that measures the methane concentration. The system allows direct and continuous measurement of GP and MP for accurate kinetic studies. The aim of the work was to test the rumen fermentation system and compare the GP and MP kinetics obtained. Barley meal (BM), alfalfa hay (AH), corn silage (CS) and soya bean hulls (SH) were used as substrates in four consecutive fermentation runs. Cumulative volumes of GP and MP and the percentage of methane on total GP were recorded continuously until 48 h and average values at 1 h intervals were fitted with an exponential model with a lag phase reaching a good fit ($R^2 > 0.992$). GP and MP reached the highest plateau levels for SH (1836 and 370 mL, respectively; $P < 0.01$) and the lowest for AH (1000 and 233 mL, respectively). The remaining substrates showed intermediate values. MP kinetics showed a discrete lag phase (from 0.09 to 1.12 h) whereas it was equal to zero for the total GP (except for SH). The methane concentration in gas flowing increased rapidly at the beginning of fermentation (from 0.35 to 0.95 /h) and reached a plateau after approximately 8-12 h.

In conclusion, the rumen fermentation system evaluated generates methane data comparable to those reported in the literature and allows simple continuous measurement of methane release throughout fermentation. Overall, because the GP and MP kinetics are different, the equipment tested is a potentially accurate metric for methane assessment.

Keywords: *in vitro* fermentation; gas production; methane.

Abbreviations: DM, dry matter; VFA, volatile fatty acids; GP, gas production; MP, methane production

1. Introduction

Emissions of greenhouse gases, such as carbon dioxide and methane, into the atmosphere, cause global warming and serious damage to the planet's ecosystem. The livestock sector is responsible for 14.5% of anthropogenic greenhouse gas emissions, according to the FAO. Of these, ruminant enteric methane production account for 40% (Gerber et al., 2013). The EU recently agreed to reduce 36% of methane emissions by 2030 compared to 2005 levels (European Commission, 2020), and as a result, new feeding strategies to reduce rumen methane emissions in livestock systems are a growing research topic.

Given the limitations of animal experiments, due to costs, time, ethical concerns, and standardization constraints, there is an increasing appreciation for experiments that do not involve animals, such as *in vitro* rumen fermentation experiments. The developed *in vitro* batch systems based on gas production (GP) and adapted to methane measurements are promising for testing many additives or comparing several dietary treatments as concerns their effects on rumen fermentability (in terms of GP) and methane production (MP) (Yáez-Ruiz et al., 2016). Pellikaan et al. (2011) and Ramin and Huhtanen (2012) suggested that the kinetics of methane production *in vitro* differ from that of GP and tested a manual batch fermentation system suitable for several samplings of gases.

In the apparatus for the simultaneous measurement of GP and MP, the gas accumulation in the fermenters can generate an increase in pressure causing a change in gas (CO_2) dissolution. This represents a potential disrupter in the fermentation process with a consequent complication in gas production assessment (Cattani et al., 2014; Alvarez Hess et al., 2018). Therefore, the methane concentration is measured at the end of fermentation in apparatus capable of accumulating gas in growing volumes (Menke and Steingass 1988) or from the gas that is regularly released from fermentation bottles in vented systems. Muetzel et al. (2014) first developed an automated batch fermentation system for methane assessment with bottles fitted with mechanized systems of valves, pressure detectors, and devices to provide gas venting and sampling.

Applying an infrared (IR) sensor to monitor MP in the outflowing gas throughout the entire fermentation process is a further advancement because gas sampling is not required and the continuous MP measure allows accurate kinetic studies. In the present work, we test a new apparatus based on IR detection, made up of independent units (fermentation bottles with gas counters and detector) installed in parallel to allow multiple simultaneous tests.

The aim of the work is to describe the batch fermentation system and to study the kinetics of MP in comparison to that of total GP. We hypothesize that the continuous measurement of methane in batch systems provides more accurate data than that obtained from a few sampling points during fermentation.

2. Material and methods

2.1. Apparatus description

The apparatus (Figure 1) is composed of fermentation glass bottles (total available capacity 750 mL) closed with an airtight cap equipped with a mixing system (rotation speed 18 rpm). Each bottle is connected with a flexible plastic tube (inner diameter 4 mm) to the gas counter (Ritter Apparatebau GmbH & Co. KG). This is composed of a bar immersed in oil which changes position at each entrance of 3 mL of gas and every bar movement was registered by a computer. After the volume measurement, the gas flows through a plastic tube into the infrared gas analyzer (RI. sens mono IR1, Ritter Apparatebau GmbH & Co. KG) for measuring the methane concentration. The analyzer is calibrated to detect methane concentrations from 0 to 30% of the total gas (accuracy ±2%) at temperatures from 5°C to 45°C. The infrared sensor works at pressures ranging from 800 mbar to 1200 mbar and it was previously tested with standard gas to guarantee measurement accuracy. When the MP was estimated, the volume of the connecting tubes (10 mL) combined with the volume in the upper part of the fermenter (250 mL) was considered as headspace volume (total headspace volume 260 mL).

In the present experiment, eight bottles were filled with filtered rumen fluid and mixed with the Menke and Steingass (1988) buffer (ratio 1:2, 500 mL in total). Substrates (3300 mg of DM) were

weighed and introduced into each bottle as ground and dry materials and then bottles were closed and immersed in a water bath at 39 °C for 48 h.

2.2 Substrates and fermentation runs

Four different feeds for ruminants, namely barley meal (BM), alfalfa hay (AH), corn silage, (CS), and soya bean hulls (SH), were tested in four consecutive fermentation runs lasting 48 h. Corn silage was dried (48 h at 60°C) and then all the feeds were milled and analyzed in duplicate for analytical DM, crude protein (CP), neutral detergent fiber (NDF; Mertens, 2002), and ash content according to the instructions of the Association of Official Analytical Chemists (1995).

The rumen fluid for all of the fermentation runs was collected in the same slaughterhouse in controlled conditions: mixed fluid was collected within 20 minutes of slaughter from 4 culled dairy cows fed with total mixed rations based on corn silage; no cow was slaughtered in an emergency; each cow was in good health, and all of the cows were transported from farms located within 50 kilometers of the slaughterhouse. The fluid was delivered, within half an hour of it being collected, to the laboratory in airtight glass bottles refluxed with CO_2 and maintained at 39° C

2.3 Fermentation fluid sampling and analysis

At the end of the incubation, pH was directly measured (GLP 22, Crison Instruments, S.A. Barcelona, Spain), while samples for NH_3 and volatile fatty acid (VFA) analyses were taken and stored at -20°C until the analyses were carried out.

Ammonia-nitrogen samples were thawed at room temperature and analyzed using an ammonia electrode (Ammonia Gas Sensing Combination Electrode, Hach Company, 2001). Samples for VFA analysis, to each of which a volume of 5 mL of 0.01 mol/L H_2SO_4 was previously added, were thawed at room temperature, centrifuged at 20,000 x g for 20 minutes at 4° C, and filtered using a polypore filter (0.45 mm, Agilent Technologies, Milan, Italy). The filtrate was injected into a high-performance liquid chromatography instrument (Perkin-Elmer, Norwalk, CN, USA) with its analysis wavelength set to 220 nm. The VFA concentration was measured as described by Martillotti and Puppo (1985).

2.4 Calculations and fitting

The cumulative MP was calculated using the following equation obtained by adapting to our system as suggested by Mengistu et al. (2017):

$$MP\ (mL) = \sum_{i=1}^{i=n+1}((C_{i+1} - C_i) * 260/100) + (\Delta V \times ((C_{i+1} + C_i)/2)/100),$$

In this equation, C_i and C_{i+1} are the methane concentrations measured at time i +1 and i, respectively, ΔV is the difference between the volume of gas (mL) produced at i +1 and that at i, and n is the total number of methane detections. The cumulative MP was calculated considering the variation of methane concentration and the increase in volume between hour intervals. Cumulative volumes of GP and MP and the percentage of methane in the total GP were recorded continuously for 48 h and average values at 1 h intervals were fitted with an exponential model with lag phase, specifically using the equation:

$$y = A \times \left(1 - exp^{(-k\times(t-L))}\right),$$

where y is the dependent variable at time t (h), A (mL) is the asymptotic GP or MP values, k (/h) is a rate constant of GP or MP production and L is the lag time (h).

2.5. *Statistical analysis*

The fermentation runs (n=4) were completed in sequenced periods (weeks) and data from two fermentation bottles within a run were averaged and the mean was used as a statistical unit (replicates among runs).

The pH, the amounts of NH_3 and VFAs, the relative percentage of single VFA, the kinetic parameters for GP, MP and methane percentage in GP, the total amount of methane calculated from concentration measured at the end of fermentation (48 h) and that calculated at multiple sampling points during fermentation (at 1, 2, 3, 4, 6, 8, 24 and 48 h) were statistically analyzed with a factorial randomized complete block (fermentation run) design using the equation:

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

Where Y_{ij} is the experimental data, μ is the overall mean, α_i is the random effect (block) of the fermentation run (i=1,4); β_j is the fixed effect of the substrate (j=1,4), and ε_{ij} is the residual error. Statistical analyses were performed with SAS software (Version 9.4, SAS Institute Inc., USA).

3. Results and discussion

In the present experiment, the rumen fluid used as inoculum in the different fermentative runs was collected at the slaughterhouse during four sessions. In each sampling, fluids from several healthy dairy cows fed similar diets were mixed to limit rumen inoculum variability. As a result, we obtained for some fermentative metrics, such as total gas or rate of gas production, a variability due to different collection sessions smaller (around one-fourth) than that attributable to various feeds.

The main aim of this work was to study the kinetics of MP and for this purpose; the total gas and methane were fitted without subtracting values from blank incubation. In fact, according to Cone (1998), blank values do not produce gas at the same rate as samples during incubations because microbial turnover begins earlier, and this phenomenon may cause an error in fermentation dynamics calculation.

The system tested differs from previously proposed in other scientific papers since the application of an infrared sensor allows direct and continuous detection of methane concentrations in the gas flowing without accumulation in the fermentation bottle. This prevents modification in CO_2 dissolution in the liquid phase and any disruption of the fermentation caused by increased pressure during fermentation, as demonstrated in the literature (Yang et al., 2002; Mould et al., 2005; Tagliapietra et al., 2010). This system appears convenient because it does not require complex equipment such as valves, pressure detectors, and related recording systems to manage outflow gas.

The proximal composition of feeds used the fermentative parameters after 48 hours of incubation and the kinetics parameters obtained for the different feeds are reported in Table 1.

Resulting from AH incubation, rumen fluid shows a higher ammonia concentration and pH after 48 hours of incubation ($P < 0.01$) compared to other feeds, owing to the high CP content.

The total VFAs concentration in the fluid resulting from the use of BM and SH (59.6-59.7 mmol/L) was approximately 20% higher than in the fluid resulting from the use of AH and CS (51.1-53.7 mmol/L). The VFA composition differed significantly between substrates, with high variations in the relative amount of butyrate (from 10.9 to 16.3% of the total VFA; $P < 0.01$), lower variations in propionate (from 12.7 to 15.3% of the total VFA; $P < 0.05$), and small but significant variations in acetate (from 65.2 to 68.9% of the total VFA; $P < 0.01$).

Representation of the kinetics is shown in Figure 2. Both the GP and MP kinetics were very well fitted with the exponential model described above ($R^2 > 0.992$). A discrete L for MP (from 0.09 to 1.12 h) was observed but no lag for the total GP was found (except for SH).

The kinetic parameters describing the changes in cumulative total GP and MP and the percentage of methane in the total gas produced are reported in Table 1. GP and MP plateaued at the highest levels when using SH as feed (1836 and 370 mL, respectively; $P < 0.01$), at the lowest for AH (1000 and 233 mL, respectively), and the remaining substrates showed intermediate values.

The asymptotic methane concentrations we observed ranged between 19.9 to 21.9% for the four feeds and were close to those obtained by Mengistu et al. (2017), who tested compound feed, grass, and corn silage at the same duration of *in vitro* fermentation. The concentrations we observed were slightly higher than the 17.0 to 21.1% values found by Pellikaan et al. (2011), who used various common types of feed for 72 h of *in vitro* fermentation. However, Maccarana et al. (2016) indicated that methane concentration measurements have been highly influenced by the methodological protocol adopted. These authors carried out a meta-analysis of 30 papers and showed the relative amount of methane in the total gas produced to be on average 17.0% but with very high variability (Standard deviation: ± 7.5% point) between the different experiments.

Navarro-Villa et al. (2011) measured the MP in straw, grass and grain and concluded that *in vitro* batch systems are not appropriate to rank feeds based on expected MP due to the biochemistry of rumen fermentation. In fact, the greater availability of H_2 for methanobacteria in fibrous feeds affects methane production (Ungerfeld, 2020). One possible explanation is that the *in vitro* systems are

highly buffered avoiding the pH drop, which normally occurs in *in vivo* conditions with starchy, feeds and is associated with reduced cellulolytic and methanogen activities.

The methane concentration in gas flowing changed during the fermentation: the percentage of methane increased rapidly (from 0.35 to 0.95 /h) initially and began to plateau at approximately 8-12 h. Furthermore, this process seemed to be substrate-dependent because plateaus were reached at different rates for the four substrates ($P < 0.01$).

The low relative methane amount detected at the beginning of the fermentation was consistent with the findings of Menci et al. (2021), who discovered a methane concentration of 9-10% after 3.5 hours of fermentation with an increase up to 28-30% after 24 hours. Colombini et al. (2020) measured only a slight increase in methane concentration (from 20-21 to 23-24 percent) during the second half of fermentation (from 24 to 48 h), whereas Muetzel et al. (2014) took 20–25 measurements of gas volume and composition, with more than 60% of the measurements taken during the first 12 hours of fermentation. These authors found a delay in the MP in the early stages of the fermentation, half of the total generated methane was produced approximately 3–4 h after half of the total generated gas was produced.

In the present experiment, a delay in the MP compared with GP was clearly observed and this phenomenon may be caused by several factors. The methanogenesis follows the hydrolytic attack of polysaccharides (in particular cellulose, hemicellulose and starch) by microorganisms and the metabolism of the resulting monosaccharides into VFAs and CO_2. While in *in vivo* and in continuous fermentation systems, there is a permanent supply of H_2 for the methanobacteria, in an *in vitro* batch system, the H_2 becomes progressively more available after the start of the polysaccharide fermentation.

Based on our experimental dataset, a comparison between the total methane calculated from a single measurement after 48 hours of fermentation (end point measure) and that obtained using multiple measurements (8 sampling points) were performed to assess the practical significance of delay in methane production. For end point measure, total methane was calculated using the concentration of

methane reached at the end of fermentation and the total gas produced, whereas for multiple measurements, the equation previously described was adopted. According to the results reported in Table 2, MP calculated from a single end-point measurement overestimates total methane produced by 5 to 28 percent when compared to MP calculated from multiple measurements. The overestimation error varied greatly between substrates, with BM having the lowest error (5%), CS and SM having intermediate error values (14-18%), and AH having the highest error (28%). Unfortunately, such variation in error makes it impossible to apply a constant correction.

In conclusion, the rumen fermentation system evaluated generates methane data comparable to those reported in the literature and allows simple continuous measurement of methane release throughout fermentation. Overall, because the kinetics of methane and total gas production are different, the equipment under study is a potentially accurate metric for methane assessment.

4. Animal Welfare Statement

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. The authors confirm that they have followed EU standards for the protection of animals used for scientific purposes.

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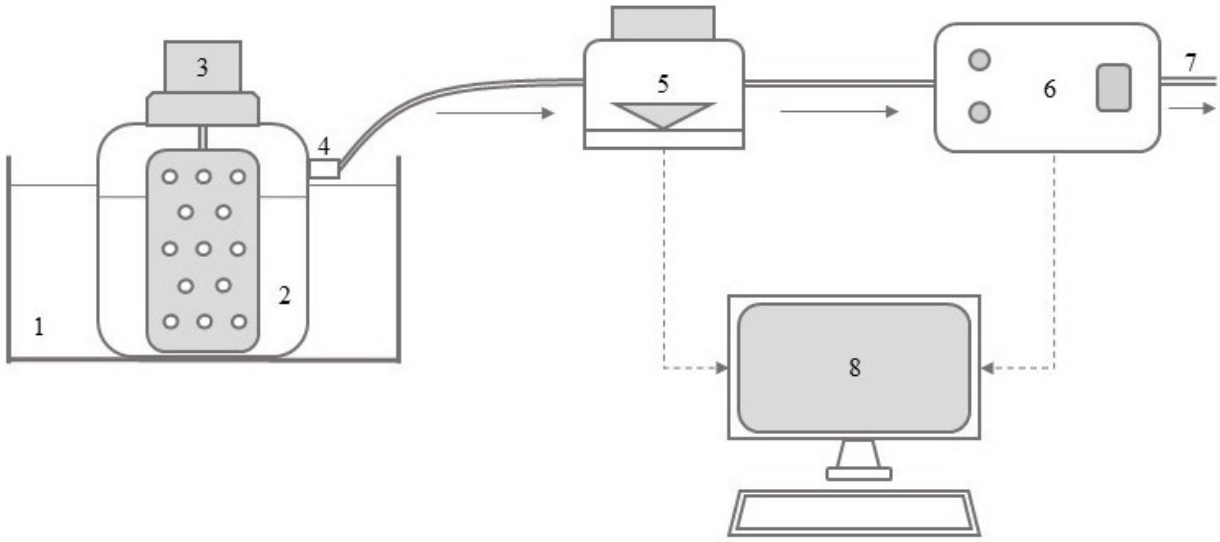

Figure 1. The layout of the fermentation system unit: 1, water bath; 2, fermentation bottle containing rumen fluid and buffer solution; 3, stirring device; 4, gas outlet hole; 5, gas flow counter; 6, methane infrared analyzer; 7, gas discharge; 8, Computer connected for the data collection. The arrows indicate the gas flow while the dashed arrows indicate the line of data acquisition.

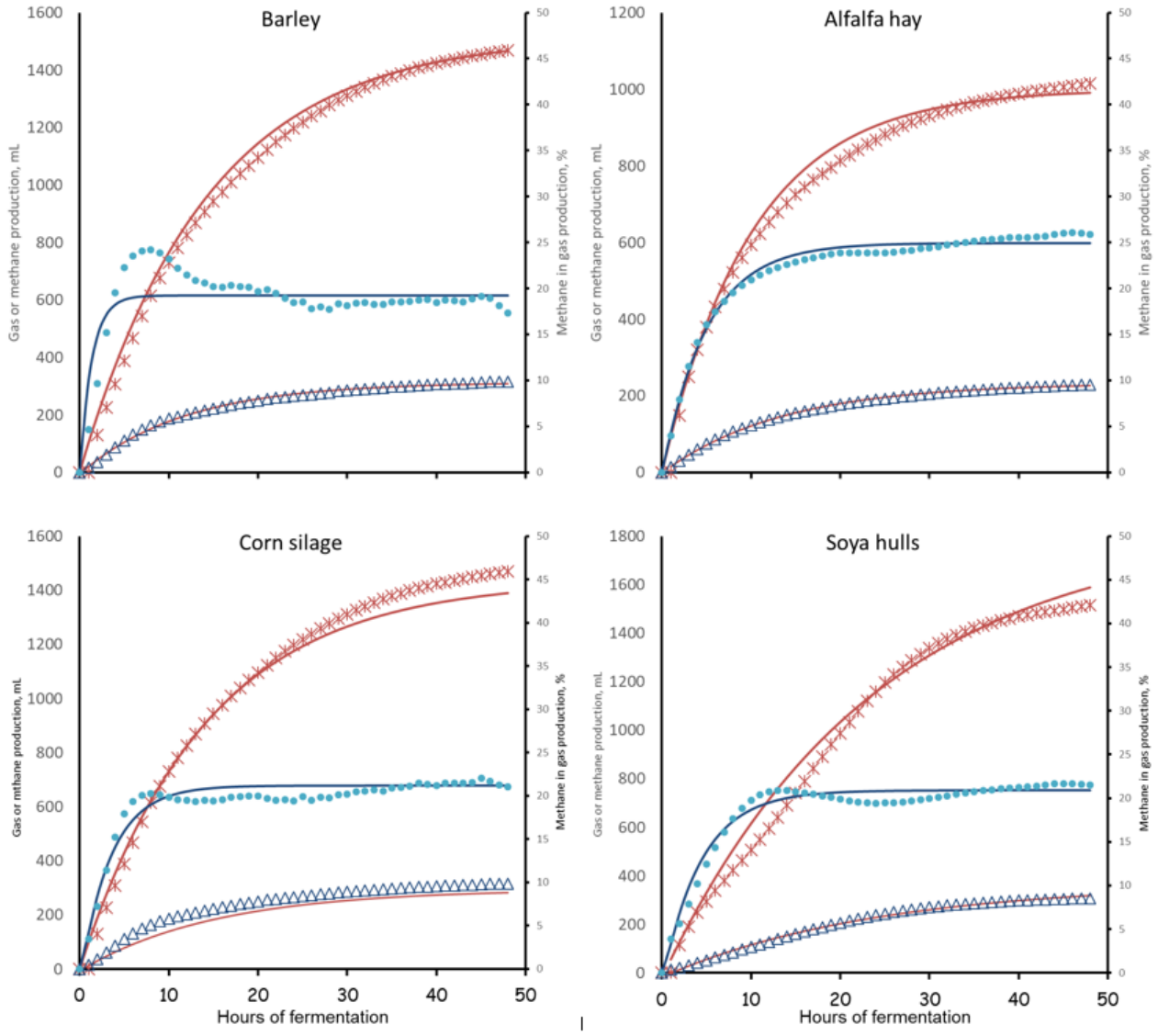

Figure 2. Kinetics (lines) and data points (symbols) of the cumulative volumes of gas and methane (left y-axis, Ɨ and Δ, respectively) and the percentage of methane in the total gas (right y-axis, ●).

Table 1. Chemical composition of substrates, fermentation data on fluids at the end (48 h) of fermentation (NH_3, pH, VFAs) and kinetic parameters of total gas and methane yields.

	Barley meal	Alfalfa hay	Corn silage	Soybean hulls	RMSE
Chemical composition:					
Crude protein(%DM)	12.4	18.3	7.6	13.3	
NDF (%DM)	17.9	48.5	36.5	67.3	
Ash (%DM)	2.4	9.4	3.9	4.7	
Fermentation parameters:					
pH	6.56^{B}	6.77^{A}	6.56^{B}	6.52^{C}	0.017
NH3 (mg dL^{-1})	52.0^{AB}	53.7^{A}	49.4^{C}	50.3^{AB}	1.124
Total VFA (mmol L^{-1})	59.62^{A}	51.06^{C}	53.71^{B}	59.72^{A}	1.537
% total VFA					
Acetate	65.18^{B}	68.95^{A}	65.50^{B}	68.67^{A}	1.274
Propionate	12.73b	13.80ab	14.63a	15.29a	0.880
Iso-butyrate	1.30	1.48	1.31	1.31	0.169
Butyrate	16.31^{A}	11.35^{C}	14.09^{B}	10.86^{C}	1.299
Iso-valerate	3.38^{ab}	3.74^{a}	3.57^{ab}	3.09^{b}	0.241
Valerate	1.12^{A}	0.97^{AB}	0.92^{BC}	0.76^{C}	0.101
Acetate:Propionate	5.22	5.06	4.52	4.54	0.369
Gas and methane production:					
Cumulative total gas					
A, mL	1520^{B}	1000^{D}	1437^{C}	1836^{A}	48.06
k, h^{-1}	0.070^{B}	0.098^{A}	0.071^{B}	0.042^{C}	0.008
Lag, h	0.000^{B}	0.000^{B}	0.003^{B}	0.278^{A}	0.096
R^2	>0.999	>0.999	>0.999	0.992	
Cumulative methane					
A, mL	314^{B}	233^{C}	295^{B}	370^{A}	16.52
k, h^{-1}	0.085^{A}	0.074^{B}	0.066^{B}	0.042^{C}	0.006
Lag, h	0.212^{B}	0.094^{B}	0.352^{B}	1.120^{A}	0.248
R^2	0.996	>0.999	0.998	0.994	
Methane (%)					
A, %	19.24^{B}	24.94^{A}	21.19^{B}	20.90^{B}	1.249
K, h^{-1}	0.743^{A}	0.200^{C}	0.293^{B}	0.230^{BC}	0.043
Lag, h	0.022	0.067	0.260	0.268	0.119
R^2	0.904	0.994	0.947	0.956	

a-b-c, A-B-C Within rows, means without a common superscript differ ($P < 0.05$, $P < 0.01$).

Table 2. MP was calculated using total GP and the percent of methane measured at the conclusion of fermentation (48 hours) or at multiple sampling points during fermentation (8 sampling points).

	Barley meal	**Alfalfa hay**	**Corn silage**	**Soybean hulls**	**RMSE**
Methane yield,					
Endpoint measure[1], mL	333^{B}	312^{B}	350^{AB}	385^{A}	22.4
Multiple measures[2], mL	316^{AB}	245^{C}	298^{B}	338^{A}	16.8
Difference, %	5.3^{C}	27.8^{A}	17.6^{B}	14.1^{B}	3.22

1 : total gas and methane measured at the end of fermentation (48 h).

2 : total gas and methane measured at 1, 2, 3, 4, 6, 8, 24, 48 h from the beginning of the fermentation.

$^{A\text{-}B\text{-}C}$ Within rows, means without a common superscript differ ($P < 0.01$).

In vitro evaluation of sodium nitrate as a rumen methane reducer

Braidot M.; Sarnataro C.; Spanghero M.

Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, Italy

Via Sondrio, 2/A, 33100 Udine, Italy

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Abstract

Methane reduction from livestock units seems to be one of the most important goals in future research in animal science. Recently, a new fully automated *in vitro* system based on an infrared sensor is proposed to study the methane production kinetic improving the ability to detect the anti-methanogenic effect of compounds in *in vitro* batch systems. In the present work, four different $NaNO_3$ inclusion levels were used to assess the relationship between dosage and methane reduction over time. Results have shown that NO_3 addition reduced CH_4 yield in a dose-dependent response. Compared to the control, the 10% and 7.5% inclusion levels demonstrated a significant impact up to 48 h of fermentation (149 vs 276 mL and 217 vs 273 mL, $P<0.01$). The inclusion level of 5% showed a significant decrement in CH_4 production until 24 h (195 vs 222 mL, $P<0.05$) while the lowest dosage demonstrate a significant effect only in the first 12 h (158 vs 173 mL, $P<0.05$). Considering the total gas, the high inclusion levels (7.5 and 10%) showed a significant reduction to relative control (1264 vs 1387 mL and 1118 vs 1381 mL respectively, $P<0.01$) while the lower levels of additive (2.5% and 5%) not affected the total production after 48 h of incubation. At the end of fermentation, the measure of pH, ammonia, protozoa counts, and total VFA production did not show differences between treatments. The experiment has confirmed the $NaNO_3$ potential to mitigate rumen methane yield and has shown that the effectiveness is both dose and time-dependent. The impact of low doses is appreciable only at the beginning of the fermentation while high doses demonstrate an effect on methane production during the whole fermentation. Assuming a duration length of 24 h it can be estimated that a safe dose could reduce 5-10% of methane.

1. Introduction

The livestock sector is critical to the food supply chain, but it has a consistent environmental impact due to greenhouse gas emissions (GHG). Methane from the enteric fermentation process accounts for 17% of global CH_4 generated by agriculture and 29% of global methane sources (Knapp et al., 2014; Lynch et al., 2021). Furthermore, CH_4 production is associated with a decrease in feed efficiency, with up to 12% dietary energy loss (Johnson et al., 1995). Reducing livestock industry emissions is a challenge for the near future, and several approaches have been proposed in this regard, including feeding strategies, rumen environment modifiers, and animal efficiency improvement (Buddle et al., 2011; Knapp et al., 2014).

Another possibility to reduce methane production is represented by the utilization of substances with an anti-methanogenic capacity (Bharanidharan et al., 2021; Honan et al., 2021). Nitrate represents an alternative electron acceptor deviating the hydrogen flow away from methanogenesis (Olijhoek et al., 2016). It is converted in the rumen to nitrite (NO_2) and then to ammonia reducing the hydrogen available for methane production. The utilization of substances that provide nitrate (NO_3) supplementation was tested in several *in vitro* studies at different inclusion levels (Patra et al., 2014; Alvarez-Hess et al., 2019; Wu et al., 2019; Almeida et al., 2022). The NO_3 supplementation impacts total methane production but its utilization must be carefully evaluated since its addition can cause possible side effects. In fact, during the conversion to ammonia, the toxic intermediate nitrite (NO_2) can accumulate and interact with the haemoglobin, altering its capacity to bind oxygen (Lee and Beauchemin, 2014). Nitrate poisoning represents a serious risk to animal health and its occurrence depends on several aspects such as NO_3 level in the diet, NO_3 consumption rate, and an incomplete conversion of NO_3 to ammonia. Considering this adverse effect, become important to investigate the impact of NO_3 at dosages able to produce a significant methane reduction without a secondary effect. *In vitro* rumen batch fermentation systems that allow CH_4 measurements are promising techniques to test a large number of additives and evaluate their effects on the fermentation process (Yáñez-Ruiz et al., 2016).

Recently, a new *in vitro* fermentation system that allows the study of methane production kinetics measuring continuously the gas and methane production during the whole fermentation process was tested (Braidot et al., 2022). This research assesses the efficacy of different $NaNO_3$ dosages over time throughout the application of an *in vitro* system suitable for the continuous measurement of methane. The innovative approach is the use of several regular dose levels of $NaNO_3$ to allow a regression study that could help predict the effects of nitrates at any low practical dietary dosage. Considering the *in vivo* adverse effect of high nitrate doses becomes important to investigate the efficacy of low NO_3 dosages able to produce a significant methane reduction, but sufficiently low to exclude secondary effects.

2 Material and methods

2.1 Experimental design

The $NaNO_3$ (Sigma Aldrich, Milan, Italy) was selected as the NO_3 source and different inclusion levels were used to assess the relationship between dosage and CH_4 reduction over time. It was added to the diet to achieve a final concentration of 2.5, 5, 7.5, and 10% of NO_3 on a dry matter basis. Each $NaNO_3$ dose was compared to a corresponding control (CTR) realized with appropriate additions of sodium chloride (Sigma Aldrich, Milan, Italy) and urea (Carlo Erba, Milan, Italy) to guarantee a comparable level of sodium and non-protein nitrogen. Table 1 reports the dosages of chemicals used both for treatments and controls.

Gas production was evaluated and fully automated equipment was adopted to detect CH_4 concentration during the whole fermentation process. The different dosages were tested in four consecutive runs of 48 h each.

2.2 In vitro experiment

The apparatus is composed of fermentation glass bottles (total available capacity 750 mL) closed with an airtight cap provided with an agitation system. The gas produced during the fermentation process flows from the fermenters to the gas counter (Ritter Apparatebau GmbH & Co. KG) which is

connected directly to a computer for data acquisition. Thereafter, the outflow passes through a plastic tube up to the infrared gas analyzer sensor (RI. sens mono IR1, Ritter Apparatebau GmbH & Co. KG) that continuously detects and registers the methane concentration.

The fermenters were filled with a 500 mL solution composed of filtered rumen fluid mixed with the Menke and Staingass (1988) buffer (ratio 1:2). The rumen fluid for all fermentation runs was collected in the same slaughterhouse in controlled conditions. Fluids from 4 culled dairy productive cows fed with total mixed rations based on corn silage were collected and mixed within 20 min from slaughter; no cow was slaughtered in an emergency; each cow was in good health, and all of the cows were transported from farms located within 50 kilometers of the slaughterhouse. The fluid was delivered, within half an hour of it being collected, to the laboratory in airtight glass bottles refluxed with CO_2 and maintained at 39 °C.

A total mixed ratio was used as substrates (3300 mg of dry matter) in all experiments. The substrate was grounded at 0.5 mm length, weighted, and introduced in each bottle as dry materials. The fermentation glass bottles were hermetically closed and incubated in a water bath at 39 °C for 48 h.

2.3 Sampling of fermentation fluid and analysis

At the end of the incubation, pH was directly measured (GLP 22, Crison Instruments, S.A. Barcelona, Spain), while samples of fermentation liquid for NH_3 (10 mL) and volatile fatty acid (VFA, 5 mL of rumen fluid added with 5 mL of H_2SO_4 0.01 N) determinations were collected and stored at -20 °C until the analyses. The NH_3 concentration was determined using an Ammonia Gas Sensing Combination Electrode (Hach Company, Colorado, USA). VFA samples were centrifuged at 20,000g for 20 min at 4° C, and the supernatant was filtered using a polypore filter (RC 0.45 µm, 25 mm, DTO Servizi Srl, Venice, Italy). The filtrate was transferred into auto sampler vials and 20 µL were injected into HPLC. The system was composed of an LC-20AT pump, a vacuum degasser, a Prominence SPD-M20A photodiode-array detector, a Prominence SIL-20AC HT auto sampler (20 µL loop) and a Prominence CTO-20AC column oven set at 40 °C (Shimadzu Corporation, Kyoto, Japan). The HPLC

separations were obtained using an Aminex HPX-87H column (300 mm x 7.8 mm) with a pre-column (Bio-Rad, Hercules, California, USA). The mobile phase used is sulphuric acid 0.008N at a flow rate of 0.6 ml min^{-1}. Full spectra were recorded in the range of 190–400 nm and the optimum wavelength detection for all VFA was found to be 220 nm. VFA standards of acetic acid, propionic acid, butyric acid, iso-butyric acid, iso-valeric acid, and valeric acid were obtained from Merck (Darmstadt, Germany). Peaks of analytes were compared with the retention times of a standard mixture and quantification was based on the external standard method.

2.4 Calculations and statistical analysis

The fermentation runs were completed in sequenced periods (weeks) and the main fermentative parameters were statistically analyzed with SAS software (Version 9.4, SAS Institute Inc., USA) with a multifactorial-factorial model design as:

$$Y_{ijk} = \mu + a_i + \beta_j + y_k + \varepsilon_{ijk}$$

where Y_{ij} is the experimental data, μ is the overall mean, α_i is the random effect (block) of the fermentation run (i=1,4); β_j is the fixed effect of the dose (j=1,4); γ_k is the effect of the type of addition (k=1,2; nitrate in treated fermenters and urea and NaCl in control fermenters, TRT and CTR, respectively) and ε_{ijk} is the residual error.

The linear effect of dose (X) was tested at different times of fermentation (6, 12, 24 and 48 h) separately for each of the two types of additions (TRT and CTR) on the methane yield (Y). The MIXED procedure of SAS Software was used as described by St-Pierre (2001) and the model applied was the following:

$$Y_{ij} = a + bX_i + Run_j + \varepsilon_{ij}$$

Where Y_{ij} is the methane produced for the inclusion level i of the j run, a represents the overall intercept, b is the overall regression coefficients, X_i is the level of nitrate inclusion (i = 82, 165, 247, and 330 mg), Run_j is random effect of the run (j = 1,4), and ε_{ij} is the residual error. The coefficient

of determination (R^2) was obtained from a single-factor regression analysis between the adjusted values derived from the mixed model and the measured values.

3. Results

Table 2 reports the principal fermentative parameters at the end of the fermentation process (48 h). The final pH increased with the dose level (P<0.01) independently from the NO_3 addition (CTR *vs* TRT). Ammonia, total VFA concentration as well protozoa counts in the fermentation fluid were not influenced by the NO_3 addition and neither by the different dosages.

Contrarily, there was a modification of the fermentation patterns. The acetate as the proportion of total VFA was significantly affected by NO_3 addition (*P*<0.01), dose (*P*<0.05), and their interaction (*P*<0.01). At the lowest dose, the concentration was similar between TRT and CTR, while there was a significant increment for TRT compared to CTR at 5.0 (67.8 *vs* 62.4%), at 7.5 (67.9 *vs* 62.9%), and 10.0% DM dose (68.1 *vs* 61.9%). Furthermore, propionate showed a lower proportion for TRT compared to CTR (on average 16.2 *vs* 17.5%, *P*<0.01) for each dose tested. Butyrate showed a significant effect of NO_3 addition (*P*<0.01) and also a significant interaction between NO_3 addition and dose (*P*<0.01). This is due to the concentration of butyrate in TRT and CTR which is similar for the lower dosage while it tends to decrease for TRT than CTR at higher doses. Finally, NO_3 addition affects significantly (*P*<0.01) the A:P ratio increasing its value for TRT compared to CTR at 5.0 (3.60 *vs* 4.47), at 7.5 (3.57 *vs* 4.15), and 10.0% (3.53 *vs* 4.05) inclusion levels.

Table 3 reports the total gas and CH_4 production at different time points. The final gas production was significantly affected both by NO_3 addition, dosage, and their interaction. The lower dosage showed a significant decrement only in the first 6 hours of fermentation (564 *vs* 538 mL, *P*<0.05) while the other dosages tested affected this fermentative trait longer. The 5% DM dosage impacts the total gas until 18 hours (983 *vs* 1022 mL, *P*<0.05) and the remaining dosages influence the total production during the whole fermentation process (1264 *vs* 1387 mL and 1118 *vs* 1381 mL for 7.5 and 10% DM respectively, *P*<0.01).

A similar trend was observed for total methane production, where the NO_3 addition reduces CH_4 yield in a dose-dependent response. CH_4 reduction peaked between 12 and 24 h after the beginning of fermentation and then gradually decreased for doses up to 7.5% DM. Compared to the corresponding CTR, the lowest dose demonstrated a significant effect only in the first 12 h (158 *vs* 173 mL, $P<0.05$) while the 5% DM level showed a significant decrement in CH_4 production (195 *vs* 222 mL, $P<0.05$) until 24 h. For the highest dose, the difference with control progressively increased up to 30 h and then was stable in fact the dosage of 7.5 and 10% DM demonstrated a significant impact up to 48 h of fermentation (217 *vs* 273 mL and 149 *vs* 276 mL respectively, $P<0.01$).

Figure 1 and Table 4 show the results of the linear regression analysis between the CH_4 production and the progressive inclusion level of $NaNO_3$ for each time considered. Regressions of the CTR bottles were not significant due to the absence of any effect due to the urea and NaCl addition whereas the regressions for TRT bottles were significant for all the fermentation times considered.

4. Discussion

Sodium nitrate ($NaNO_3$) was selected for the experiment because, has shown the highest efficacy against methanogenesis among salts that contain NO_3 (Latham et al 2016).

The present work is designed to estimate by a regression approach the impact on the *in vitro* rumen fermentation of low nitrate dosages (e.g., around 1% DM), assumed compatible with *in vivo* health conditions. The continuous measurement of gas and methane permits the assessment of the nitrate effect throughout the whole *in vitro* fermentation. Finally, the use of specific control fermenters for each dose tested allows us to take into account the effect of the progressive nitrogen and sodium inclusion caused by the sodium nitrate addition in treated fermenters.

4.1 Fermentative parameters

Usually, the reduction of NO_3 generates an increment in the NH_3 concentration (Bozic et al 2009; Patra et al. 2015 Nguyen et al 2016), but in the present experiment, no increase was found even at the highest concentrations. In contrast, Guo et al. (2009) tested *in vitro* different dietary treatments (including $NaNO_3$) and found a reduction in the NH_3 concentration but an increment in the microbial

nitrogen. The NO_3 reduction to ammonia follows two main steps: NO_3 reduction to NO_2 and subsequently NO_2 reduction to NH_3. The rumen environment conditions might alter this reaction pathway with a consequent accumulation of the intermediate NO_2 instead of a complete conversion to NH_3 (Yang et al 2016). Furthermore, the presence of nitro-reducing bacteria able to use NO_3 as a nitrogen source can decrease the amount available to be converted into NH_3 with an impact on its final concentration (Latham et al 2016).

Anyway, the pH showed a small but significant increase with the NO_3 additions as a result of the buffering effect of NH_3 and other compounds upon the fermentation liquid acidity.

The NO_3 addition was associated with a shift in the VFA profile, namely an increase in acetate and a decrease in propionate. These results are consistent with previous findings both from *in vitro* and *in vivo* studies (Li et al., 2012; Lin et al., 2011; Nolan et al., 2010). The NO_3 reduction reaction is favoured over propionate synthesis since is thermodynamically more favourable (van Zijderveld et al., 2010). Moreover, the increment in acetate production generates a further decrease in the H_2 available for propionate production (Nolan et al., 2010) and a similar effect was also observed on butyrate. The NO_3 supplementation causes a decrement in butyrate proportion and this outcome is in agreement with the results of Lin et al. (2011).

4.2 Gas and methane

In general, the NO_3 additions generate a decrement in the total gas produced at the end of fermentation. The lower doses determined a reduction only in the first hours of fermentation while the higher doses determined a depletion of gas during the whole fermentation. From the regression analysis results that in our conditions the inclusion of 1% of NO_3 generates a reduction in total gas of 2.5 % after 48 h of fermentation. Depression of the rumen fermentative process caused by NO_3 supplementation has been observed both *in vitro* (Guo et al., 2009) and *in vivo* (Zhao et al., 2015). The main reason for this phenomenon is related to the negative effect of NO_3 on the microbial population (Sakthievel et al 2012; Patra et al 2013, Yang et al 2016).

At the lowest NO_3 dose, the CH_4 was significantly lower than the respective CTR within the first 6 h of fermentation and at the medium dose of 5.0 % within the first 12 h. For longer fermentation lengths, the difference with the CTR progressively diminished and was not significant.

Overall, these variations can be explained by taking into account the balance between the H_2 generated during fermentation and the available NO_3. For doses of 2.5 and 5.0% DM, H_2 consumes all the available NO_3 in 6 or 12 h respectively while 24 and 30 h are required for doses of 7.5 and 10% DM. It means that at short times the limiting factor is the H_2 availability but as fermentation proceeds, the limitation becomes the NO_3 accessibility. However, the fermentation time at which the limitation shift from H_2 to NO_3 is dose-dependent: it is included within 6 h of fermentation for the two lowest dosages, while it requires 24 h for the 7.5 % DM dose and 30 h for the 10 % DM dose.

The previous consideration help to understand the outcomes of the regression analysis calculated at four distinct fermentation times (e.g. 6, 12, 24, and 48h, table 4 and figure 1). It appears that the dose-effect increases according to the fermentation time considered.

From a stoichiometric calculation, 1 mg of NO_3 completely transformed in NH_3 allows a reduction in CH_4 yield of 0.409 mL. Considering the equation obtained from linear regression at different times, the slope achieved is very lower than the expected value for the 6h fermentation (0.069 mL/mg). The slope increases to 0.226 mL/mg at 12 h and at 24 h it is close to that expected (0.407 mL/mg). A further increase in fermentation time overestimates the CH_4 reduction (0.517 mL/mg).

The variation described corresponds to previous observations on the time dependency of this phenomenon. Our interpretation is that at large doses, the limiting component for short fermentation times is H_2, while for low doses the limit is NO_3 and this results in a low slope value for the regression line. On the contrary, NO_3 is the limiting component during long fermentation times for low doses but not for high doses and this causes a large drop in the line, as a consequence low doses have to be tested for short fermentation times, and high doses for long fermentation times.

Given the conclusion, 24 hours appears to be a realistic time to evaluate the methane reduction in our circumstances, and the effect of a nitrate addition of around 1% of the fermented substrate (e.g. 33

mg/bottle) was estimated in methane of about 5%. In the review of Lee and Beauchemin (2014), it is reported that around 1% DM of NO_3 can be considered a safe *in vivo* dosage. However, our *in vitro* data were not corrected by blanks and the concentration calculated on the substrate added does not represent the actual concentration of NO_3 for the whole fermentable material within the fermentation bottle. We estimated that the supply of material from the rumen inoculum is almost equivalent to the substrate added and therefore according to this calculation the expected reduction will be double (e.g. -10% in CH_4 yield).

5. Conclusion

The experiment confirmed the potential of NaNO3 to mitigate rumen methane yield. However, the dose-effect results are dependent on the fermentation time. The impact of low doses is appreciable only at the beginning of the fermentation, while high doses demonstrate an effect during whole the fermentation process. Assuming a duration length of 24 h it can be estimated that a safe dose could reduce 5-10% of methane.

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Table 1 Dosages of the chemicals used in the treatments tested

	Sodium nitrate dosages (% DM)							
	2.5		5.0		7.5		10	
	CTR	TRT	CTR	TRT	CTR	TRT	CTR	TRT
Chemicals (mg/bottle)								
Urea (CH_4N_2O)	39	-	79	-	118	-	158	-
Sodium Cloride (NaCl)	76	-	152	-	228	-	305	-
Sodium Nitrate ($NaNO_3$)	-	112	-	224	-	336	-	448
Nitrate (NO_3)	-	82	-	165	-	247	-	330

Table 2 Main fermentative *in vitro* traits achieved for the different inclusion levels of nitrate and corresponding controls at the end of the fermentative process (48 hours)

	Dosage (%DM)								NO_3	Dose	NO_3*Dose
	2.5		5.0		7.5		10				
	CTR	TRT	CTR	TRT	CTR	TRT	CTR	TRT			
pH	6.71	6.71	6.73	6.75	6.75	6.76	6.76	6.75	NS	**	NS
NH_3	67.6	66.9	71.1	74.3	71.6	74.9	76.7	72.4	NS	NS	NS
Total protozoa 10^3 cell/mL	86.0	77.2	82.4	80.4	86.8	95.2	90.0	91.6	NS	NS	NS
Total VFA (mmol/L)	79.5	92.4	86.6	94.0	89.4	86.4	91.6	94.9	NS	NS	NS
% total VFA											
Acetate	62.6	63.9	62.4^{B}	67.8^{A}	62.9^{B}	67.9^{A}	61.9^{B}	68.1^{A}	**	*	**
Propionate	17.1	16.2	17.4^{A}	15.2^{B}	17.7	16.4	17.7	16.9	**	NS	NS
Iso-butyrate	1.07	1.09	0.96	0.91	0.95	0.90	0.98	0.85	NS	*	NS
Butyrate	13.9	13.2	13.8^{A}	11.26^{B}	13.3^{A}	9.67^{B}	14.0^{A}	8.98^{B}	**	**	**
Iso-valerate	4.00	4.22	4.03^{a}	3.68^{b}	3.90	3.98	4.06	3.98	NS	NS	NS
Valerate	1.19	1.20	1.29^{a}	1.08^{b}	1.23	1.10	1.23	1.09	**	NS	NS
A:P	3.76	3.96	3.60^{B}	4.47^{A}	3.57^{B}	4.15^{A}	3.53^{B}	4.05^{A}	**	NS	NS

a-b-c, A-B-C Within rows, means without a common superscript differ ($P < 0.05$, $P < 0.01$)

Table 3 Total gas and methane generated from nitrate addition and corresponding controls at different time intervals

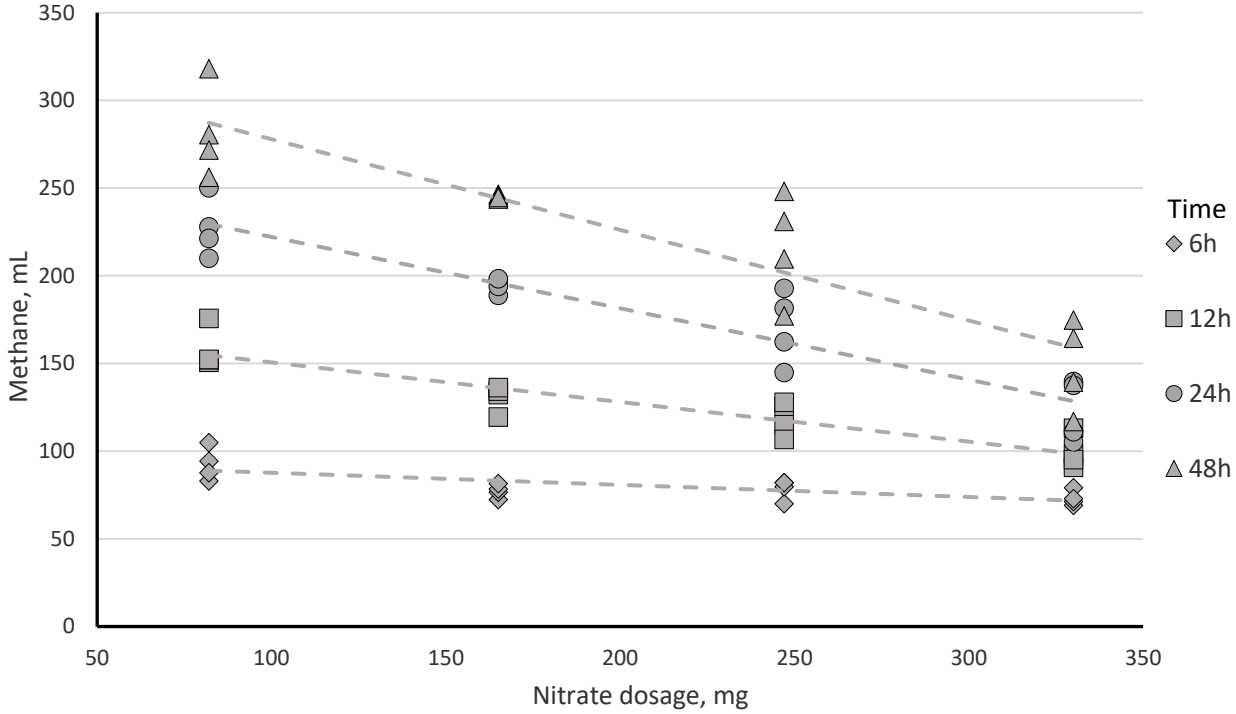
	Dosage (%DM)								NO_3	Dose	NO_3*Dose
	2.5		5.0		7.5		10				
	CTR	TRT	CTR	TRT	CTR	TRT	CTR	TRT			
Total gas											
6	564^{a}	538^{b}	543^{A}	513^{B}	530^{A}	479^{B}	520^{A}	473^{B}	**	**	NS
12	859	836	838^{A}	795^{B}	820^{A}	711^{B}	813^{A}	641^{B}	**	**	**
18	1044	1023	1022^{a}	983^{b}	1005^{A}	884^{B}	998^{A}	789^{B}	**	**	**
24	1180	1160	1155	1121	1139^{A}	1015^{B}	1133^{A}	909^{B}	**	**	**
30	1281	1263	1254	1224	1237^{A}	1113^{B}	1233^{A}	996^{B}	**	**	**
36	1349	1330	1322	1295	1307^{A}	1183^{B}	1300^{A}	1052^{B}	**	**	**
42	1395	1379	1369	1342	1354^{A}	1231^{B}	1348^{A}	1089^{B}	**	**	**
48	1430	1413	1404	1379	1387^{A}	1264^{B}	1381^{A}	1118^{B}	**	**	**
Methane											
6	109^{A}	92.5^{B}	99.2^{A}	77.3^{B}	98.8^{A}	78.5^{B}	99.3^{A}	73.2^{B}	**	**	NS
12	173^{a}	158^{b}	159^{A}	131^{B}	161^{A}	119^{B}	163^{A}	99.4^{B}	**	**	**
18	212	198	195^{A}	167^{B}	197^{A}	148^{B}	201^{A}	113^{B}	**	**	**
24	241	227	222^{a}	195^{b}	225^{A}	171^{B}	228^{A}	124^{B}	**	**	**
30	263	251	241	216	244^{A}	188^{B}	248^{A}	132^{B}	**	**	**
36	276	264	252	229	257^{A}	200^{B}	260^{A}	138^{B}	**	**	**
42	282	270	257	234	262^{A}	208^{B}	265^{A}	142^{B}	**	**	**
48	292	282	269	245	273^{A}	217^{B}	276^{A}	149^{B}	**	**	**

a-b-c, A-B-C Within rows, means without a common superscript differ ($P < 0.05$, $P < 0.01$)

Table 4. The linear regressions (within fermentation run) at different fermentation lengths (6, 12, 24 and 48 hours) between methane yield (mL) and level of nitrate inclusion (mg) or at equivalent controls (inclusions of urea and NaCl).

Time (hours)	Intercept (±SD)	Slope (±SD)	Significance		R^2
			Intercept	Slope	
Equivalent controls					
6	108.5 (±10.6)	-0.053 (±0.053)	<0.01	NS	0.30
12	170.93 (±9.57)	-0.034 (±0.026)	<0.01	NS	0.12
24	237.39 (±13.78)	-0.042 (±0.026)	<0.01	NS	0.09
48	288.55 (±18.03)	-0.053 (±0.053)	<0.01	NS	0.08
Nitrate inclusion					
6	94.57 (±9.57)	-0.069 (±0.021)	<0.01	<0.01	0.49
12	173.23 (±11.65)	-0.226 (±0.030)	<0.01	<0.01	0.84
24	262.85 (±18.56)	-0.407 (±0.048)	<0.01	<0.01	0.86
48	329.58 (±26,15)	-0.517 (±0.073)	<0.01	<0.01	0.81

Figure 1 Linear regression of the CH_4 production (mL) for different nitrate levels (82, 165, 247, and 330 mg) inclusion within each time considered (6, 12, 24, and 48 hours).



Appendix 1

Continuous monitoring of methane production in an *in vitro* rumen batch system after nitro ethanol addition

1. Introduction

Lowering the livestock sector environmental impact, particularly in terms of gaseous emissions, is one of the main goals in the coming years and several strategies have been proposed in this sense. Among them, dietary additives with anti-methanogenic capacity seem to be useful tools to reach the goal of methane reduction. Nitroderivates have demonstrated their ability to reduce methane production by altering the electron transfer in methanogenesis, modifying enzyme activity, and influencing the methanogen population (Anderson et al., 2003; Zhang et al., 2018). Recently, a new fully *in vitro* system based on an infrared sensor is proposed to study the kinetics of methane production and this research aims to assess the applicability of that device to test the anti-methanogenic efficacy of additives. The 2-Nitroethanol (2-NeOH), a nitrocompound with documented anti-methanogenic properties, was applied to reduce methane generated during the *in vitro* fermentation process. The goal of this work was to analyze the alteration caused by 2-NeOH addition to the methane kinetic obtained through the continuous measurements of methane.

2. Material and methods

2.1 Experimental design

The 2-NeOH (Sigma Aldrich, Milan, Italy) was tested in a final concentration of 10 mM, in agreement with previous studies (Gutierrez-Bañuelos et al., 2008; Chagas et al., 2019; Zhag et al., 2020) whereas the control treatment was realized with buffered rumen fluid with no addition. During the experiment, gas production and CH_4 concentration were evaluated continuously during the whole fermentation process with automated equipment. The duration of fermentation is 48 hours and treatment was tested in three consecutive runs.

2.2 In vitro experiment

The apparatus is composed of fermentation glass bottles (total available capacity 750 mL) closed with an airtight cap provided with an agitation system. The gas produced during the fermentation process flows from the fermenters to the gas counter (Ritter Apparatebau GmbH & Co. KG) which is

connected directly to a computer for data acquisition. Thereafter, the outflow passes through a plastic tube up to the infrared gas analyzer sensor (RI. sens mono IR1, Ritter Apparatebau GmbH & Co. KG) that continuously detects and registers the methane concentration.

The fermenters were filled with a 500 mL solution composed of filtered rumen fluid mixed with the Menke and Staingass (1988) buffer (ratio 1:2). The rumen fluid for all fermentation runs was collected in the same slaughterhouse in controlled conditions. Fluids from 4 culled dairy productive cows fed with total mixed rations based on corn silage were collected and mixed within 20 minutes from slaughter; no cow was slaughtered in an emergency; each cow was in good health, and all of the cows were transported from farms located within 50 kilometers of the slaughterhouse. The fluid was delivered, within half an hour of it being collected, to the laboratory in airtight glass bottles refluxed with CO_2 and maintained at 39° C.

A total mixed ratio was used as substrates (3300 mg of DM) in all experiments. The substrate was grounded at 0.5 mm length, weighted, and introduced in each bottle as dry materials. The fermentation glass bottles were hermetically closed and incubated in a water bath at 39 °C for 48 h.

2.3 Sampling of fermentation fluid and analysis

At the end of the incubation, pH was directly measured (GLP 22, Crison Instruments, S.A. Barcelona, Spain), while samples of fermentation liquid for NH_3 (10 mL) and volatile fatty acid (VFA, 5 mL of rumen fluid added with 5 mL of H_2SO_4 0.01 N) determinations were collected and stored at -20 °C until the analyses. The NH_3 concentration was determined using an Ammonia Gas Sensing Combination Electrode (Hach Company, Colorado, USA). VFA samples were centrifuged at 20,000g for 20 min at 4° C, and the supernatant was filtered using a polypore filter (RC 0.45 µm, 25 mm, DTO Servizi Srl, Venice, Italy). The filtrate was transferred into autosampler vials and 20 µL was injected into HPLC. The system was composed of an LC-20AT pump, a vacuum degasser, a Prominence SPD-M20A photodiode-array detector, a Prominence SIL-20AC HT autosampler (20 µL loop) and a Prominence CTO-20AC column oven set at 40 °C (Shimadzu Corporation, Kyoto, Japan). The HPLC separations were obtained using an Aminex HPX-87H column (300 mm x 7.8 mm) with a pre-column

(Bio-Rad, Hercules, California, USA). The mobile phase used is sulphuric acid 0.008N at a flow rate of 0.6 ml min^{-1}. Full spectra were recorded in the range of 190–400 nm and the optimum wavelength detection for all VFA was found to be 220 nm. VFA standards of acetic acid, propionic acid, butyric acid, iso-butyric acid, iso-valeric acid, valeric acid, and lactic acid were obtained from Merck (Darmstadt, Germany). Peaks of analytes were compared with the retention times of a standard mixture and quantification was based on the external standard method.

2.5 Calculations and statistical analysis

The fermentation runs were completed in sequenced periods (weeks) and data from two fermentation bottles within a run were averaged and used as statistical units.

The main fermentative parameters were statistically analyzed with SAS software (Version 9.4, SAS Institute Inc., USA) with a mono-factorial model design as:

$$Y_{ij} = \mu + a_i + \beta_j + \varepsilon_{ij}$$

where Y_{ij} is the experimental data, μ is the overall mean, α_i is the random effect (block) of the fermentation run (i=1,3); β_j is the fixed effect of the treatment (j=1,4), and ε_{ij} is the residual error. The cumulative methane production was calculated as reported by Braidot et al. (2022).

The data obtained from measurements of methane and gas production were fitted with an exponential model with a lag phase, specifically using the equation

$$y = A \times \left(1 - exp^{(-k \times (t-L))}\right),$$

where *y* is the dependent variable at time *t* (h), A (mL) is the asymptotic GP or MP values, k (/h) is a rate constant of gas production or methane production and L is the lag time (h). The kinetic parameters (A, k and L), that describe the changes in cumulative volumes of GP and MP, and the percentage of methane in the total GP, were estimated from data collected during the fermentation process and analyzed with a mono-factorial model to study the differences in kinetics.

3. Results

The 2-NeOH is an additive that has demonstrated its efficacy against methane production, interacting directly with methanogenesis and also affecting the microbial population. Therefore, the application of this additive guarantees a significant decrement in methane generated during fermentation. Because 2-NeOH shows high efficacy it was selected to evaluate the system's response to additive addition and the effect of this compound on kinetics parameters. The dosage applied is similar to that used in other scientific papers to allow a comparison between results achieved at the end of fermentation.

The 2-NeOH treatment affects a few fermentative parameters as reported in Table 1. Additive addition did not influence the ammonia concentration or the pH at the end of fermentation. The additive did not determine a change in the total VFAs production after 48 hours of incubation but has involved a shifting in VFA composition as a percentage of total VFAs. In fact, a significant increase was reached for the propionate fraction (17.50 *vs* 42.68, $P<0.05$) while a decrease was achieved in the acetate percentage (65.56 *vs* 41.06, $P<0.05$) leading to a significant decrease in the A:P ratio (4.08 *vs* 1.27, $P<0.01$). Moreover, a statistically significant decrement was obtained for the iso-butyrate fraction (*1.25 vs* 1.11, $P<0.05$) was observed in the treated sample. The gas and methane kinetics for the 2-NeOH treatment were reported in Figure 1. The figure shows that the methane produced during the fermentation process was lowered by 95% with the 2-NeOH treatment. Table 2 reported the kinetics parameters obtained. Accordingly to this result, the treatment has shown an effect on total methane (14 *vs* 279 mL, $P<0.01$) and methane as a percentage of total gas (1.95 *vs* 18.38 %, $P<0.01$). Moreover, the 2-NeOH has affected the total gas production with a significant reduction (1423 *vs* 1064 mL, $P<0.01$). Finally, the additive addition has caused a significant increment in the rate of production both for gas (0.08 *vs* 0.11 h^{-1}, $P<0.01$) and methane (0.07 *vs* 0.58 h^{-1}, $P<0.01$).

4. Discussion

In general, the 2-NeOH addition has generated a 95% reduction in total cumulative methane. This result is in agreement with previous studies that show a reduction capacity of this nitrocompound from 90% to 98% (Zhang et al., 2011; Zhang et al., 2012; Zhang et al., 2019; Zhang et al., 2019b). Considering this result, the system has shown a response comparable to the outcomes obtained in previous studies confirming that the apparatus is suitable to study antimethanogenic additives.

It should be noted that 2-NeOH inclusion has negatively affected total gas production in agreement with the outcomes achieved by other authors in previous studies (Zhang et al., 2011; Zhang et al., 2012). This result may depend on two main causes: a possible negative effect of 2-NeOH against microorganisms or an increment in hydrogen concentration. As reported in other studies (Latham et al., 2016; Teng et al., 2021), the nitrocompounds have been demonstrated to affect different microbial species and consequently, a modification in the microbial population has not been excluded. Moreover, the hydrogen not consumed by the hydrogenotrophic pathway of methanogenesis tends to accumulate in the environment with a consequent negative effect on the fermentation activity and a consequent reduction of feed digestibility. (Zhang et al., 2018). The total VFA production has not changed but there was a shift in the VFA composition. The variation observed was similar to that reported in the previous experiments where this additive was tested in a similar concentration (Zhang et al 2011; Zhou et al 2011; Zhang et al 2020). Usually, the reduction of methane formation is related to an increment of some VFA production in specific propionate and butyrate at the expense of acetate. To prevent an increment in the H_2 concentration, more reduced VFAs were produced as a direct consequence of electron redirection away from methane production (Anderson et al., 2006). Consequently, a reduction in the A:P ratio was expected and rightly observed in the results obtained.

4. *Conclusion*

The system has demonstrated its capacity to detect methane reduction caused by additive addition. Even though the reduction in methane production can be clearly observed during the whole fermentation process, the system allows an accurate kinetic study with an evaluation of kinetic parameter differences.

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Table 1 Main fermentative parameters obtained after 48 hours of incubation

	CTR	2-NeOH	RMSE
pH	6.69	6.68	0.02
NH_3 (mg dL^{-1})	47.73	46.87	5.27
Total VFA (mmol L^{-1})	51.49	77.18	20.07
% total VFA			
Acetate	65.56^{a}	41.06^{b}	5.60
Propionate	17.50^{b}	42.68^{a}	8.06
Iso-butyrate	1.25^{a}	1.11^{b}	0.25
Butyrate	11.78	13.50	1.86
Iso-valerate	3.88	4.37	0.69
Valerate	1.17	1.45	0.36
A:P	4.08^{A}	1.27^{B}	0.44

$^{a\text{-}b\text{-}c,\ A\text{-}B\text{-}C}$ Within rows, means without a common superscript differ ($P < 0.05$, $P < 0.01$).

Table 2 Kinetics parameters of cumulative gas, cumulative methane, and methane percentage estimated for 2-NeOH treatment and relative control

	CTR	2-NeOH	RMSE
Cumulative total Gas			
A, mL	1423^{A}	1064^{B}	80.86
K, h^{-1}	0.08^{B}	0.11^{A}	0.01
L, h	0.00	0.12	0.10
R^2	0.999	0.997	
Cumulative methane			
A, mL	279^{A}	14^{B}	38.69
K, h^{-1}	0.07^{B}	0.58^{A}	0.11
L, h	0.24	0.03	0.19
R^2	0.998	0.994	
Methane %			
A, %	18.38^{A}	1.95^{B}	1.21
K, h^{-1}	0.39^{B}	1^{A}	0.05
L, h	0.03	0.00	0.06
R^2	0.989	0.786	

$^{a\text{-}b\text{-}c,\ A\text{-}B\text{-}C}$ Within rows, means without a common superscript differ ($P < 0.05$, $P < 0.01$).

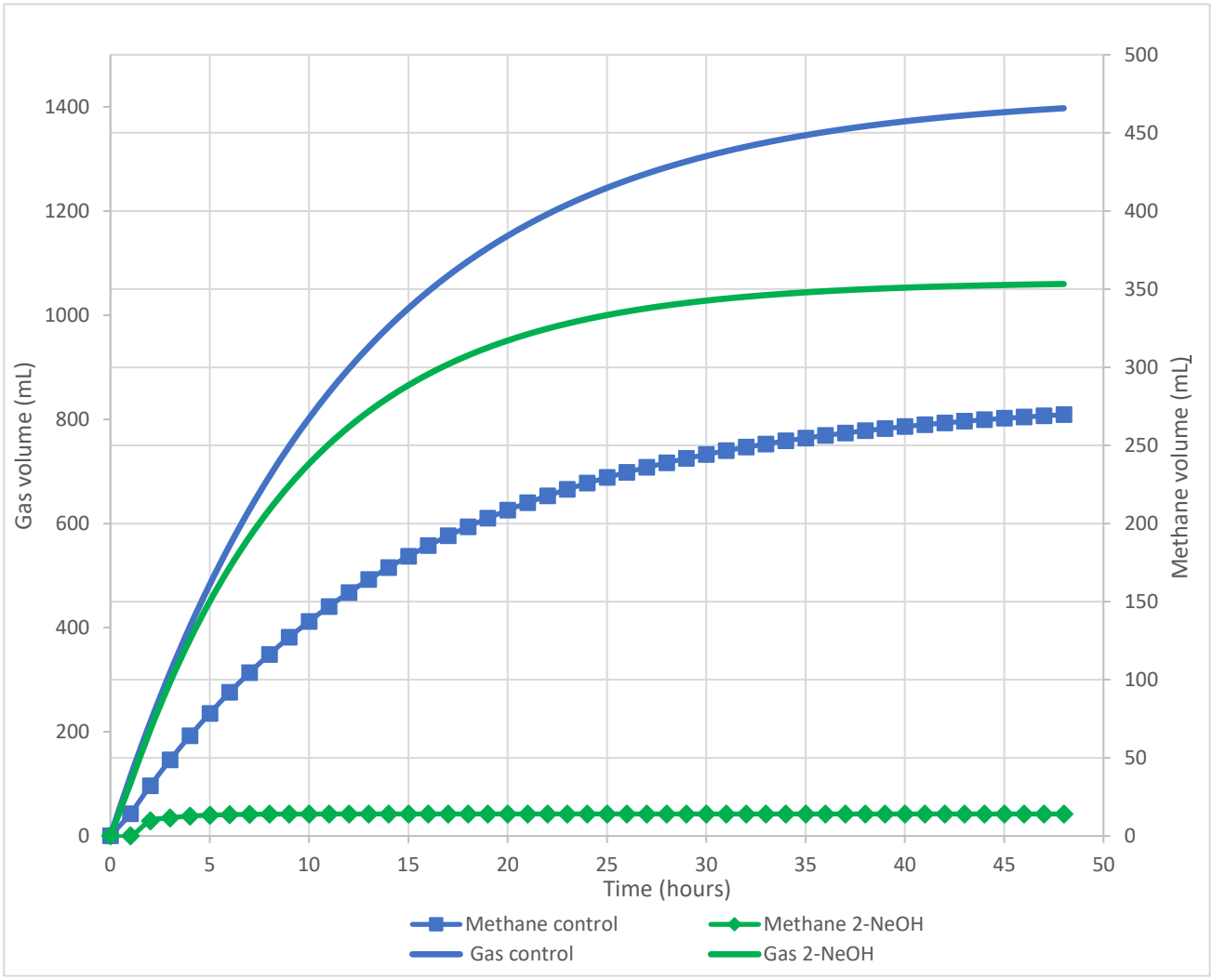

Figure 1 Kinetics of gas and methane production achieved for 2-NeOH treatment after 48-hour fermentation. The blue and green lines represent the total gas production (left axe) while the blue and green lines whit symbols represent the total methane volume (right axe)

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A meta-analysis on the relationship between rumen fermentation parameters and protozoa counts in *in vitro* batch experiments

Mauro Spanghero, Matteo Braidot *, Carla Fabro, Alberto Romanzin

Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, Via Sondrio, 2/A, 33100 Udine, Italy

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ABSTRACT

Present meta-analysis examines recent *in vitro* rumen batch experiments where a significant change in the protozoa population was measured after the additions of different substances thought to modify *in vitro* rumen metabolism. The aim is to study how protozoa number is relating to methane yield and rumen fermentation parameters.

Approximately 80 % of the 46 trials from the selected 27 papers (for a total of 201 dietary treatments) tested plant-derived substances, either alone or in mixtures, and 24 trials used rumen fluid from cattle and 22 from sheep. *In vitro* fermentations with sheep fluid used slightly larger bottles (113 *vs* 94 mL, $P < 0.05$), but the inoculum volume, substrate amount, and fermentation duration (38.2 mL, 328.9 mg, and 32.3 h on average, respectively) did not differ ($P > 0.05$). Only ammonia concentration in cattle fluids was higher compared to sheep (274.2 *vs* 137.3 mg/L, $P < 0.01$).

Within each trial, we calculated the percentage variation of protozoa compared to control bottles and the majority of the experimental treatments reduced the number of protozoa, by an average of −27.5 %. The relationship between fermentation parameters and variations of protozoa was studied by linear regressions, adjusted for trial effect. In general, protozoa variation in fermentation liquids did not affect total gas production or the sum of volatile fatty acids concentration. Contrarily, a decrease in protozoa numbers was associated with a significant decrease in methane production, both in absolute terms ($R^2 = 0.604$) and as a proportion of total gas ($R^2 = 0.528$). In terms of individual volatile fatty acids, there was a slight decrease in acetate ($R^2 = 0.298$), an increase in propionate ($R^2 = 0.388$), resulting in a decrease in the acetate: propionate ratio ($R^2 = 0.379$), and no effect of the protozoa on butyrate. Finally, there appeared to be a positive relationship ($R^2 = 0.580$) between protozoa counts and NH_3 concentration.

In conclusion, rumen protozoa counts of *in vitro* batch systems are positively associated with methane and ammonia yields, as found *in vivo*, and this regression study predicts that the complete removal of protozoa results in a reduction of about 25 % and 36 % of total ammonia and methane yields, respectively.

Batch fermentation systems appear to be appropriate for testing substances that can modulate protozoa counts and related fermentative characteristics.

* Corresponding author.
E-mail address: braidot.matteo@spes.uniud.it (M. Braidot).

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Mauro Spanghero, Matteo Braidot*, Carla Fabro, Alberto Romanzin

Department of Agricultural, Food, Environmental and Animal Sciences, University of Udine, Italy

Via Sondrio, 2/A, 33100 Udine, Italy;

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In conclusion, rumen protozoa counts of *in vitro* batch systems are positively associated with methane and ammonia yields, as found *in vivo*, and this regression study predicts that the complete removal of protozoa results in a reduction of about 25 and 33% of total ammonia and methane yields, respectively.

Batch fermentation systems appear to be appropriate for testing substances that can modulate protozoa counts and related fermentative characteristics.

1. Introduction

Protozoa are an important component of the rumen microbiota because they account for up to 50% of the rumen microbial mass (Newbold et al., 2015). They aid in fiber degradation, engulf starch granules, and act as bacteria predators (Firkins et al., 2020; Nagaraja, 2016; Newbold et al., 2015). Despite these essential functions, rumen fermentation continues even after protozoa have been completely removed (e.g., defaunation), as several *in vivo* experiments with protozoa-free animals have demonstrated (Guyader et al., 2014). Protozoa are involved in rumen CH_4 production, engulfing symbiotic methanogenic bacteria, which use the hydrogen produced by the hydrogenosomes of protozoa to reduce CO_2 to methane (Embley et al., 2003). Free-living methanogen, on the other hand, contributes little to methane output, given their low relative abundance in the rumen (Tapio et al., 2017, López-Garcia et al., 2022).

The positive link between rumen protozoa and methane was reported in the meta-analysis of Guyader et al. (2014) and Newbold et al. (2015) based mainly on *in vivo* experiments of defaunation and was recently verified *in vivo* by López-Garcia et al. (2022) by a large survey on productive dairy cows. Li et al. (2018) also validated the beneficial effects of *in vivo* defaunation on methane output, but a reduction in the effects was observed after an adaption period. In addition, the meta-analysis of *in vivo* experiments of Dai et al. (2022) has recently stated that isotrichids are more important than entodiniomorphids in methanogenesis. Even though the relationship between protozoa and methane production is complex and "not a simple cause-effect relationship (Morgavi et al., 2012), significant research effort has been continued to study the impact of protozoa on rumen methanogens, because developing feeding strategies to reduce ruminant emissions is a growing research topic (Tricarico et al., 2022). Given the limitations posed by animal experiments (e.g., costs, time, ethical issues), much research has recently shifted its focus to the cheaper and more practicable short *in vitro* fermentation

experiments (Yáñez-Ruiz et al., 2016) to test additives or rumen conditions to modulate rumen fermentation efficiently. Recently, Tedeschi et al. (2022) included the *in vitro* tests in the list of techniques to assess enteric methane, citing their advantages in terms of low costs, high reproducibility, and suitability for evaluating a large number of feeds and rumen conditions.

In these experiments, there is often also the measure of the protozoa population and overall these researches are valuable information to contribute to understanding their impact on methane output. Protozoa are particularly sensitive to the laboratory conditions of fermentation, and continuous culture systems (CC), in particular, are frequently not designed appropriately to keep protozoa populations alive for long periods (Teather and Sauer, 1988; Muetzel et al., 2009; Hristov et al., 2012). Our hypothesis is that batch rumen fermentation systems, which have a short fermentation time, are suitable for proving the protozoa-methane relationship that has already been established *in vivo*. As a result, the present meta-analysis collects and analyzes recent *in vitro* rumen batch experiments where researchers measured a significant change in the protozoa population after the additions of different substances thought to modify *in vitro* rumen metabolism. The purpose is to determine how the number of protozoa is related to methane yield and rumen fermentation parameters.

2. Materials and methods

2.1. Database development

A comprehensive search of the scientific literature was conducted using the following search engines: Scopus (https://www.scopus.com/), PubMed (https://pubmed.ncbi.nlm.nih.gov), and Web of Science (https://www.webofscience.com). The keywords ‘protozoa’, ‘rumen’, ‘*in vitro*, and ‘methane’ were used to identify publications suitable for further examination. Among the around 800 publications retrieved, only research articles written in English and published in the period 2012-2022 were considered, totaling 574 scientific works. Of the papers that were selected, only those that satisfied the following predetermined inclusion criteria were included in the meta-analysis:

(1) trials conducted using an *in vitro* batch rumen system (not *in vivo* or *in vitro* continuous systems) with direct measures of total gas and methane yield,

(2) trials in which the number of protozoa was directly counted using a microscope in the fermentation medium of batch *in vitro* systems,

(3) trials based on the addition of substances to a control substrate determined a significant difference in protozoa counts.

A flowchart detailing the searching and screening procedures for eligible studies is shown in Figure 1 and the papers are listed in the Appendix.

After the screening phase, a total of 46 trials from 27 papers were integrated into the database. Ruminant species used as liquid donors were cattle and sheep. Fermentative parameters included total gas production, methane, total volatile fatty acids (VFA), acetic, propionic and butyric acids, and ammonia. Organic matter digestibility (OMD) was also taken into account, and for trials that only reported DM digestibility (DMD), the DMD was converted to OMD (McLeod and Minson, 1974). To account for different levels of accuracy across experiments, the treatment means of each experiment were weighted (St-Pierre, 2001; Roman-Garcia et al., 2016) using the standard error of the means (SEM). For each variable, the mean SEM across experiments was calculated, and variables with a SEM lower than 0.5 x the mean SEM were trimmed to half the mean SEM (Firkins et al., 2001) to prevent overweight. Then, the 1/SEM was calculated for each variable in each experiment, and then the average 1/SEM across experiments (e.g., mean 1/SEM) was computed for each variable. The 1/SEM was standardized (i.e., divided by the mean 1/SEM) to normalize weighting factors to 1 and was used in the WEIGHT statement of the PROC MIXED of SAS Software (Version 9.4, SAS Institute Inc., USA).

2.2. Statistical analysis

The main characteristics of the trials and the fermentative parameters were statistically analyzed with SAS Software with a mono-factorial model design as:

$$Y_{ij} = \mu + a_i + \varepsilon_{ij} \quad [1]$$

where: Y_{ij} is the predicted value of the dependent variable for the j^{th} dietary treatment (j = 1, … ki; ki is the number of treatments in trial i) of the i^{th} trial (i = 1, …, 46), μ is the overall mean, a_i is the fixed effect of animal species of rumen fluid donor, and ε_{ij} is the residual error.

The relationship between fermentation parameters (total gas, methane, methane as a proportion of total gas, total VFA, proportions of acetate, propionate and butyrate on total VFA, acetate: propionate ratio and NH_3) and the percentage of protozoa counts in the fermentation bottles (calculated assuming the control, within each experiment, equal to 0%) was analyzed using a linear model:

$$Y_{ij} = a_0 + a_1 x_{ij} + \beta_i + \varepsilon_{ij} \quad [2]$$

where a_0 is the overall intercept among trials (fixed effect); a_1 is the overall regression coefficient for the linear effect of X (fixed effects); X_{ij} is the percentage of protozoa counts of the j^{th} dietary treatment of the i^{th} trial (i = 1, …, 46; j = 1, …, k_i; k_i is the number dietary treatments in trial i); β_i is the random effect of the i^{th} trial (0, σ_s^2); and ε_{ij} is the residual error, considered to be approximately normal. Adjusted values for the trial effect, calculated according to St-Pierre (2001), were used to create two-dimensional graphs. Significance was assessed at *p*-values of 0.05 and 0.01.

Outliers were removed using a procedure that involved calculating the residual standard deviation (SD) and deleting data points with residual absolute values more than 3 times the SD. After removing the outliers, the above regression model was reused, and the final regression was considered the definitive result.

3. Results

Approximately 80% of the trials from the 27 publications tested plant bioactive substances either alone or in most cases in mixtures (Table 1). In general, these most studied additives were equally included in experiments that used rumen fluid from both large and small ruminants. Table 2 summarizes the main traits and results of the *in vitro* trials considered, divided according to the donor species of the rumen fluid (cattle n. 24 trials and sheep n. 22 trials).

In vitro fermentations with sheep fluid resulted in slightly larger bottles ($P<0.05$). However, the inoculum volume, substrate amount, and inoculum duration (328.9 mg, 38.2 mL, and 32.3 hours on average, respectively) did not differ ($P > 0.05$).) Among the main results considered, the only significant difference ($P< 0.01$) was reached for NH_3, the mean value for cattle was about twice that of sheep (274.2 vs 137.3 mg/l).

The majority of the experimental treatments reduced the number of protozoa by an average of 27.5 percent when compared to the control. *In vitro* fermentation yielded an average of 59.0 mL of gas, 8.2 mL of which was methane (14%). The average VFA concentration was 76.0 mmol/L, with 61.7% acetate, 21.7% propionate, and 11.6% butyrate, with no significant differences between groups. *In vitro* digestibility was the least reported value among those considered in the publications examined (less than 60% of the trials), accounting for 57.3% of the OM on average.

Figures 2, 3 and 4 show the linear regressions (adjusted for trial effect) between fermentation traits and protozoa count variation in fermentation bottles, assuming the count of control bottles in each experiment is equal to 0%. In general, protozoa variation in fermentation liquids did not affect gas production or total VFA concentration. Contrarily, a decrease in protozoa numbers was associated with a significant decrease in methane production, both in absolute terms ($R^2 = 0.604$) and as a proportion of total gas ($R^2 = 0.528$). In terms of individual VFAs, there was a slight decrease in acetate ($R^2 = 0.298$), an increase in propionate ($R^2 = 0.388$), resulting in a decrease in the acetate: propionate ratio ($R^2 = 0.379$), and no effect of the protozoa on butyrate.

Finally, there appeared to be a positive relationship ($R^2 = 0.580$) between protozoa counts and NH_3 concentration.

4. Discussion

The majority of substances used in the experiments were natural constituents found in plants. That's because there is public concern about the use of chemical additives, while natural substances derived from plants were generally regarded as safe. Moreover, there has been a lot of research on rumen

methanogens focused on finding new molecules like biologically active compounds (such as saponins, tannins, and essential oils) that can affect either directly or indirectly methanogenesis (Cieslak, et al. 2013).

To increase the number of trials for our meta-analysis, we gathered trials conducted with rumen liquor of sheep and cattle, and the first question concerned the feasibility of examining all the trials together given the significant differences in rumen capacity, function, and physiology (e.g., intake, mean retention time, protozoa counts). *In vitro* comparisons of rumen liquor from cattle or sheep revealed no differences in fermentative capacity (e.g. total gas, methane and VFA yield) and comparable reductions of protozoa counts. The only exception was the higher ammonia yield in cattle fermentation fluids than in sheep fermentation fluids. In the absence of relevant fermentative differences, and given that *in vitro* conditions do not directly account for differences in rumen physiology and rumen functioning between species (such as feed intake level, ruminal degradation, passage rate, and mean retention time), we thought it was correct to analyze data from both species together. Furthermore, Van Gastelen et al. (2019) recently compared the effectiveness of methane mitigation strategies in cattle and sheep. They concluded that if the mode of action is linked to methanogenesis-related fermentation pathways (rather than ruminant-specific factors, such as feed intake or rumen physiology) the strategy is applicable to all ruminant species. In fact, a recent meta-analysis (Dai et al., 2022), which studied *in vivo* the relation between CH_4 emissions and protozoa counts in rumen fluid, used a mixed data set composed of bovines and small ruminants.

In the present meta-analysis, we selected trials where protozoa were quantified in the fermentation liquid of batch *in vitro* systems by direct microscopic identification and counting, which has been demonstrated to be accurate. In fact, larger protozoa like *Epidinium* spp or *Polyplastron* spp are overrepresented when the microscopic enumeration is compared to DNA pyrosequencing, while tiny *Entodinia* spp are underrepresented (Firkins et al., 2020). Based on this consideration, microscopic counting is still the gold standards for assessing ciliate protozoa in the rumen (Newbold et al., 2015).

Another drawback of our work was the inability to distinguish between protozoa (for example, entodiniomorphids vs isotrichids) that may have distinct relationships with methanogens (Firkins et al, 2020; Dai et al., 2022).

Concerning the *in vitro* systems, protozoa are sensitive to fermentation conditions, and CC systems, in particular, are unable to maintain the original protozoa population of rumen inoculums (Tether and Sauer, 1988; Muetzel et al., 2009). Hristov et al. (2012) found that only 227 of 1074 experiments with CC systems reported protozoa data. This is most likely because CC are not designed to keep protozoa populations alive for extended periods. The short duration of batch fermentation systems, on the other hand, allows for sufficient maintenance of a metabolically active protozoa population.

Expressing the average gas production (55-63 mL) as a function of the incubated substrate amount, the approximate gas yield determined was around 200 mL per g of DM. This value is slightly lower than the average (250 mL/g DM) value obtained with the Menke and Steingass procedure in a cross-lab test (Getachew et al., 2002) that included 16 different feeds (from straw to cereal meals) at 24 h incubation. The average methane yields were 7-10 mL, which is approximately 6-7% of the gross energy of the substrate (assuming the gross energy of the substrate is equal to 18 kJ/g DM). Methane concentration in total gas ranged between 13 and 15%, which is slightly lower than the 17 to 21% found by Pellikaan et al. (2011) after 72 hours of *in vitro* fermentation in several common types of feeds. However, CH_4 concentrations in fermentation gas are highly dependent on the methodological protocol adopted and Maccarana et al. (2016) in a meta-analysis of about 30 papers report an average value of 17% and a coefficient of variation of 44% (between the different experiments). The proportions of the main VFA were in the expected ranges, while despite the high variability the ammonia yield was higher in cattle fermentation fluids than in sheep.

Overall, the inclusion of various substances resulted in a wide variation of protozoa counts which ranged from a minimum of -60% to a maximum of +30% to control concentrations. On average there was a decrease in protozoa (- 27.5 % lower than the controls), because several important plant secondary metabolites, such as saponins and, to a lesser extent, some types of tannins and essential

oils, have been shown to have a deleterious interaction with protozoa (Patra and Saxena, 2009, Dai and Faciola, 2019). Less frequently have been reported increments in protozoa (Colombini et al, 2021). Despite the batch system greatly simplifying the rumen function and data having to be translated *in vivo* with caution, we were able to study several progressive different protozoa concentrations without complete removal of protozoa.

Our dataset allowed us to investigate the relationship between protozoa variations and the major *in vitro* parameters. Both total gas and VFA concentrations, which are an expression of the fermentation intensity, were not associated with protozoa variations. The digestibility data, which we do not present in figures due to the small number of data points, also confirmed this ($R^2 < 0.10$).

These results support the findings of a large number of *in vivo* trials in which defaunation, despite the numerous actions of protozoa in rumen metabolism, is not responsible for variation in rumen fermentation and total tract dry matter digestibility, apart from a possible depression in fiber digestibility (Newbold 2015; Dai and Faciola, 2019). Indeed, the authors suggest that the relative increase of propionic acid is the consequence of a reduction of cellulolytic activity.

Contrary to fermentation intensity, the current meta-analysis revealed a relatively close negative link between protozoa counts with methane and ammonia yields ($R^2 = 0.64$ and 0.61, respectively). This result was expected given the large prevalence of specific metabolic active compounds (saponins, condensed tannins and essential oils) or their mixtures in experiments of our meta-analysis. In fact, Cieslak, et al. (2013) in a review of *in vitro* experiments have reported several actions against methane production for plant metabolites. Saponins mitigate methanogenesis mainly by reducing the number of protozoa, condensed tannins both by reducing the number of protozoa and by a direct toxic effect on methanogens, whereas essential oils act mainly by a direct toxic effect on methanogens. Our results are also consistent with recent findings from Lopez- Garcia et al. (2022), who conducted an *in vivo* assessment on a large number of lactating dairy cows and found that methane emissions were linked to rumen ciliates (and also fungi and pseudo fungi). In the same work, methane yield was not associated with rumen *Archaea*, as previously found in large datasets (Tapio et al. 2017), and is

explained by a low abundance of free-living methanogens in the rumen compared to the high presence of engulfed methanogenic bacteria in protozoa.

Despite the statistically significant link between protozoa and methane, the impact on methane yield is limited (e.g. 0.33 mL of methane for each 10% protozoa reduction, Figure 2): a complete defaunation (-100% protozoa counts) results in a reduction of about 33% in total methane yield. However, this impact is higher than that that observed in *in vivo* experiments (Hegarty, 1999; Morgavi et al., 2010), where the defaunation resulted in 12% reduction in methane production. In our investigation, a decrease in protozoa was linked to an increase in propionate and a decrease in acetate (e.g. R^2 = 0.35 and 0.44, respectively), and these trends are supported by stoichiometry, as acetic fermentation produces more H_2. Indeed, a reduction of fiber degradability was reported by Newbold et al. (2015) and by Dai and Faciola (2019) after a complete or partial defaunation. Moreover, Firkins et al. (2020) report that the decreased NDF digestibility *per se* should decrease the relative fermentation process by affecting acetate or butyrate production with a consequent depression of methanogenesis.

The reduction of ammonia after rumen defaunation, first observed by Newbold et al (2015), was confirmed by Dai and Faciola (2019), who considered it the most consistent of the observed consequences of protozoa elimination, and attributed it to a decrease in microbial protein breakdown and feed protein degradability.

The effect of protozoa variation on ammonia is similar in size to that on methane, and based on the equation in Figure 4, ammonia reduction in the event of total protozoa removal is roughly 25%.

Both the relationship with methane and ammonia confirm literature data where the symbiosis of protozoa with methanogenic *Archaea* and the predation of bacteria have been utilized to explain the phenomena. From the present results, we confirm these associations and the novelty of our findings is that also batch *in vitro* fermentation systems are suitable to provide further information about protozoa involvement in rumen metabolism.

5. Conclusions

According to the current study, rumen protozoa counts of *in vitro* batch systems are positively associated with methane and ammonia yields, and these findings are comparable with what was discovered *in vivo*. As a result, batch fermentation methods appear to be suitable for testing compounds (e.g., plant-derived substances) in their ability to alter protozoa numbers and related fermentative properties.

In conclusion, the regression study predicts that the complete removal of protozoa results in a reduction of about 25 and 33% of total ammonia and methane yields, respectively.

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Appendix

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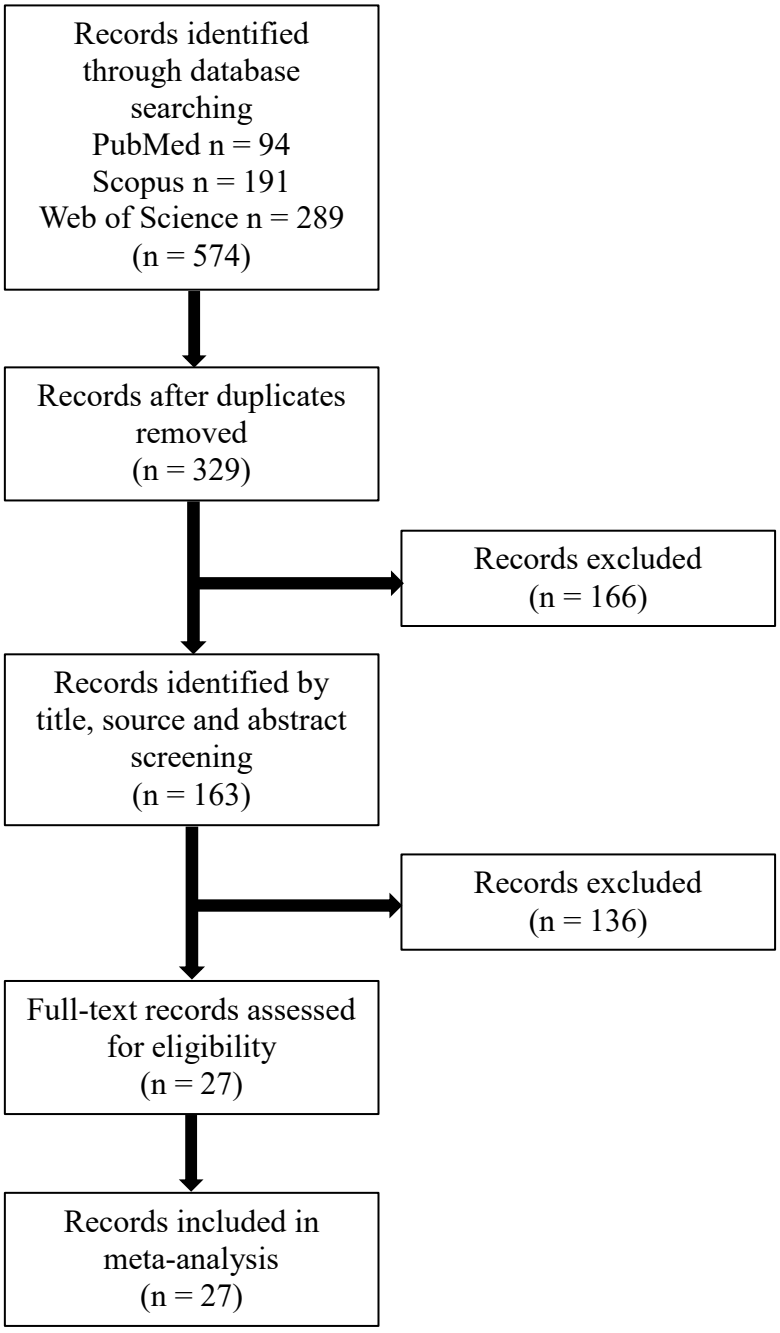

Figure 1. Data flow diagram shows the literature search, screening and selection of papers, which were considered for the meta-analysis.

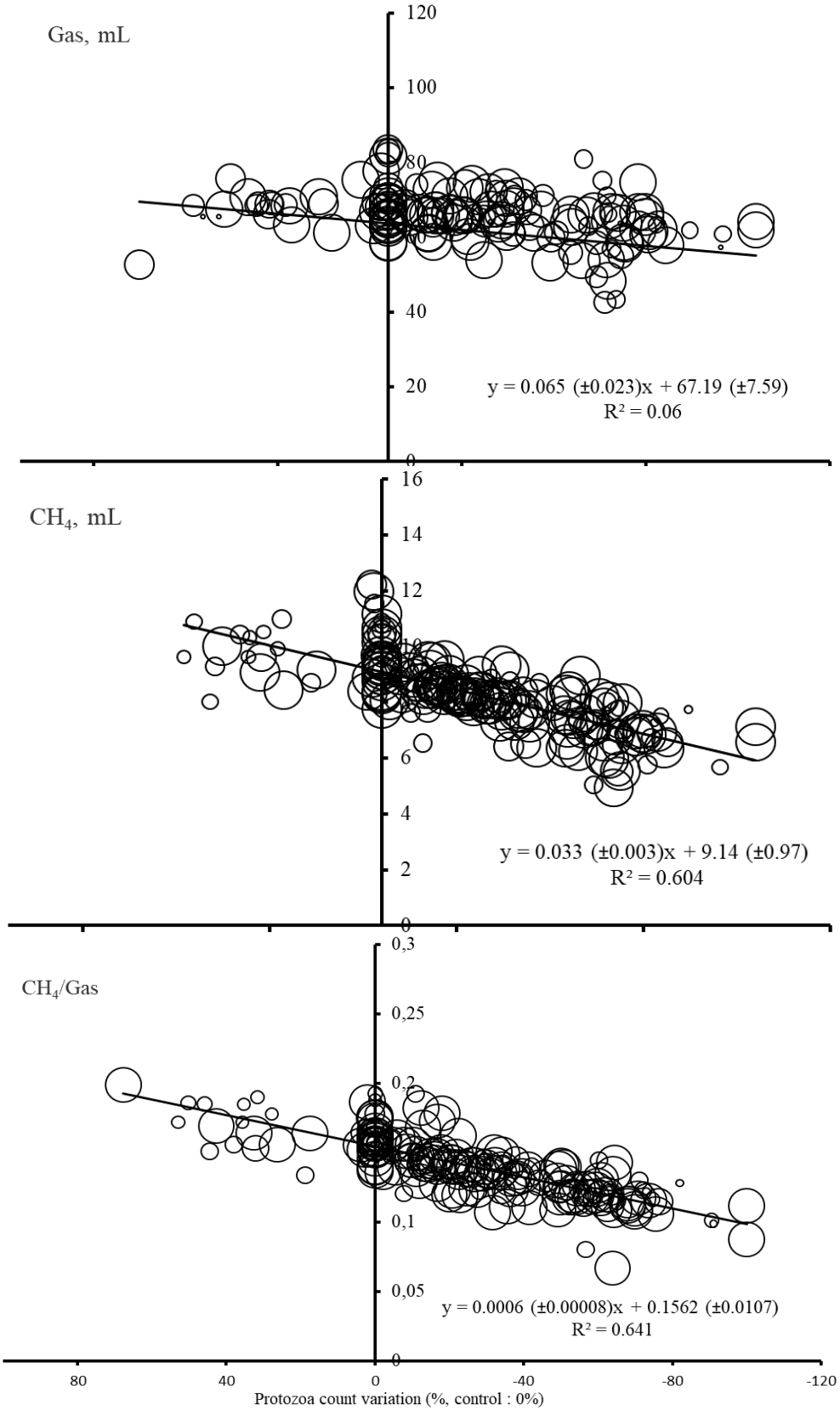

Figure 2. Linear regressions between the fermentation traits (Gas, CH4, CH4/Gas) and protozoa count in the fermentation bottles (assuming the control count within each experiment equal to 0%). All intercepts and slopes have $P < 0.01$. The size of the symbols represents the weight of trial mean.

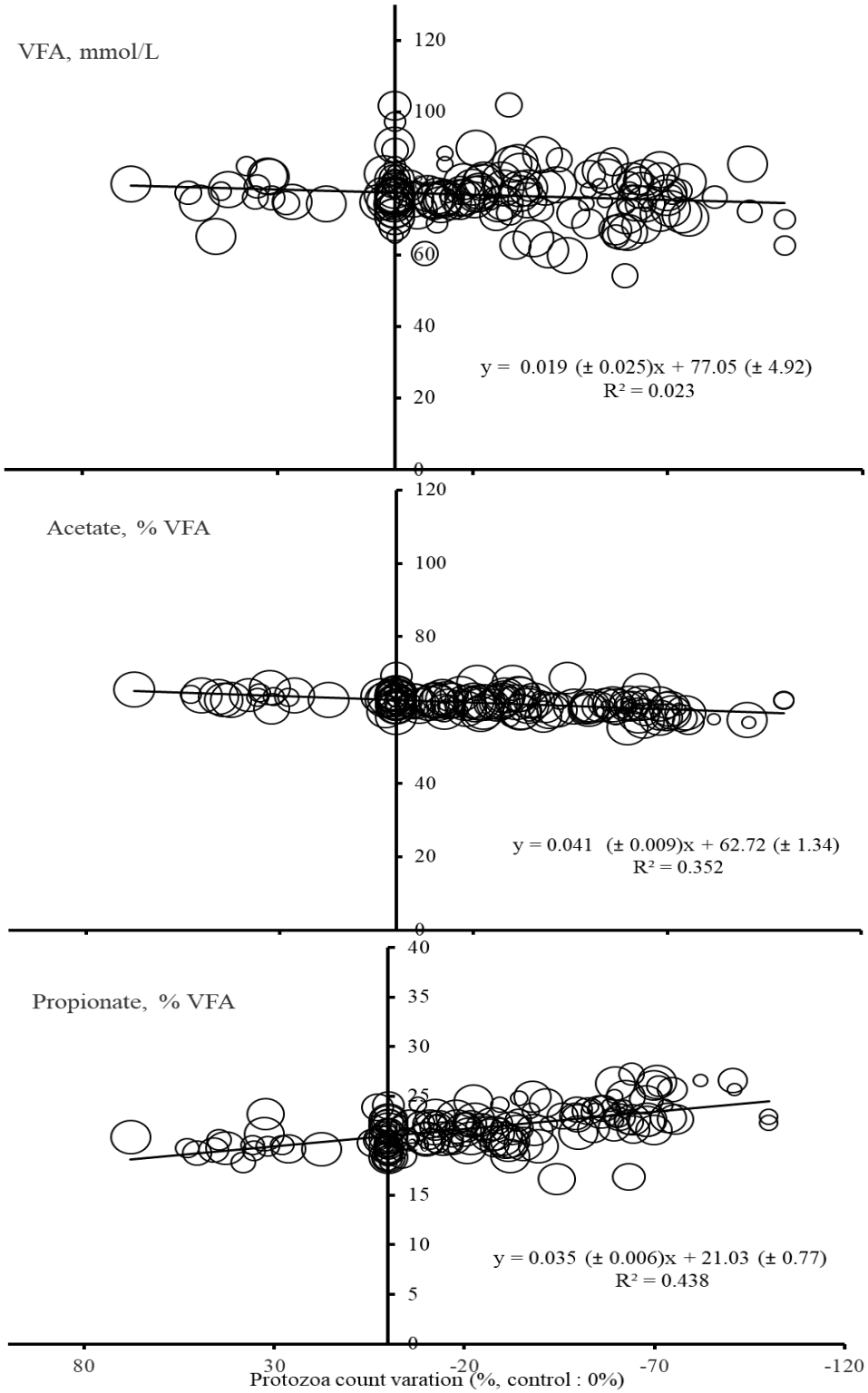

Figure 3. Linear regressions between the fermentation traits (VFA; acetate; propionate) and protozoa count percentage in the fermentation bottles (assuming the control count within each experiment equal to 0%). All intercepts and slopes have $P < 0.01$, except the slope of the regression of VFA ($P = 0.456$). All intercepts and slopes have $P < 0.01$. The size of the symbols represents the weight of trial mean

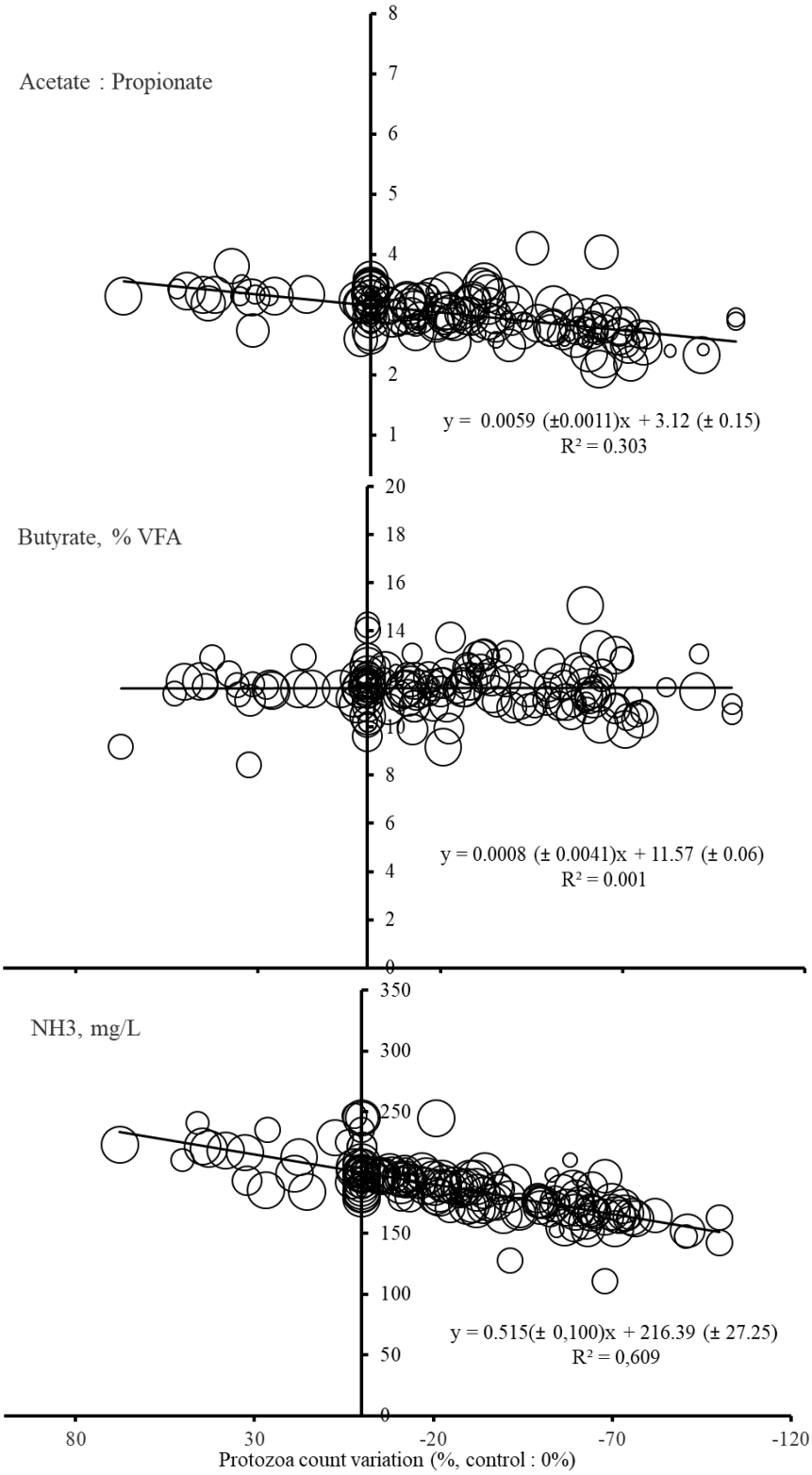

Figure 4. Linear regressions between the fermentation traits (butyrate, acetate:propionate, NH_3) and protozoa count percentage in the fermentation (assuming the control count within each experiment equal to 0%). All intercepts and slopes have $P < 0.01$, except the slope of the regression of butyrate ($P = 0.848$). All intercepts and slopes have $P < 0.01$. The size of the symbols represents the weight of trial mean.

Table 1. List of categories of additives, secondary plant compounds and extracts used in *in vitro* fermentation experiments.

Additives	Trials	Source
Condensed tannins	10	Seresinhe et al., 2014 (S); Rajkumar et al., 2015 (C); Batista et al., 2021 (C)
Essential oils	6	Nooriyan Soroor et al., 2017 (S); Colombini et al., 2021 (C); Golbotteh et al., 2021(S); Daning et al., 2022 (C)
Saponins	6	Budan et al., 2013 (C); Cieslak et al., 2014 (C); Manatbay et al., 2014 (S); Guyader et al., 2017 (C)
Secondary plant metabolites	14	Zmora et al., 2012 (C); Wencelová et al., 2014 (S); Inamdar et al., 2015 (C); Anantasook et al., 2016 (C); Boussaada et al., 2018 (C); Yanza et al., 2018 (C); Purba (a) et al., 2020 (S); Purba (b) et al., 2020 (S); Khejornsart et al., 2021 (C); Saheb Ala et al., 2021 (C)
Silkworm pupae oil	4	Thirumalaisamy et al., 2020 (C)
Nitrate	2	Nguyen et al., 2016 (C)
Propionic acid	1	Kara, 2018 (C)
Fish oil	1	Sondakh et al., 2017 (C)
Monensin	1	Hartanto, 2018 (S)
Probiotics	1	Hassan et al., 2020 (S)

Table 2. Main *in vitro* traits and results derived from dataset, divided by rumen liquor donors.

	Cattle		Sheep		RMSE[1]
	n.[2]	Mean ± SD	n.[2]	Mean ± SD	
In vitro traits					
Substrate (mg)	24	334.6 ± 201,05	22	322.9 ± 207.93	204.36
Inoculum volume (mL)	24	40.9 ± 35.56	22	35.4 ± 9.87	26.6
Bottle volume (mL)	24	94.4^{b} ± 34.51	22	112.9^{a} ± 21.53	29.04
Incubation time (h)	24	29.7 ± 14.78	22	34.9 ± 12.23	13.62
In vitro results					
Protozoa count variation (%)	24	24.0 ± 24.86	22	30.9 ± 25.24	25.04
Gas (mL)	23	62.8 ± 38.73	22	55.2 ± 36.42	37.62
CH_4 (mL)	24	9.6 ± 7.69	22	6.7 ± 5.18	6.61
CH_4/Gas	23	0.15 ± 0.08	22	0.13 ± 0.06	0.071
VFA (mmol/L)	17	83.2 ± 27.42	14	68.7 ± 25.77	26.69
Acetate (%)	17	63.4 ± 6.34	14	59.9 ± 8.21	7.27
Propionate (%)	17	22.6 ± 4.42	14	20.8 ± 4.65	4.53
Butyrate (%)	17	11.2 ± 3.32	14	12.0 ± 4.06	3.67
Acetate:Propionate	17	2.96 ± 0.81	14	3.1 ± 0.96	0.88
NH_3 (mg/L)	16	274.2^{A} ± 206.47	18	137.3^{B} ± 62.58	148.54
OM digestibility (%)	15	54.0± 14.40	12	60.6 ± 12.62	13.64

a, b = $P < 0.05$; A, B = $P < 0.01$;

1 = RMSE = root-mean-square error;

2 = Number of trials;

5. Discussion

The discussion section follows the three aims of the experimental work of the thesis and focuses on (i) the innovative *in vitro* apparatus used to study the kinetics, (ii) the trials conducted to investigate the effects of nitrous compounds in methane mitigation, and (iii) the relationship between protozoa and methane in batch fermentation systems.

5.1 The new apparatus to study methane kinetics

A first and important novel aspect of this thesis is the validation of a new *in vitro* rumen fermentation batch equipment that presents peculiar structural and functional characteristics.

First, the fermentation system employed allows for the use of high quantities of both liquid and substrate, whereas most *in vitro* rumen studies use systems with fermentation apparatus of limited dimensions (e.g., substrates of less than 1000 mg and volumes of less than 100 mL). Usually, low amounts of additives (generally lower than 1% of DM) are included in the diet to study their effects on ruminal processes and this involves some difficulties or some inaccuracy in weighting very small dosages when their efficacy is assessed *in vitro*. For example, from a recent meta-analysis by Kebreab et al. (2023) emerges that the antimethanogenic additive 3-NOP is used in an average dose of 70.5 mg/kg DM: this can be translated in an amount of additive in our conditions of around 0.2 mg/ fermentation bottle. In the case of 100 ml bottles having 5 times less than those of our apparatus and consequently the amount to include reaches very low levels. Moreover, the system presents a large final volume of fermentation fluid (approx. 500 mL) which can be easily sampled for several analyses and determinations. In contrast, a possible restriction is the overall dimensions of the apparatus, which probably is not suitable for very large numbers of contemporary fermentation units. Furthermore, the volumes of rumen inoculums required (1.2 L for 10 fermentation bottles) could represent a possible limitation.

A further advantage of the equipment tested is the management of gas outflow. Compared to previous systems, the equipment proposed does not require a complex system (valves, pressure detectors, and

related systems of recording) for each bottle to control the gas outcome. In our apparatus gas yield and methane concentrations are measured directly and continuously from the gas vented without any accumulation in the fermenters. The possible accumulation of the gases generated during the substrate degradation can alter the fermentation process causing an error in gas and methane production assessment (Cattani et al., 2014; Alvarez-Hess et al., 2018).

However, the most relevant and novel aspect of the apparatus described in this thesis is represented by the possibility to study the methane kinetic yield. In previous work, Ramin and Huhtanen (2012) suggest that methane has a kinetic production different from that of total gas, and consequently the endpoint measure (24 or 48 h) often used *in vitro* is not the best approach to its evaluation. Therefore, the continuous measurement of methane generated during the whole fermentation and the study of its kinetics instead of endpoint CH_4 concentration assessment represents a useful approach to improve the whole accuracy. This aspect was confirmed in Chapter 2 of the experimental part. Analyzing our results it has been possible to estimate the difference between these two different approaches by comparing the methane reached at the end of fermentation with the methane calculated using the kinetics of production. It was calculated that the endpoint measurements tend to overestimate the total methane, in the specific case, in a range from 5 to 28%. We assume that the extent of this difference depends on the methane production kinetic shape because the endpoint measurements do not consider the behavior of methane production. Our results suggest that methane concentration is low in the first hours of fermentation and tends to grow in the following hours. This trend was already observed by Muetzel et al., (2014), that have related this methane production behavior to the degradation process of cell wall which probably causes a lag in H_2 production with a consequent effect on the methane generated. This aspect should be considered when testing methane production in *in vitro* systems.

As reported by Wang et al. (2013; 2014) the H_2 produced in the fermentation medium of *in vitro* batch systems is presented in two major different fractions: hydrogen dissolved in the liquid (dH_2) and the hydrogen present in the gas (gH_2). The gH_2 represents the sum of the hydrogen in the headspace of the bottle (hH_2) and the part that is vented (aH2) from the system.

Usually, in batch fermentation systems, a fraction of the hH_2 is sampled and used for the analysis. Figure 7 reports a schematic representation of the different hydrogen fractions in the fermenter.

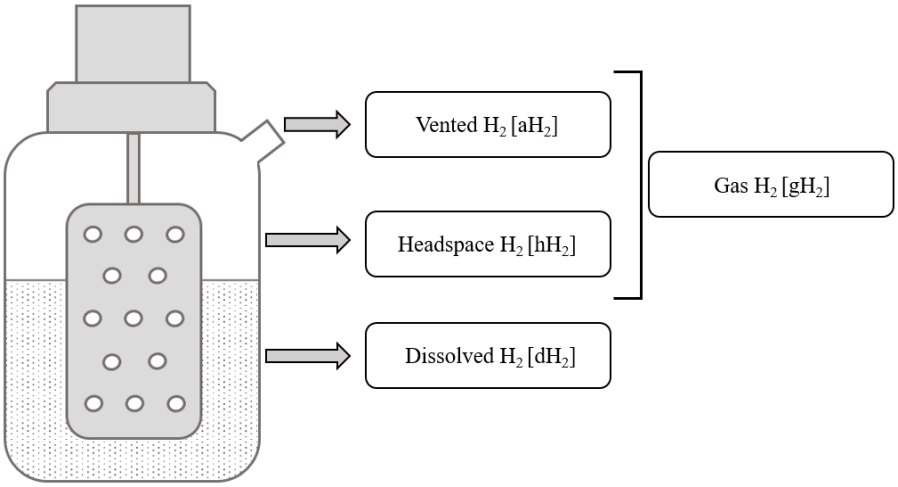

Figure 7 Forms of H_2 present in the in vitro fermentation system (Wang et al., 2013).

The whole H_2 amount is represented by the equilibrium between the liquid and gaseous phases. Wang et al (2014) stated that "…. Only the dH_2 form of H_2 is available for methanogens. H_2 will be released into the gas phase as dH_2 increases and then re-dissolve into the liquid phase if the dH_2 pool is decreased by the activity of methanogens"(Figure 8).

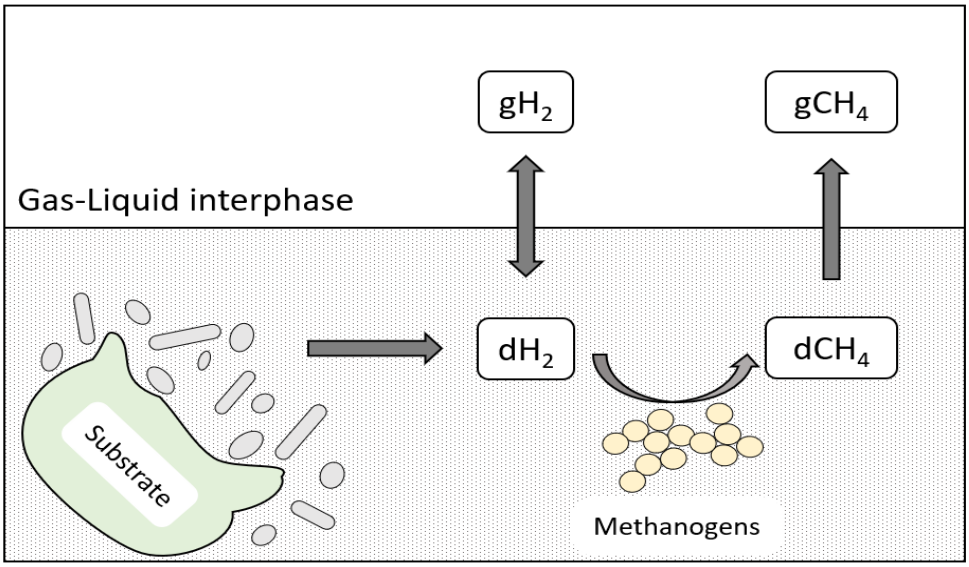

Figure 8 Degradation of the substrate by bacteria, fungi, and protozoa (grey) generates dH_2 that is converted to methane by methanogens (yellow). Dissolved CH_4 (dCH_4) is released and enters in the gas phase (gCH_4).

An increment in the pressure in the headspace can alter the equilibrium between the two hydrogen pools promoting the re-dissolution of hH_2 in the liquid phase. As result, there is a growth in the hydrogen available for methane production modifying the amount generated during the fermentation. In contrast with other systems, in our apparatus, there is no gas accumulation and therefore the gH_2 is continuously removed from the fermenter. We should hypothesize that this aspect affects the equilibrium between dH_2 and hH_2 shifting it towards the formation of hH_2 and consequently reducing the re-dissolutions process decreasing the H_2 available for the methanobacteria. Unfortunately, H_2 was not measured in our experiments and therefore we have no data to support the interpretation of the phenomena. Despite this, the continuous elimination of gas generated allows for excluding any modification of gases dissolution in the liquid and any disturbance to fermentation, as well demonstrated in the literature (Tagliapietra et al., 2010; Yang et al., 2002; Mould et al., 2005a).

5.2 Nitrous dietary additives

The nitrous dietary additives examined in this thesis are a nitrocompound (2-nitroethanol, 2-NeOH) and a nitrate salt (sodium nitrate). Nitrocompounds are derivatives of hydrocarbons, alcohols, fatty acids, and esters that contain one or more nitro functional groups ($-NO_2$) while nitrate is a polyatomic inorganic ion. Both can reduce rumen methane but with a completely different mechanism of action: as described in the state of the art of this thesis nitrocompounds exhibit probable toxicity against microorganisms and specific enzymatic toxicity while nitrate provides an alternative H_2 sink in the rumen environment. As a direct consequence, methanogenesis is definitively reduced when an appropriate dose of nitrocompound is reached while nitrate causes a continuous and progressive dose-dependent effect. In the last years, several nitrocompounds were tested to reduce methane production. The 3-nitroxypropanol has been largely studied, demonstrating a strong antimethanogenic activity without compromising animal performances in *in vivo* trials (Haisan et al., 2014; Hirstov et al., 2015). Other studies have focused their attention on the application of nitroethane and also in this case positive results were achieved (Gutierrez-Bañuelos et al., 2008; Brown et al., 2011). In recent years,

some studies have focused their attention on the evaluation of the antimethanogenic capacity of 2-NeOH both *in vitro* (Chagas et al., 2019; Zhag et al., 2020) and *in vivo* (Zhang et al., 2021) showing promising results. This nitrocompound is the alcohol that derives from the alkane nitroethane (Figure

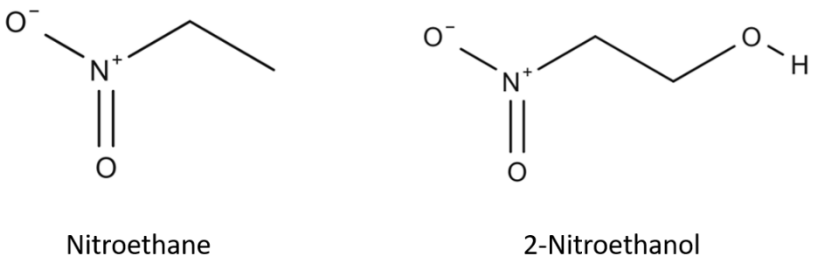

Figure 9 Chemical structure of Nitroethane and 2-Nitroethanol.

9) and a previous study demonstrated that these two molecules have a similar antimethanogenic capacity. (Anderson et al., 2003).

Given this result, 2-NeOH was selected to perform an *in vitro* experiment with our apparatus. We used a dose of 2-NeOH, which can produce a very significant effect, as a positive control to test the system response to the addition of an antimethanogenic substance. We have found that a dose of 10 mM of 2-NeOH which was similar to that evaluated in previous experiments (Gutierrez-Bañuelos et al., 2008; Chagas et al., 2019; Zhag et al., 2020) was able to generate a 95 % of reduction in agree with previous outcomes. Overall results were consistent with what was expected suggesting that the apparatus appears suitable to study the antimethanogenic capacity of additives.

The successive application of the apparatus involved the evaluation of sodium nitrate efficacy as reported in Chapter 3. It is confirmed that sodium nitrate can decrease methane production but the results suggest that extent of the phenomenon depends on the dose and the time of fermentation.

The graph reported in Figure 10, shows the reduction of methane as a percentage of the relative controls (one specific for each dose) summarizing the results achieved in the experiment. It can be noted that the effect of different doses is influenced by the time considered. At short times (e.g. 6 h)

the differences between doses are limited, tent to increase at 12 h and further increase at the end of fermentation.

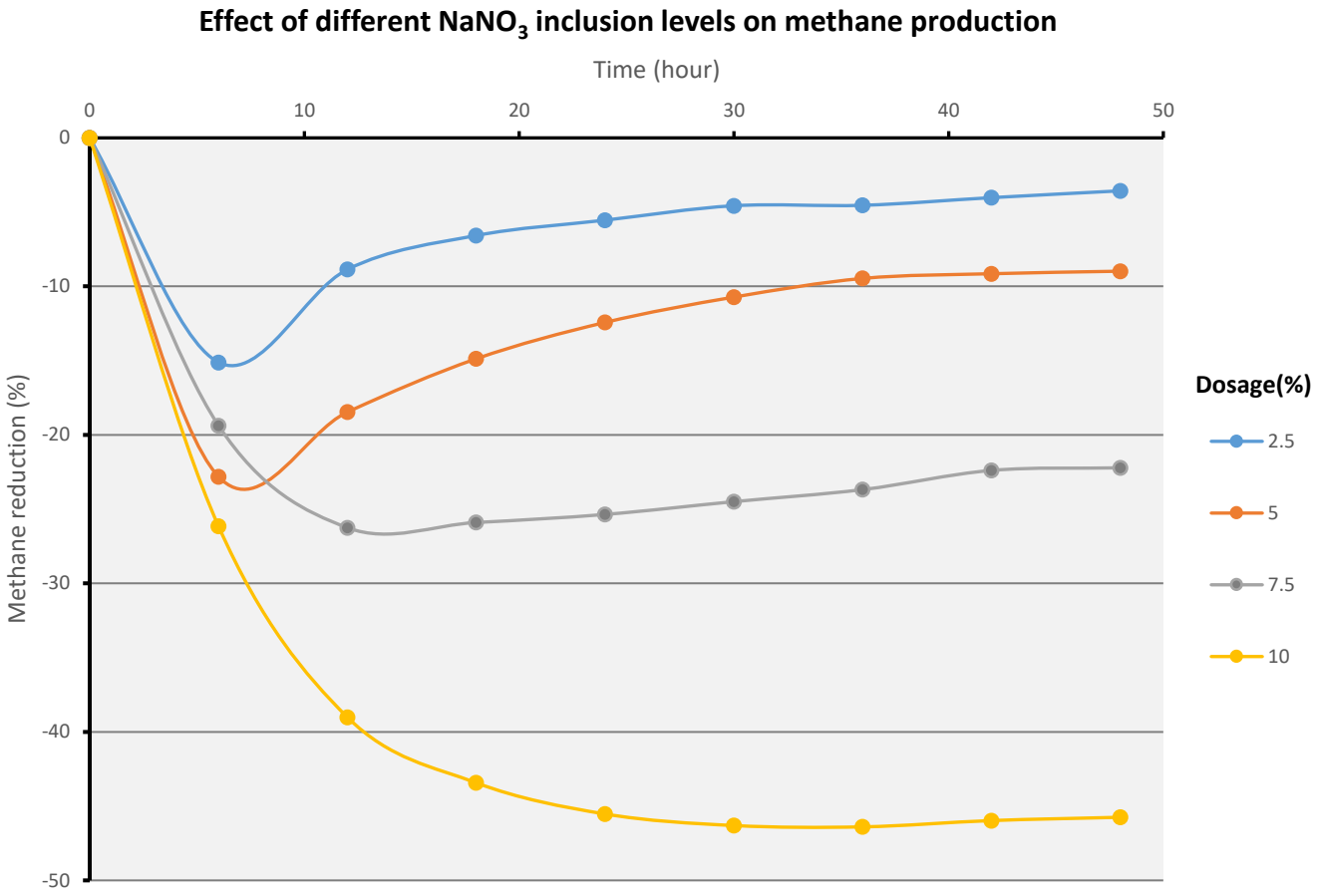

Figure 10 Methane reduction as a percentage of the relative controls for the different nitrate inclusion levels obtained at different fermentation times.

This behavior can be explained by considering two main factors: hydrogen availability and nitrate concentration. Considering the equilibrium between these two parameters is possible to better understand how the effect of nitrate addition on methanogenesis changes over time. A more complete discussion is reported in the appropriate section.

Furthermore, this approach highlights a useful consideration for estimating the effect of a specific dose since it is not constant over time. Considering the results obtained, it has been observed that different regression lines are obtained depending on the incubation time considered causing a complication in the estimation of the result of a specific level of inclusion. To overcome this problem,

in our experiment we have applied the regression line that produces values close to that from stoichiometric calculations is employed to estimate the effect of a specific dosage.

5.3 Protozoa and methane yield in batch fermentation systems

The meta-analysis has confirmed the *in vivo* data concerning the role of protozoa in the ammonia and methane yield. The regression derived from the studies considered predicts that the complete removal of protozoa results in a reduction of about 25 and 33% of total ammonia and methane yields, respectively. The results demonstrated that also *in vitro* rumen batch systems are suitable to study substances able to mitigate methane production through a protozoa reduction.

A first comment on our results regards the impact on methane (-33% assuming a total removal of protozoa) which is greater than that found *in vivo.* In two recent meta-analyses Dai and Faciola (2019) and Dai et al. (2022) have evaluated the impact of protozoa on total methane production. From the data presented it is possible to estimate the methane reduction due to a complete protozoa removal *in vivo*. In both cases, the complete defaunation generates a reduction of around -18% in the methane yield. Comparing results from our study and those derived from other work appears to be difficult since the conditions used are very different. Moreover, the *in vitro* systems are a practical tool to evaluate rumen metabolism but they are an exemplification of the real processes that occur in the rumen.

Furthermore, the method used for rumen inoculum collection is another aspect that can influence the outcomes of *in vitro* trials. Hook et al. (2012) in a modeling study, demonstrated that the majority of protozoa are attached to rumen materials and/or rumen walls and that only a limited fraction (from 10 to 37%) are free in the liquid fluid. They hypnotize that the collection of rumen inoculum with procedures that keep only the liquid fluid in the rumen (through tubes, for example) could result in an underestimation of the true rumen protozoa population because the protozoa attached to rumen materials and/or rumen walls are not collected. Therefore, given the proven role of protozoa in methane yield due to their symbiotic link with methanogens, it can be assumed that the collection

procedure influences the protozoal population concentration and, consequently, could alter the results of methane yield in *in vitro* batch fermentation systems. Furthermore, collecting only the liquid part of the rumen content can generate a selection of the protozoal population and as some species are more related to methane production than others as reported by Dai et al (2022) this leads to an additional source of variability in the results obtained. In our experiments, we kept rumen inoculums from slaughtered animals, as reported by Fortina et al. (2022), by collecting solid rumen samples from various sites and squeezing them to get rumen fluid. As a result, we consider that the adoption of this procedure allows a high recovery because we collect also a relevant portion of the protozoa attached to the solid fraction.

However, it is important to emphasize that the correlation found *in vivo* is confirmed *in vitro* even if whit a different scale. Moreover, the outcome is in agreement with previous studies in which the effect of defaunation is estimated in a CH_4 reduction ranging from 11 to 35% (Hegarty, 1999; Morgavi et al., 2008, 2012; Newbold et al., 2015).

A second remark of our outcomes concerns the protozoal population composition. In the dataset used to study the correlation it was not possible to distinguish between the main families of protozoa such as isotrichids and entodiniomorphids. Therefore, we were not able to confirm recent results from Dai et al (2022) indicating that isotrichids are more important than entodiniomorphids in methanogenesis.

A decrement in total methane production related to protozoa number is observed both *in vivo* and *in vitro* confirming their interaction with methanogenesis. Despite this general result, the interaction with methanogenic *archaea* is still unclear and further studies are needed. Moreover, the protozoa involve only a step of the whole methane production and a complete defaunation can promote other compensation mechanisms. Consequently, several aspects have to be considered to evaluate their impact on methanogenesis.

A further limitation of our meta-analysis was the lack of information about the specific effects (and the extent) of the main categories of biologically active substances used in the papers to modulate the protozoa. Unfortunately, the available data set was not suitable for a deep investigation of single

compounds given the limited number of scientific papers for a specific class of compounds. Based on the criteria adopted for article selection the number of papers available to run regression analysis within main categories of biologically active substances was modest (6 for saponins and essential oils, 10 for tannins). Contrary there was a larger body of studies (14 papers) where the identification of a single compound is difficult. For instance, some papers (Boussaada et al., 2018; Saheb Ala et al, 2021) report general “plant extracts” and do not give information about the composition whereas in other papers (Inamdar et al., 2015; Yanza et al., 2018; Khejornsart et al., 2021) the treatments are represented by the contemporary presence of tannins, essential oils, saponins, and others. This last group of papers in our study was formerly referred "secondary plant metabolites".

Nevertheless, the macro classes of compounds that influence protozoa number are presented and widely discussed in the literature. Essential oils are molecules derived from various parts of plants that have shown antimicrobial efficacy against a large number of microorganisms including protozoa (Cobellis et al., 2016). The effect on ruminal protozoa depends on inclusion levels and also on the source used since oils extracted from different origins have demonstrated various results (Hart et al., 2008; Colombini et al., 2021). The main effects caused by essential oil additions are modification of the cytoplasmatic membrane, alteration in the electron transfer, modification of ion gradient, and variation in the enzyme activity (Patra 2012). Taking into account these effects is difficult to understand if the methane reduction is related only to a decrement in the protozoal population or depends on other factors. Tannins are a subclass of polyphenols that show an antimethanogenic capacity. Their mechanism of action is not clear yet and different hypotheses are proposed. Considering their effect on ruminal protozoa, a possible explanation considers a probable alteration in the hydrogen transfer between protozoa and methanogenic *archaea* (Ku Vera et al., 2020). In general, appears that they impact more rumen bacteria abundance and metabolisms instead of rumen protozoa number (Patra et al., 2017). Saponins are a group of high molecular weight glycosides widely diffused in plants. These secondary metabolites are widely studied as rumen modifiers. It is demonstrated that the somministration of saponins extract or saponins-containing plants affects the

ruminal protozoa both *in vitro* (Hu et al., 2005) and *in vivo* (Hristov et al., 2003). The overall efficacy varies in the different studies and the lack of antiprotozoal effect can derive from several factors such as the type of saponin, the inclusion level in diets, and the method used for their extraction (Patra and Saxena, 2009). Furthermore, it seems that not all species are equally sensitive to the addition of saponins. Lipids that have exhibited an impact both on growth and the total number of rumen protozoa are usually medium and long-chain fatty acids (Enjalbert et al., 2017). Furthermore, Oldick and Firkins, (2000) have indicated a relationship between the degree of unsaturation and the effect on protozoal count suggesting that the increment in the unsaturation generates a greater decrease in the number of protozoa. As reported by Fouts et al. (2022), lipids can also reduce the production of methane through a process of bio-hydrogenation of unsaturated fatty acids that consumes the hydrogen produced during the fermentation.

Considering these main classes of substances can be noted that their effect is not related only to the protozoal population but they also interact with different mechanisms of methanogenesis. As result, this phenomenon complicates the study of the relationship between protozoa number and methane, and consequently, further studies are needed to fully understand this interaction.

6. Conclusion and implications

The results obtained in the experiments of this thesis confirm that *in vitro* batch systems are a valuable practical tool for methane assessment. Furthermore, the utilization of an apparatus that allows a continuous measure of the methane concentration has proven to increase the overall accuracy. The outcomes promote the use of methane production kinetics as an appropriate method to accurately estimate the proportion of methane generated in *in vitro* trials. Furthermore, the system was demonstrated to be especially useful when the antimethanogenic capacity of dietary additives must be detected throughout the fermentation and simple end-point measure is not accurate enough.

Given these results, we promote the application of our apparatus based on an infrared sensor for methane assessment as a rapid, precise, and reliable method that can be adopted in different applications.

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