THE EFFECTS OF GRASS SILAGE TREATED WITH EM-SILAGE ON METHANE AND VOLATILE FATTY ACID PRODUCTION IN THE RUMEN

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1. INTRODUCTION

Methane, the second main greenhouse gas, contributes almost to 18% of the greenhouse effect. Its production in the digestive tract of farm animals is estimated to be responsible for 22% of the anthropogenic sources and is mainly due to the rumen fermentation. Moreover, the methane produced reduces the energy available for the animal. Up to 10% of the gross energy in the feed is lost as methane (Jouany, 1994). Thus managing the microbial activity of the rumen to reduce the methane production is an important goal for nutritionists.

Grass silage is the major ingredient in the ration of dairy cows. A good quality grass silage can be obtained by a proper silage fermentation. EM-silage is an inocculant which is added to the grass during the ensiling process. It contains selected species of microorganisms including predominant populations of lactic acid bacteria and yeasts and smaller numbers of photosynthetic bacteria, actinomycetes and other types of organisms.

The object of the study is to carry out an experiment to determine the effect of grass silage treated with EM-silage on the volatile fatty acid and methane production in vitro.

2. METHANE PRODUCTION IN RUMINANTS

2.1 Production of methane in ruminants

As a consequence of the degradation of carbohydrates in the rumen, the end products are primarily hexoses and pentoses. Subsequently they are used by the microbes either to supply carbon skeletons for the microbial biomass or to provide energy for maintenance and growth requirements.

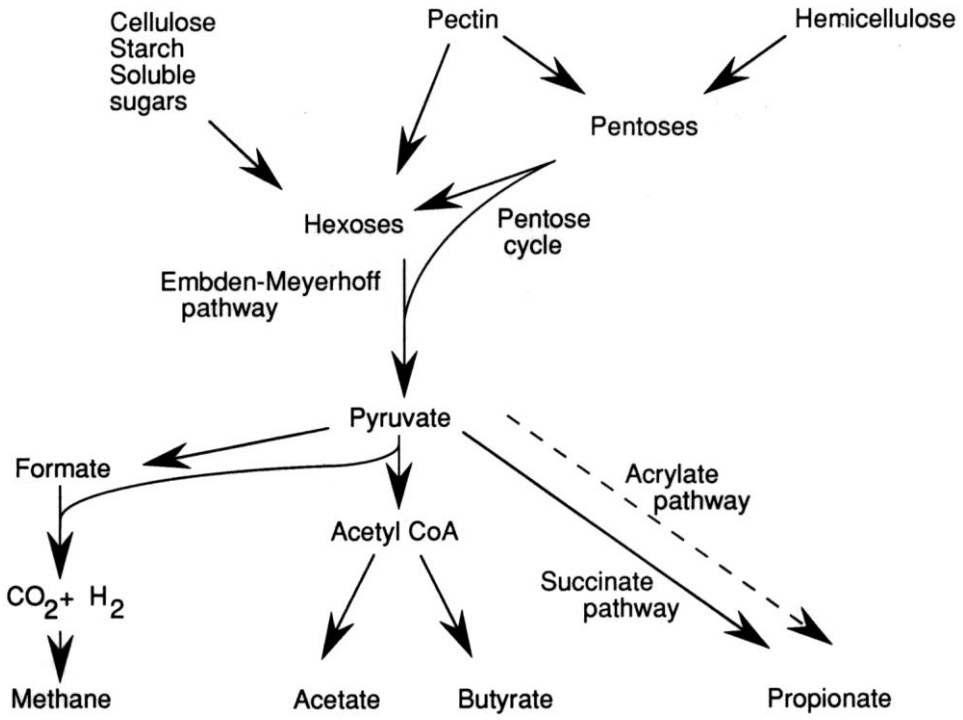

Fig. 5.1. A schematic representation of the major pathways of carbohydrate metabolism in the rumen.

Energy is generated by the degradation of the hexoses and pentoses in VFA. The primary pathway of hexoses fermentation is the Embden-Meyerhoff pathway. The oxidation of one molecule of glucose leads to the production of two molecules of pyruvate and the reduction of two co-factors NAD. The pyruvate is then degraded to VFA, mainly acetate (HAc), propionate (HPr) and butyrate (HBr).

The stochiometric reactions of the fermentation of one molecule of glucose are as follows (Hungate, 1966):

1 mol Hexose + 2 H_2O → 2 Acetate + 2 CO_2 + 4 H_2
1 mol Hexose + 2 H_2 → 2 Propionate + 2 H_2O
1 mol Hexose → 1 Butyrate + 2 CO_2 + 2 H_2

The formation of propionic acid is the only reaction that requires the uptake of H_2. The other reactions lead to the production of hydrogen. The ratio in which the VFA are produced is

important since the VFA will not have the same utilisation later in the body. All the VFA can be used to generate ATP in the intermediary metabolism, but unlike acetic and butyric acid, propionic acid can be used later as a glucose precursor (Dijkstra, 1993).

Also H_2 is a major end product of the fermentation by protozoa, fungi and bacteria, it does not accumulate in the rumen because it is immediately used by other bacteria which are present in the rumen. The stoichiometry of the methane formation in the rumen is:

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

Looking at this equation, it appears that the methane produced by the methanogenic bacteria can be seen as a disposal route for the hydrogen fermentation. It is assumed that about 90% of the hydrogen is used for the production of methane. Other major routes are the propionate production and the hydrogenation of unsaturated fatty acids.

2.2 Possible effect of the use of inocculants in grass silage on methane production in the rumen

Both Cushnahan (1995) and Huhtanen et al. (1997) compared the addition of a fermentation inhibitor (formic acid) with the addition of a fermentation enhancer (lactic acid bacteria) in grass silage. In both experiments the propionic acid production in the rumen of dairy cows increased and the acetic acid production decreased with the inocculant treated grass silages.. It has to be noticed both experiments were conducted with grass silages with, compared to Dutch practices, low dry matter percentages. The methane production in the rumen was not measured in these two experiments, however an lower methane production is expected through the shift in volatile fatty acid production which leads to a decreased hydrogen surplus in the rumen

3. MATERIAL AND METHODS IN VITRO EXPERIMENT

The objective of this experiment is to study the effects of grass silage treated with EM-silage on the methane production and the VFA production. The kinetics of fermentation will be determined using the cumulative gas production technique. In addition, the gas and the VFA composition will be determined at different time points by gas chromatography.
The principle of the cumulative gas technique is to measure the gas produced in the head space of a sealed serum bottle (Williams, 2000) using a pressure transducer in a manual run. The procedure requires milled substrate as energy source, an anaerobic medium as a nutrient source and a mixed microbial population as an inoculum. The pre-weight substrate is suspended in a medium, the mixture warmed at 39°C and a freshly collected sample of rumen fluid added as inoculum. From that moment onwards the gas production is recorded at different time points. The experiment was conducted at, and in cooperation with, Wageningen University, department Animal Nutrition.

Sample preparation

Two representative grass silages were obtained from ID-Lelystad. One control grass silage and one grass silage treated with EM-silage. 0.5±0.0001 g of each substrate was weighed in each bottle.

The media

The medium B is a complex semi-defined medium (some components are defined but not all of them) that should provide most of the micro-organisms with their requirements except for energy that is provided by the substrate (Williams, 2000). This medium is designed for bacteria that are strictly anaerobic, so it needs to be prepared under anaerobic conditions and the components have to be kept sterile before use.

Serum bottles of 100 ml, where the substrates was already been weighed in, were filled, under CO_2, with the 82 ml of media which composition was:
- 76 ml of basal solution
- 1 ml of vitamin/phosphate solution
- 4 ml of bicarbonate buffer
- and 1 ml of reducing agent

The Inoculum

On the day of the inoculation, one litre of rumen fluid with feed particles was collected before the morning feeding from 3 different dry cows. It was stored in a warmed thermos and filled with CO_2 in order to keep anaerobic conditions. The rumen fluid of the three different cows was mixed in a large beaker filled with CO_2. This rumen fluid was filtrated trough a double layer of cheesecloth sitting in a large funnel and squeezed out into a beaker, with CO_2 flowing into it to extract as much as possible of liquid.

Inoculation of the bottles

Before the inoculation the bottles with the substrate and the media were warmed at 39°C and some reducing agent was re-added to the bottles in which the colour indicator was still pink. The bottles were inoculated at t0 with 5 ml of rumen fluid. It must be noticed that the

inoculation started with the 72h bottles and ended with the 0h bottles. Due to the large number of bottles, and the fact that the rumen fluid temperature was not kept constant 39°C, the temperature of the rumen fluid was around 25°C at the end of the inoculation.

The schedule

For the VFA and the bottles were autoclaved at each time point. The gas reading was realised on 3 other bottles at 19 time points and the gas composition was determined on the same bottle. These bottles were autoclaved at the end of the run to determine the VFA. Every treatment has been carried out in three replicates at each time point.

Table 3.1: Schedule

Time points	***1***	*2*	*3*	***4***	*5*	***6***	*7*	***8***	*9*	***10***	*11*	*12*	***13***	*14*	***15***	*16*	*17*	*18*	***19***
Hours (h)	**0**	2	4	**6**	9	**12**	15	**18**	21	**24**	28	32	**36**	42	**48**	54	60	66	**72**
VFA analysis	√			√		√				√									√
Gas composition				√		√				√									√
Gas reading	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√	√

The manual total cumulative gas technique was used. Thanks to the transducer, the pressure in the bottle was determined at each time point and registered automatically under the software. Moreover, the volume of gas was measured with a syringe (when the pressure in the bottle was equal 0, the volume was read on the syringe). Unless the gas composition needed to be determined, the gas was released in order to bring back the pressure and the volume of gas in the bottle to 0.

Gas composition

After the gas reading, the gas was left in the bottle and using a syringe, 2 ml of gas were exactly collected and released in a vacutainer (No 367614 with HenGard® safety closure; Becton Dickinson) to be analysed (H_2, CH_4, CO_2) by gas chromatography.

VFA analysis

The bottles were incubated at 39°C with a needle in the stopper in order to let the gas go out. At each time point two bottles per substrate were autoclaved during 20 min at 120°C. It must be noticed that the autoclave was cold when the bottles were put inside. So, mainly at the beginning, the fermentation has gone on during the warming up of the autoclave. The bottles were then cooled down and stored in a cooling room.

For the sampling, the bottles were desealed. With a syringe 5 ml of rumen fluid were taken out for the VFA analysis and 5 ml for the NH_3 in the fluid taking care not to take any feed particle. For the VFA, 0.250 ml of phosphoric acid 85% and 5 ml of trichloroacetic acid (TCA) 10% for the NH_3 were added to the sample. Those samples were then frozen. Moreover at the same moment the pH of the solution was measured.

VFA were analysed by gas chromatography. The samples were defrozen and 1 ml was collected and centrifuged at 12300 rpm during 10 min. 0.5ml of supernatant phase were taken with an automatic pipette and mixed with 0.2 ml of water and 0.3 ml of the internal standard (caproic acid). The VFA were then determined by gas chromatography (ref: HRGC mega 2 from Carbo Erba; the column was 6 feet long with an internal diameter of 2 mm and an outside diameter of 0.25 inch; the column was filled with chromabsorb and its temperature was 190°C; direct injection in the column with flame ionisation detector (FID); the temperature of the injection was 185°C and of the detector 225°C; the carrier gas was very pure nitrogen saturated with formic acid and the data analysis was realised with chromcard from Fison instrument)

Dry matter and organic matter left

The content of the bottles was filtered on crucibles (N2) with treated sand that have previously been washed, dried and weighed. The crucibles were washed with hot demineralised water. They were dried for 4h at 120°C and cooled down in a dessiccator for 1 hour before being weighed to determine the DM left. Finally the crucibles were ashed at 550°C for 2 hours and cooled down in a dessiccator for 1h30 for being weighed to determine OM left.

Statistical analysis

The data are analysed with the statistical package SAS. The analysis of differences are carried out by the Tukey-Kramer procedure. Differences of $P< 0.05$ were considered to be significant. The model for each dependent variable was the following:

- $Y_{ijk} = U + a_i + b_j + w_{ij} + e_{ijk}$

  With: a_i, effect of the substrate i = (1,2)
  b_j, effect of the times j = (1,.., 4)
  w_{ij}, interaction between the two factors
  And e_{ijk}, the error term (k = 1,2)

The dependant variables (Y_{ijk}) were:

- Acetate, propionate, butyrate, produced per gram of OM incubated
- VFA produced per gram of OM consumed (yield)
- NGR Ratio: (HAc + HiBu + HBu + HiVal + HVal)/(HPr + HiVal + HVal)
- The acetate: propionate ratio
- Methane calculated;
  CH_4= 0.5 HAc – 0.25 HPr + 0.5 (HBu + HiBu) – 0.25 (HVal + HiVal)
- Ammonia at 72 hours
- Organic matter break down
- Methane analysed
- Hydrogen and carbondioxide analysed

4. RESULTS

Two representative grass silage samples were obtained from ID-Lelystad who previously conducted an experiment to determine the effect of EM-silage on the ensiling process and aerobic stability of the silage after opening (report nr. 2165, ID-Lelystad). In the next table the results of this experiment regarding the composition of the grass silages are given.

Table 4.1 Effect of EM-silage on characteristics of silage after two months incubation (Wikselaar et al., 2000)

	Control	+ EM-Silage
Dry matter (g/kg)	451	440
Weight loss (g/kg)	11.5	24.0
PH	5.11	4.42
Yeasts (log kve / g)	2.15	< 2
Moulds (log kve / g)	< 2	< 2
Lactic acid (g / kg dm)	41.9	79.3
Acetic acid (g / kg dm)	7.6	36.2
Ethanol (g / kg dm)	11.2	17.7
1,2-Propanediol (g / kg dm)	0	10.0
2,3-Butanediol (g / kg dm)	0.3	0.3
Propionic acid (g / kg dm)	2.2	2.4
1-Propanol (g / kg dm)	0	2.3
Ammonia (g / kg dm)	2.5	3.5

Compared to the control silage, the silage treated with EM-silage showed a higher weight loss and higher levels of lactic and acetic acid. The pH was consequently lower of the silage treated with EM-silage.
The results of the chemical analysis of the silages conducted by the Wageningen University are listed in the following table.

Table 4.2 Chemical analysis of the grass silages (in g/kg d.m.)

	Control	+ EM-silage
Dry matter (g / kg)	905.4	928
N	30.15	31.99
NDF	369	391
Crude fat	29.1	36.6
Ash	86.9	93.0

In the next tables the results of the in vitro experiment are presented in table 4.3 and 4.4. The results are an average of the measurements at time points 6, 12, 24 and 72 hours (unless otherwise stated).

Table 4.3 Effect of the use of EM-silage in grass silage on the production of individual VFA, the OM break down after 72 hours, the NGR ratio, Ammonia level after 72 hours and the calculated methane production.

Parameter	Unit	Grass silage	
		Control	+ EM-silage
Hac	mmol/ g om	3,76	3,39 *
Hpr	mmol/ g om	1,40	1,48 *
Hibr	mmol/ g om	0,060	0,064
Hbr	mmol/ g om	0,52	0,57 *
Hival	mmol/ g om	0,13	0,11
Hval	mmol/ g om	0,12	0,10
Hac	%	64,3	60,9 *
Hpr	%	23,0	25,4 *
Hbr	%	8,5	9,7 *
Hac/Hpr		2,85	2,42 *
Organic matter break down	%	87	85
NGR		3,33	3,02 *
NH_3	Mg/l	429	438
CH_4 calculated	mmol/ g os	1,75	1,59 *

: difference with control silage is significant (: p<0,01).

Tabel 4.4 Effect of grass silage treated with EM-silage on the analysed content of H_2, CO_2 and CH_4 per g incubated (inc) and degraded (deg) organic matter. The results are calculated per g incubated (inc.) and degraded (deg) organic matter

Parameter	Unit	Grass silage	
		Control	+ EM-Silage
H_2	µl/ 2 ml/ g om inc	22,7	28,2 #
H_2	µl/ 2 ml/ g om deg	26,1	33,3
CO_2	µl/ 2 ml/ g om inc	3929	3803
CO_2	µl/ 2 ml/ g om deg	4464	4492
CH_4	µl/ 2 ml/ g om inc	444	406
CH_4	µl/ 2 ml/ g om deg	510	479
CH_4/CO_2	%	10,2	9,6

#: difference with control silage is significant (#: p<0,1).

Both the production of acetic acid in mmol/g om and the molar percentage acetic acid were significant lower in the EM-silage treated grass silage. The production of butyric and

propionic acid (in mmol/g om and the molar percentage) was significant higher. As a consequence of this shift in production of volatile fatty acids the acetic acid / propionic acid ratio and the NGR decreased. Also the methane production calculated from the production of volatile fatty acids decreased significantly.

In the measured gas composition only the hydrogen production from the silage treated with EM-silage was significantly different from the control silage. However not significantly different, a clear effect of EM-silage on the measured methane production was seen (expressed per gram incubated organic matter and per gram degraded organic matter).

5. CONCLUSIONS AND RECOMMENDATIONS

- In the literature indications are found that a shift in volatile fatty acid production in the rumen by using an inocculant consisting of lactic acid bacteria can be achieved. The reason for this shift is not clear yet, the higher level of lactic acid in the silages treated with the inocculant could play a role.

- The grass silage treated with EM-silage showed a significantly higher propionic acid production and a significantly lower acetic production compared to the control silage. This resulted in a decrease of the calculated methane production based on the volatile fatty acid production and a (not significantly different) decrease in the measured methane production by gas analysis.

- In many rations for dairy cows (particularly rations with a high percentage of grass silage) the glucogenic nutrients are limiting. An increase in the production of propionic acid has positive effects on milk and milk protein production.

- From this in vitro research it can be concluded EM-silage has a positive effect in decreasing the methane production in the rumen and increasing the production of glucogenic nutrients. Because of the large practical potential of the results of this experiment, it is important to obtain more knowledge about the effects of the use of EM-silage in grass silages.