UNIVERSITÀ DEGLI STUDI DI MILANO
DIPARTIMENTO DI SCIENZE VETERINARIE
PER LA SALUTE, LA PRODUZIONE ANIMALE
E LA SICUREZZA ALIMENTARE

Lodi, 01th July 2020

"Effect of Anavrin (Vetos Europe SAGL) on in vitro methane production" – Survey on the effect of a pool of the essential oils, bioflavonoids and tannins on the production of methane through Biochemical Methane Potential (BMP) assays in organic matrix

Introduction

Livestock sustain the livelihood of millions of people in the world in both developing and developed countries, and the demand of animal product is constantly increasing following the increase in the world population, and also the improvement in the economic status in some developing countries. Beef producers must satisfy this increase in the demand. But now, because of the growing attention and importance of the problems related to climate change and sustainability, livestock producers, especially beef farmers, are under pressure to reduce the total greenhouse gas emissions per unit of output, commonly known as carbon footprint (CO_2–eq).

The agro-zootechnical sector, including also the production of all vegetable crops for human consumption (vegetables, cereals, fruit etc.), contributes 20% to the overall greenhouse gas emissions, expressed in CO_2 equivalents. Within this 20%, animal husbandry impacts 10%, or 14.5% if we also consider the emissions deriving from changes in land use (FAO, 2017).

Herrero et al. (2013), estimated that the total emissions from livestock were in the range of 5.6 – 7.5 giga tons (Gt) of CO_2–eq/year between 1995 and 2005. Between all the livestock production categories, ruminants are the most impactful. Havlík et al. (2014), opined that ruminants represent more than 80% livestock emissions. Particularly, beef and dairy sector contribute to about 60% (Bellarby et al., 2013). The high impact of ruminants on climate change is mainly due to their specific physiology and anatomy, that lead to a great production, in the rumen, of methane, the most impactful greenhouse gas due to its higher global warming potential (GWP), that reached the value of 25-28 in a 100-year time horizon. In fact, Herrero et al. (2013), found out that methane is the main cause (alone 32% of the total emissions) of environmental pollution arising from the livestock sector. The mixed rumen microbiota and the specific ruminal environment and fermentation patterns that allows ruminants to convert non-usable forages, has the negative aspect related to the methane production. In fact, methane (CH4) is the main sink of metabolic hydrogen (H) in rumen fermentation. Metabolism of carbohydrates by the fermentative microbiota of bacteria, protozoa and fungi, reduces co-factors, which are re-oxidized mostly by transferring electrons to protons. Dihydrogen (H2) so formed, that if it accumulates in large quantities can alter, is transferred to methanogenic Archaea bacteria into CH4, to balance the hydrogen pressure in the rumen, and then released mainly through eructation (Russel et al., 1997).

In addition to being a cause of environmental impact, methane production is also an energy loss for the animal, which results in reduction of production efficiency. For these reasons, scientific research is focusing on identification and development of strategies to optimize ruminal functions in order to reduce methane emissions and, simultaneously, to achieve the best feed conversion efficiency.

Particular attention has been paid to essential oils and natural products as they are able to modulate rumen activity by improving efficiency and reducing methane production (Broudiscou et al., 2000, 2002; Cardozo et al., 2004, 2005; Mohammed et al., 2004; Busquet et al., 2005, 2006, Calsamiglia et al., 2006). Essential oils are, together with saponins and tannins, the secondary metabolites produced by the volatile fraction of some varieties of plants and spices (Balandrin and Klocke, 1985), whose antimicrobial activity towards different categories of bacteria and microorganisms (Gershenzon and

Croteau, 1991), has been known since antiquity (Davidson and Naidu, 2000). The essential oils and natural products, are able to manipulate the rumen microbial population and to alter the rumen fermentation pathways toward a reduction in methane production and, parallelly, toward a more efficient use of the feed, with an increase in the production of volatile fatty acids, in particular butyrate. Essential oils derived from thyme, oregano, cinnamon, garlic, horse radish, rhubarb and frangula, have decreased CH_4 production in vitro in a dose dependent manner. Some essential oils, such as garlic, cinnamon, rhubarb and frangula, may exert a direct effect on methanogens (Benchaar et al., 2011).

Aim of the project

This project aims at evaluating, through two different in vitro scientific trials (Table 1), the effect of the inclusion of Anavrin (Vetos Europe SAGL, via delle Industrie 18, 6593 - Cadenazzo (TI), Switzerland), a pool of essential oils plus bioflavonoid and tannins, on methane and biogas, production, in a situation that mimics the rumen environment, considering a retention time of 16, 20 and 24 hours, through the use of the Biochemical Methane Potential (BMP) assay methodology. This timing comes from the evidence that the principal rumen fermentation activity in high-producing dairy cows and fattening beef cattle fed with high nutritive levels, occurs during the first 24 hours post feeding. Indeed, the most recent scientific approaches pointed out to evaluate degradability, fermentability and digestibility at the rumen level, are focalized on the first 24 hour.

The pool of essential oil, bioflavonoid and tannins, were included in the BMP at different concentrations, to evaluate the potential different activity on rumen microbiota and methane and volatile fatty acids production.

Table 1: The two scientific trials

Trial	Title	Duration
First trial	"Effect of Anavrin on in vitro methane production in a 20 hours Biochemical Methane Potential assay"	20 hours
Second trial	"Effect of Anavrin on in vitro methane production in a 16- and 24-hours Biochemical Methane Potential assay"	16 hours 24 hours

The Biochemical Methane Potential (BMP) assay

The Biochemical Methane Potential (BMP) assay is a biological test that allows to evaluate, in batch mode, the maximum quantity of methane and/or biogas producible from a specific organic matrix.

For the purpose of the assay, a known quantity of the testing product, inoculated with an anaerobic mud preventively acclimatized at 35°C for at least 48 h, was placed in anaerobic conditions with constant temperature (37°C). The quantities of biogas produced by the biological process was measured, determining also the quantities of methane and carbon dioxide produced, expressed as percentage of the total biogas production. Subsequently, the collected data were processed to calculate the production of biogas and/or methane attributable to the organic substance present in the inoculation mud, as well as, to the endogenous decay of the biomass.

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First trial

"Effect of Anavrin (Vetos Europe SAGL) on in vitro methane production in a 20 hours Biochemical Methane Potential assay" – Survey on the effect of a pool of essential oils, bioflavonoids and tannins on the production of methane through Biochemical Methane Potential (BMP) assay in organic matrix in a 20 hours fermentation assay

The purpose of the first assay was to measure at 20 hours, the total production of methane and biogas consequent to the addition of different concentration of Anavrin (Vetos Europe SAGL, via delle Industrie 18, 6593 - Cadenazzo (TI), Switzerland), in a biomethane reactor using as substrate the anhydrous glucose.

Materials and methods

In this assay the Anavrin was in a powder form with a concentration of 10%. The assay consisted in 5 different tests (Table 2), one without any addition (Test 1, white control), and the others (Test 2 to 5) with four different concentrations of Anavrin on the dry matter basis of the reference substrate (glucose anhydrous).

Table 2: Experimental protocol

Parameter	Unit	TEST 1	TEST 2	TEST 3	TEST 4	TEST 5
Anavrin	% $DM_{glucose}$	0,000	0,005	0,050	0,250	0,500

Times and methods of tests execution

Each one of the five tests were conducted in duplicate (two replicates). Each test was done in a specific close reactor, maintained in anaerobic conditions with constant temperature (37°C) for the entire time period of the test, and opened at the 20^{th} hour. The quantity of glucose and Anavrin used, were defined referring to the following conditions that have to be respected for the preparation of the reactors:

- Reactor's final volume 750 mL
- Glucose concentration in the reactor 2,7 g/L
- Ratio M/F (glucose/inoculum) 0,25

The quantities of glucose and Anavrin used for the different tests are reported in table 3.

Table 3: Quantities of glucose and product Anavrin used in the different tests

Substance	units	TEST 1	TEST 2	TEST 3	TEST 4	TEST 5
Glucose anhydrous	g	2,0	2,0	2,0	2,0	2,0
Anavrin	g	0,00000	0,00010	0,00100	0,00500	0,01000

Preparation of the glucose solution

The needed dosage of glucose, equal to 2,0 g for all the tests, was administered adding, in every reactor, 20 mL of a solution at 10% prepared based glucose anhydrous.

Preparation of the solutions of the product Anavrin

Considering the small quantities used, for the dosage of essential oil, diluted solutions were prepared by providing the following dosages:

- 1: 50.000 for test 2 and 3
- 1: 2.500 for test 4 and 5

Results

The results of the 5 tests are shown in Table 4.

Table 4: Production of methane and biogas and speed of methane production at 20 hours (in the brackets are reported the percentual difference compared to the "white" control test 1):

	Anavrin dosage [% DMglucose]	BMPf [Nm^3CH_4 t_{TQ}^{-1}]	Fmax [Nm^3 t_{TQ}^{-1} d^{-1}]	Biogas [Nm^3 t_{TQ}^{-1}]
Test 1	-	188,89	227,5	304,34
Test 2	0,005	162,21 [-14,12%]	199,5 [-12,31%]	250,00 [-17,86%]
Test 3	0,05	151,11[-20,00%]	186,9 [-17,85%]	245,65 [-19,28%]
Test 4	0,250	155,56 [-17,65%]	191,5 [-15,82%]	262,22 [-13,84%]
Test 5	0,5	171,11 [-9,41%]	209,8 [-7,78%]	256,52 [-15,72%]

The inclusion of the product Anavrin at the concentration of 0.005% (Test 2) of the dry matter of the reference substrate (glucose), reduced the methane production (BMP_f) by 14% after 20 hours of incubation, compared with the white test (Test 1). It reduced also the speed of methane production by 12,31% after 20 hours of incubation.

The inclusion of the product Anavrin at the concentration of 0.05% (Test 3) of the dry matter of the reference substrate (glucose), reduced the methane production (BMPf) by 20% after 20 hours of

incubation, compared with the white test (Test 1). It reduced also the speed of methane production by 17,85% after 20 hours of incubation.
The inclusion of the product Anavrin at the concentration of 0.250% (Test 4) of the dry matter of the reference substrate (glucose), reduced the methane production (BMPf) by 17,65% after 20 hours of incubation, compared with the white test (Test 1). It reduced also the speed of methane production by 15,82% after 20 hours of incubation.
The inclusion of the product Anavrin at the concentration of 0.5% (Test 3) of the dry matter of the reference substrate (glucose), reduced the methane production (BMPf) by 9,41% after 20 hours of incubation, compared with the white test (Test 1). It reduced also the speed of methane production by 7,78% after 20 hours of incubation.

Conclusions

Analysing the data in a time range representative of the rumen kinetics, stipulated as 20 hours, the inclusion of Anavrin has led to a sharp decrease in both methane and biogas production. The speed of methane production (Fmax) was also considerably lower following the use of essential oils to highlight their ability to reduce methanogenesis.
Specifically, the better results, in terms of either methane and biogas production and speed of methane production, merged in the Test 3, which corresponds to the concentration of Anavrin identified as ideal for the inclusion in the diet of dairy cows, beef cattle and ruminants in general.
A lower effect of the product was instead observed in test 5 presumably due to the excessive activity of limiting ruminal microorganisms in the presence of a dosage 10 times higher than that considered ideal.
In conclusion, the inclusion of Anavrin made it possible to reduce methane production up to 20%.

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Second trial

"Effect of Anavrin (Vetos Europe SAGL) on in vitro methane production in a 16- and 24-hours Biochemical Methane Potential assay" – Survey on the effect of a pool of essential oils, bioflavonoids and tannins on the production of methane through Biochemical Methane Potential (BMP) assay in organic matrix in a 16- and 24-hours fermentation assay

Considering the positive results obtained from the first assay, the aim of the second one was to measure at 16 and 24 hours, the total production of methane, biogas, and volatile fatty acids, consequent to different concentration of Anavrin (Vetos Europe SAGL, via delle Industrie 18, 6593 - Cadenazzo (TI), Switzerland), and also in comparison to monensin sodium, a proved antibiotic able to reduce methanogenesis and increase propionic volatile fatty acid production.
These timings were chosen to better understand the beginning of methanogenic bacteria inhibition and his entity after 24 h of incubation.

Materials and methods

In the second assay the Anavrin was pure and in a liquid form. The assay consisted in 5 different tests (Table 5), first without any addition (Test 1, white control), second with the addition of monensin sodium (with a concentration of 20% of the active ingredient), and the others (Test 3 to 5) with three different concentrations of Anavrin on the dry matter basis of the reference substrate (glucose anhydrous).

Table 5: Experimental protocol

Parameter	Unit	TEST 1	TEST 2	TEST 3	TEST 4	TEST 5
Anavrin	% $DM_{glucose}$	-	-	0,0025	0,005	0,025
Monensin sodium (antibiotic)	% $DM_{glucose}$	-	0,006	-	-	-

Times and methods of tests execution
Each one of the five tests were conducted in duplicate (two replicates). Each test was done in a specific close reactor, maintained in anaerobic conditions with constant temperature (37°C) for the entire time period. For each test, one of the duplicates was opened at the 16th hours while the second one at the 24th hour. The quantity of glucose, Anavrin and monensin used were defined referring to the following conditions that have to be respected for the preparation of the reactors:

- Reactor's final volume 750 mL

- Glucose concentration in the reactor 2,7 g/L
- Ratio M/F (glucose/inoculum) 0,25

The quantities of glucose, Anavrin and monensin used for the different tests are reported in the following table (Table 6).

Table 6: Quantities of glucose, essential oils and monensin used

Substance	units	TEST 1	TEST 2	TEST 3	TEST 4	TEST 5
Glucose anhydrous	mg	2000	2000	2000	2000	2000
Anavrin	mg	-	-	0,05	0,1	0,5
Monensin (antibiotic)	mg	-	0,12	-	-	-

Preparation of the glucose solution

The needed dosage of glucose, equal to 2,0 g for all the test, was administered adding, in every reactor, 20 mL of a solution at 10% prepared based glucose anhydrous.

Preparation of solutions of the essential oil and monensin

Considering the small quantities used, for the dosage of essential oils and antibiotic diluted solutions were prepared by providing the following dosages:

- 2,4 mL of a solution 1: 20.000 for the test 2;
- 2,5 mL of a solution 1: 50.000 for the test 3;
- 2,5 mL of a solution 1: 25.000 for the test 4;
- 12,5 mL of a solution 1: 25.000 for the test 5.

The test 1 represent the reference (white test), and therefore for this test neither the addition of essential oils or monensin sodium was foreseen.

Analysis of the volatile fatty acids (VFA) production on the digestate

At the end of the BMP assays, samples of digestate were collected. Subsequently, on those samples, the concentrations of volatile fatty acids (VFA) were determined with a mass gas chromatography analysis (MP 58C/2018 rev.0).

RESULTS

Production of methane and biogas

The results about the production of methane and biogas are reported below in Table 7.

Table 7: Production of methane and biogas in the five tests (in the brackets are reported the percentual difference compared to the "white" control test 1)

	Anavrin dosage [% $DM_{glucose}$]	Antibiotic dosage [% $DM_{glucose}$]	BMP_f [Nm^3CH_4 t_{TQ}^{-1}]	Biogas [Nm^3 t_{TQ}^{-1}]
Test 1	-	-		
16h			142,7	236,5
24 h			228,9	368,9
Test 2	-	0,006		
16h			131,3 [-7,99%]	206,8 [-12,56%]
24h			155,6 [-32,02%]	255,6 [-30,71%]
Test 3	0,0025	-		
16 h			138,2 [-3,15]	224,1 [-5,24%]
24h			210,5 [-8,03%]	330,1 [-10,52%]
Test 4	0,005	-		
16h			130,3 [-8,69%]	210,6 [-10,95%]
24 h			177,7 [-22,37%]	280,2 [-24,04%]
Test 5	0,025	-		
16 h			129,6 [-9,18%]	208,3 [-11,92%]
24 h			149,1 [-34,86%]	246,2 [-33,26%]

Analysis of the volatile fatty acids (VFA) production on the digestate

At the end of the BMP assays, samples of digestate were collected. Subsequently, on those samples, the concentrations of volatile fatty acids (VFA) were determined with a specific analysis.

Acetic and propionic acids were detected in all the five tests, while the other acids were absent or present in concentrations lower than the detection limit of 50 mg/L.

Volatile fatty acids (VFA) concentrations are reported in Table 8.

Table 8: Volatile fatty acids concentrations (in the brackets are reported the percentual difference compared to the "white" control test 1)

	Anavrin dosage [% DMglucose]	**Antibiotic dosage** [% DMglucose]	**Acetic acid** [mg/l]	**Propionic Acid** [mg/l]
Test 1	-	-		
16h			228	180
24 h			178	194
Test 2	-	0,006		
16h			191 [-16,2%]	202 [+12,22%]
24h			127 [-28,7%]	228 [+17,55%]
Test 3	0,0025	-		
16 h			211 [-7,45%]	188 [+4,44%]
24h			167 [-6,18%]	204 [+5,15%]
Test 4	0,005	-		
16h			205 [-10,1%]	195 [+8,33%]
24 h			133 [-25,3%]	222 [+14,43%]
Test 5	0,025	-		
16 h			62 [-72,8%]	90 [-50,0%]
24 h			36 [-79,8%]	67 [-65,5%]

The results highlight that the use of monensin sodium (at 20% of the active ingredient), at the concentration on 0.006% of the dry matter of the reference substrate (glucose), that is equal to a real provision of 0.120 g/head/d in beef cattle with an average feed intake of 10 kg of dry matter, reduced the methane production (Table 7) (BMP_f) by 8% after 16 hours of incubation and by 32% after 24 hour. It also reduced the production of acetic acid by 16% after 16 hours of incubation and by 29% after 24 hours, while the production of propionic acid resulted to be enhanced by 12% after 16 hour of incubation and by 18% after 24 hours (Table 8).
The inclusion of Anavrin at the concentration of 0.0025% of the dry matter of the reference substrate (glucose), that is equal to a real provision of 0.1 g/head/d in beef cattle with an average feed intake of 10 kg of dry matter, reduced the methane production (Table 7) (BMP_f) by 3% after 16 hours of incubation and by 8% after 24 hours. It reduced also the production of acetic acid by 7% after 16 hours of incubation and by 6% after 24 hours, while the production of propionic acid resulted to be enhanced by 4% after 16 hour of incubation and by 5% after 24 hours (Table 8).
The inclusion of Anavrin at the concentration of 0.005% of the dry matter of the reference substrate (glucose), that is equal to a real provision of 0.5 g/head/d in beef cattle with an average feed intake of 10 kg of dry matter, reduced the methane production (Table 7) (BMPf) by 9% after 16 hours of incubation and by 22% after 24 hours. It reduced also the production of acetic acid by 10% after 16 hours of incubation and by 25% after 24 hours, while the production of propionic acid resulted to be enhanced by 8% after 16 hour of incubation and by 14% after 24 hours (Table 8).
The inclusion of Anavrin at the concentration of 0.025% of the dry matter of the reference substrate (glucose), that is equal to a real provision of 2.5 g/head/d in beef cattle with an average feed intake of 10 kg of dry matter, reduced the methane production (Table 7) (BMP_f) by 9% after 16 hours of incubation and by 35% after 24 hours. It reduced also the production of acetic acid by 73% after 16 hours of incubation and by 80% after 24 hours, and also the production of propionic acid resulted to be lowered by 50% after 16 hour of incubation and by 66% after 24 hours (Table 8).

Conclusions

The use of Anavrin at the concentration of 0.005% of dry matter of the reference substrate (glucose), which corresponds to the concentration of Anavrin identified as ideal for the inclusion in the diet of dairy cows, beef cattle and ruminants in general, has an efficacy in the reduction of methane and acetic acid and in the increasing of the propionic acid that is similar to the efficacy of monensin sodium.
The use of Anavrin at the highest concentration, equal to the 0.025%, strongly inhibit the methane production.
Considering the two different times (16 and 24 hours) separately, it seemed that the inhibition of the methanogenesis, at the 16^{th} hours, has just begun and increase up to 24^{th} hour. The difference in inhibition capacity between the different dosage of the product, followed the same trend between the two different times.
In conclusion, the inclusion of Anavrin made it possible to reduce at 24 hours of incubation the methane production up to 34,86%.

General conclusions

Considering the results of the two different experimental studies, the Anavrin (Vetos Europe SAGL, via delle Industrie 18, 6593 - Cadenazzo (TI), Switzerland) has been effective in inhibiting methanogenesis, reducing it in each test and up to the maximum percentage of 34.86%. Moreover, the product maintained satisfactory inhibition levels in each of the concentrations used. The modulating action of the Anavrin product on rumen fermentations towards more efficient and sustainable fermentative pathways, is also clearly showed by the increase in the production of propionic acid, more energetic and less methanogenic. Indeed the addition of Anavrin leaded to an increase in the propionic acid up to 14.43%, while acetic acid, less energetic and main source of hydrogen ions at rumen level, has resulted always significantly reduced. The product has therefore shown, in vitro, to inhibit the methanogenesis and, to positively modified the rumen population and fermentations.

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