Development and evaluation of an *in vitro* procedure to assess enteric methane production

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***Abstract*—Livestock production systems are estimated to emit 14.5% of the global anthropogenic greenhouse gases (GHGs). Methane (CH_4) emissions from enteric fermentation of cows is the main source of GHGs emissions from the livestock sector, contributing for the 44% of the total. Nowadays research is focusing on finding technical solutions to mitigate these emissions. One strategy is the addition of natural additives in the cows diet, especially since the use of antibiotics as feed additives was banned in EU. *In vitro* fermentation trials are a useful strategy to simulate a ruminal digestion process and to test the effect of several compounds in reducing enteric CH_4 production. The aim of this study was to develop a method to assess an *in vitro* enteric fermentation process. An automatic system (Ankom RF Gas Production System) for gas production (GP) measurement was used. The set-up protocol was validated by investigating the effect of two feed additives on CH_4 and volatile fatty acids (VFAs) production. The developed procedure has shown to be appropriate to perform enteric fermentation trials. The additives did not show any significant ($p>0.05$) effect on the tested parameters. Keywords—ruminal fermentation, gas emissions, additives.**

I. INTRODUCTION

Enteric methane emitted by ruminants is one of main concern related to livestock activity. Enteric fermentation, in fact, is responsible for 44% of global greenhouse gases (GHG) emissions from the sector [1]. Methane (CH_4) is produced into rumen as a by-product of ruminal feedstuff fermentation. Once ingested, feedstuff is broken into monomers by ruminal microorganisms, which later anaerobically ferment them producing volatile fatty acids (VFAs), mainly acetate, propionate and butyrate and CH_4 [2]. VFAs are absorbed as they are a main energy source for the animal, while CH_4, produced by the Archaeal community, is erupted as it represents a waste. Enteric CH_4 production is both an environmental issue and a loss of energy for the animal [3]. For these reasons it is of primary importance to find effective ways to reduce enteric CH_4 production. *In vitro* ruminal fermentation trials are used to evaluate ruminal fermentation processes, such as gas production (GP), VFAs concentration and CH_4 production. Feed, ruminal fluid and a buffer solution are incubated in anaerobiosis at a constant temperature of 39°C, to reproduce the ruminal environment. The most recent techniques use automatic systems to detect total gas production, coupled with analytical instruments to analyze the CH_4 content of the produced gas and VFAs content of fermented ruminal fluids.

In recent years, several studies have been performed to assess the effectiveness of natural compounds in reducing enteric CH_4 production, since the use of antibiotics as feed additives was banned in EU in 2003 [4].

The aim of this study was to develop an analytical procedure to perform *in vitro* rumen fermentation trials. Furthermore, this method was validated testing the effect of two commercial feed additives in reducing enteric CH_4 production. The first additive (GAR) contains garlic extracts, while the second (SFO) contains sunflower oil. Both, garlic and sunflower oil are known to be effective compounds in reducing *in vitro* ruminal methanogenesis [4; 5; 6]. In particular, garlic extracts have demonstrated to alter metabolic pathways of rumen microbes [7] and interfere with Archea cell membrane structure. Furthermore, lipids have been shown to decrease CH_4 production from dairy cows when added as dietary supplements [8]. Crude fat concentration in the diet is a factor known to decrease enteric CH_4 emissions in ruminants, while unsaturated fatty acids are compounds able to compete for H^+ ions in the rumen during hydrogenation, wich would otherwise be used to reduce CO_2 with the final formation of CH_4 [9].

II. MATERIALS AND METHODS

Incubation trials were performed using an automatic system (Ankom RF Gas Production System) designed to monitor the amount of gas pressure produced during a fermentation process (cumulative and absolute pressure). This system consists of 24 modules (250 mL glass bottles) equipped with temperature and pressure sensors. Furthermore, each module continuously communicates information to a computer using radio frequency transmission.

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For the trials a typical dairy cow total mixed ration (TMR) was incubated with rumen fluid and buffer solution for 24 hours.

The total mixed ration (TMR) was sampled in a dairy farm in Candiolo (Torino, Italy), while rumen fluid (RF) was collected at the slaughterhouse. The dry matter (DM) content of the TMR was 45.8%. The chemical composition of TMR is shown in table I.

Table I. chemical composition of TMR

Parameter	% DM
Crude protein	14.0
NDF	37.6
ADF	19.8
NFC	36.9
Ether extracts	2.9
Ashes	8.6

NDF=neutral detergent fiber; ADF=acid detergent fiber;

NFC= non-fiber carbohydrates

RF was collected at a slaughterhouse from 16-18 months old animals fed a TMR with a forage:concentrate ratio of 40:60; RF was obtained by squeezing the content of the rumen and avoiding as much as possible the inoculum devitalization, according to the [10].

A. Incubation procedure

The day before the incubation, buffer solution was prepared according to the Kansas state method [11]. Obtained pH values ranged from 6.8 to 6.9. Furthermore, each module was filled with 1.0 ± 0.0010g of TMR and the corresponding amount of additive.

Buffer solution, modules and laboratory glassware used during the experiment were pre-heated at 39°C inside a climatic chamber for the whole night before the incubation.

Once the RF arrived at the laboratory, it was immediately filtered through an appropriate cheesecloth bag under a constant N_2 flow. Each module was quickly filled with 50 mL of the filtered RF and 100 mL of the buffer solution. The ratio between TMR and buffered RF (150 mL/g DM) was chosen to ensure the correct buffering action and the fluid pH maintenance during the incubation [12]. After filling the module with the buffered RF, anaerobiosis was set insufflating N_2 in the headspace of each module. Lastly, each bottle was closed and inserted inside the climatic chamber.

During the incubation, the gas produced inside the headspace of the bottles was vented when the pressure reached the value of 1 PSI [4].

At the end of the incubation, analyses were performed to assess total gas production, CH_4 concentration of the gas, VFAs (acetic, propionic and butyrric) concentration of the fermented fluids and acetic/propionic ratio. GP and CH_4 production were expressed as ml per g of incubated dry matter (DM), while VFAs concentration as ppm.

Total GP was computed with the ideal gas law (1) as follows:

$$P * V = R * n * T \qquad (1)$$

$$GP = n * 22.4 * 1000/\ \mathrm{DM}$$

Where:

- P is the cumulative pressure in the headspace of the bottle after 24 hours (kPa);
- V is the bottle headspace volume (l)
- R is the gas constant (8.314472 $l \times kPa \times K^{-1} \times mol^{-1}$);
- n is the gas produced in moles (mol);
- T is the average temperature of the bottle during the 24 hours (K);
- 22.4 is the volume occupied by 1 mol of gas (l);
- DM is the incubated dry matter (g);

Gas samples were collected with a 10 mL gas-tight syringe from the headspace of the bottles. At each sampling, the syringe was flushed to allow the collection of a homogeneous sample, which was immediately inserted into 30 mL vials to be analyzed with gas chromatography technique to assess CH_4 concentration. Total CH_4 concentration was computed from a single gas sampling, following an equation (2) proposed by [4; 13].

$$\textit{total } CH_4 = -0.0064 \times [CH_4 \textit{ concentration in the headspace} \times (\textit{headspace volume} + \textit{total GP volume})]^2 + 0.9835 \times [CH_4 \textit{ concentration in the headspace} \times (\textit{headspace volume} + \textit{total GP volume})] \qquad (2)$$

For VFAs concentration analysis, 5 mL of fermented ruminal fluid were collected from each module and acidified with 5 mL of sulfuric acid (H_2SO_4) 0.1 N. samples were centrifuged at 3000 rpm for 15 minutes and the supernatant was filtered with syringe filters (0.2 µm diameter) into vials to be analyzed with HPLC technique.

B. Validation trials

Once the appropriate procedure was found, it was validated with two incubation runs, testing the efficacy of the additives. For each run the experimental design was as follows: 6 treatments in 3 replications, plus 3 blanks (bottles with only buffered RF) and 3 controls (CTR, bottles with buffered RF and TMR, but without additive).

The two additives were tested at 3 increasing dosages: 0.05 g/g DM (1), 0.1 g/g DM (2) and 0.2 g/g DM (3).

C. Statistical analysis

The effect of the two additives on the gas and CH_4 production and VFAs concentration was assessed through a one-way ANOVA procedure (significancy level was $P<0.05$), with a Bonferroni post-hoc test.

III. RESULTS AND DISCUSSION

The developed analytical procedure has shown to be appropriate to simulate the fermentation process in ruminant animals. The average CH_4 production (30.43 mL/g DM) data are in line with those presented in other studies [4; 14; 15].

No effects of the tested additives were highlighted during the validation trials. Table II shows the ANOVA results.

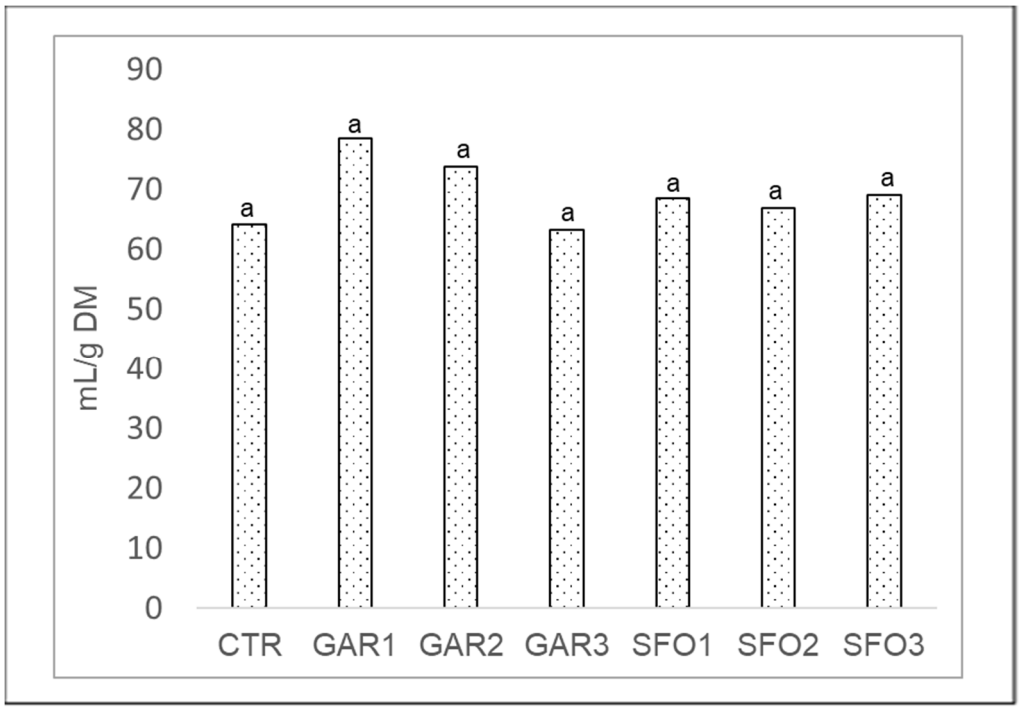
In particular, there was no statistically significant difference among treatments and control on GP (Fig.I), with an average value of 69.28 mL/g DM. The same results have been obtained in terms of CH_4 production (Fig.II). No significant difference was noticed on VFAs concentration in the fermented fluids among treatments (Fig. III). VFAs concentrations were on average 4104.23 ppm, 2272.99 ppm and 1837.90 ppm respectively for acetic, propionic and butyric acid. Acetic/propionic ratio was on average 1.8.

Table II. ANOVA results

Parameter	Mean		DF	Sum sq	Mean sq	F value	*P*-value
GP (mL/g DM)	69.28	Treatment	6	1007.7	167.94	0.88	0.52
		Residuals	34	6476.8	190.49		
CH_4 (mL/g DM)	30.43	Treatment	6	52.88	8.81	0.6	0.72
		Residuals	33	482.16	14.6		
Acetic acid (ppm)	4104.23	Treatment	6	1003343	167224	0.85	0.54
		Residuals	35	6915425	197584		
Butyrric acid (ppm)	1837.90	Treatment	6	178602	29767	0.99	0.45
		Residuals	35	1058769	392251		
Propionic acid (ppm)	2272.99	Treatment	6	344632	57439	0.84	0.55
		Residuals	35	2395441	68441		

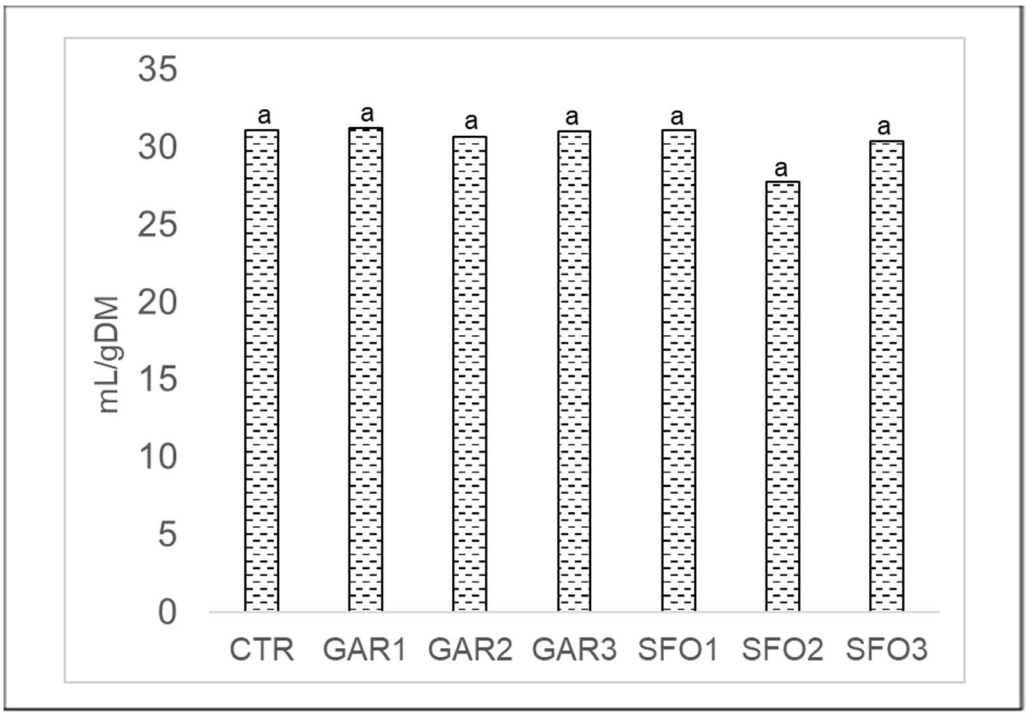
DF= degree freedom; Sum sq= sum of squares; Mean sq= mean square; Treatment= between treatments; Residuals= within treatment; DM= dry matter

Figure I. Average gas production values measured at the end of the incubations



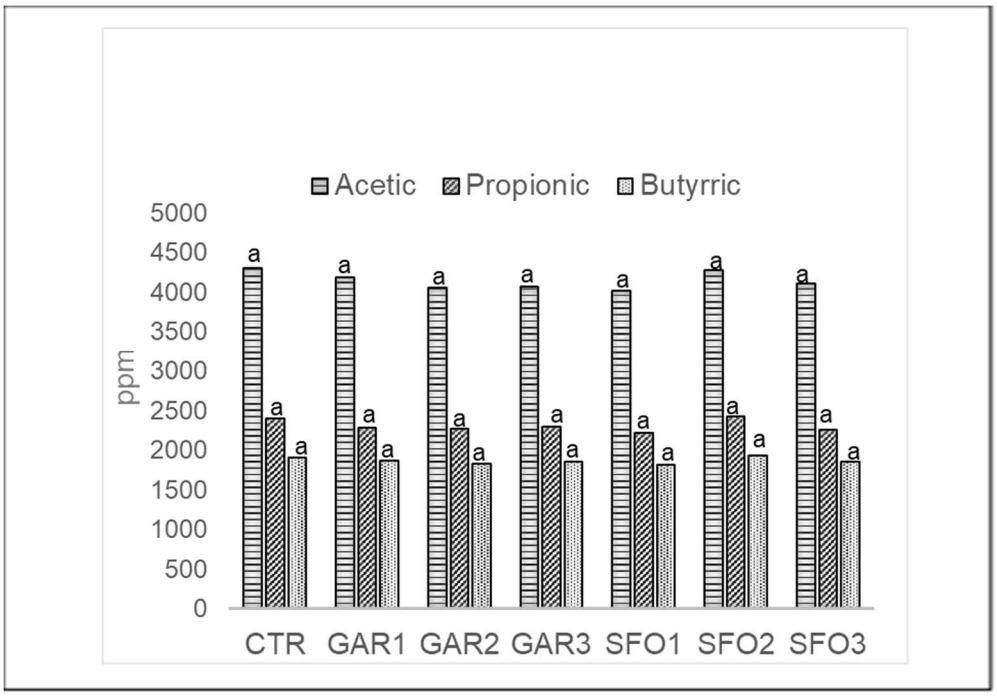
GAR= Garlic additive; SFO= Sunflower oil additive;
1= 0.05 g of additive per g of dry matter; 2= 0.1 g of additive per g of dry matter;
3= 0.2 g of additive per g of dry matter; CTR = no additive, DM= dry matter.

Figure II. Average CH_4 production measured at the end of the incubations



GAR= Garlic additive; SFO= Sunflower oil additive;
1= 0.05 g of additive per g of dry matter; 2= 0.1 g of additive per g of dry matter;
3= 0.2 g of additive per g of dry matter; CTR = no additive, DM= dry matter.

Figure III. Average VFAs concentrations values of the ruminal fluids measured at the end of the incubations



GAR= Garlic additive; SFO= Sunflower oil additive;
1= 0.05 g of additive per g of dry matter; 2= 0.1 g of additive per g of dry matter;
3= 0.2 g of additive per g of dry matter; CTR = no additive, DM= dry matter.

IV. CONCLUSIONS

The set-up protocol for ruminal fermentation analysis has shown to be useful to collect reliable data about GP, CH_4 and VFAs production, simulating a ruminal digestion process. Validation trials have shown no effect of the tested additives on the analyzed parameters. The two additives did not influence the fermentation process at any tested doses. Although they both contain compounds with known antimethanogenic effects, it is not specified neither the type, neither the concentration of the compounds. It could be hypothesized for both additives, that the active antimethanogenic molecules are too low-concentrated to determine any kind of visible effect during the *in vitro* fermentation trials.

Further trials will be performed using other types of ruminal fluid and additives and doses, in order to find effective solutions to mitigate enteric CH_4 emissions.

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