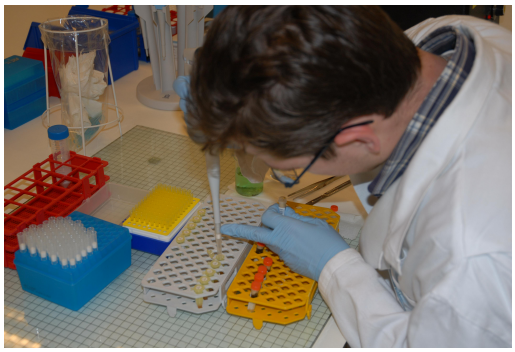
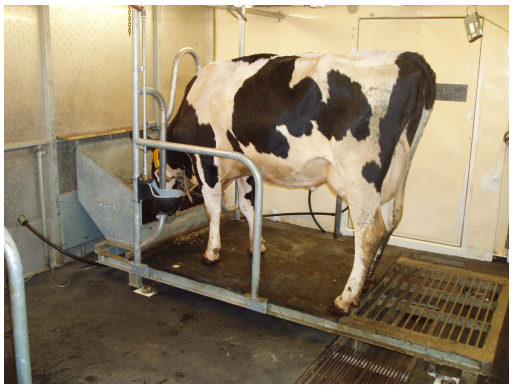
Research Report

PROTOZOA INHIBITORS TO REDUCE METHANE EXCRETION FROM DAIRY COWS





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Aan dit project is in het kader van het Besluit milieusubsidies, Subsidieregeling milieugerichte technologie een subsidie verleend uit het programma Reductie Overige Broeikasgassen dat gefinancierd wordt door het Ministerie van Volkshuisvesting, Ruimtelijke Ordening en Milieubeheer. SenterNovem beheert deze regeling.

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1a. SHORT SUMMARY

Effects of feed additives on enteric methane emission and rumen protozoa in dairy cows were measured in two short-term experiments. A second objective was to evaluate alternative methods for quantifying methane emissions and rumen protozoa.

Emissions of methane were reduced by 10-15% and this was associated with a 28% fall in protozoal population size. Improvements in bacterial N efficiency resulting from the fall in protozoa may explain the reduction in milk urea concentration seen in response to the additives. Dry matter intake, milk yield and milk protein production were not affected by the feed additives, but milk fat concentration was reduced. The proportion of undesirable saturated fatty acids in milk fat increased: this was probably due to the specific dietary fats used (lauric and myristic acids).

Results confirm that methanogenic bacteria physically associated with protozoa make a significant contribution to enteric methane emissions. Progress was made in the development of new genetic methods for rapid quantification of rumen protozoa but refinement and validation is needed. Two methods of methane measurement yielded similar estimates of the response to the feed additives, although measurements in a commercial tie-stall barn were, on average, higher than those in respiration chambers.

Feed additives have potential to significantly reduce methane production from Dutch animal production. For example, application of these results to all 1.43 million Dutch dairy cows would generate a reduction of between 0.44 and 0.66 Mton CO_2-equivalents per year. Greater savings would be achieved by applying such additives to other ruminants or by developing more effective additives.
Cost-effectiveness and persistency of response were not measured in this 'proof of principle' study. Further work is required to identify optimal combinations of feed additives that give cost-effective reductions in methane emissions over long periods, without detrimental effects on milk fatty acid profile.

1b. KEYWORDS

Methane reduction, dairy cows, proof of principle, feed additives, dietary fatty acids, milk urea, protozoa, genetic profiling and quantification, rumen fermentation

2a. KORTE SAMENVATTING

In dit project zijn de effecten van voederadditieven op de methaanemissie en pensprotozoa van melkkoeien onderzocht in twee korte termijn experimenten. Een tweede doel van dit project was om alternatieve methoden te ontwikkelen om methaanemissie en pensprotozoa te kwantificeren.

Er werd een reductie van de methaanuitstoot gerealiseerd van 10-15%. Dit ging gepaard met een afname van 28% van protozoa in de pens. Een verbetering van de bacteriële N efficiëntie, wat een gevolg is van de daling van het aantal pensprotozoa, is een mogelijke verklaring voor de waargenomen daling van het ureumgehalte in de melk. Droge stofopname, melkproductie en melkeiwitproductie waren niet beïnvloed door de gevoerde additieven. Het vetgehalte in de melk was gedaald. Het gehalte aan ongewenste verzadigde vetzuren in het melkvet nam toe: dit wordt toegeschreven aan bepaalde vetzuren in het rantsoen (laurine- en myristinezuur).

Deze resultaten bevestigen dat de methanogene bacteriën die fysiek gekoppeld zijn aan protozoa een significante bijdrage leveren aan methaanemissie door melkvee. Er is vooruitgang geboekt bij de ontwikkeling van nieuwe genetische methoden voor snelle kwantificering van pensprotozoa, maar verfijning en validatie is noodzakelijk. De twee gebruikte methoden om methaanemissie te meten gaven beiden een vergelijkbare schatting van de relatieve methaanreductie. Methaanmetingen in de aanbindstal gaven echter een hoger absoluut niveau.

Voederadditieven hebben de potentie om de methaanuitstoot van de Nederlandse melkveestapel significant te verlagen. Als bijvoorbeeld de resultaten van dit project worden toegepast op alle 1,43 miljoen melkkoeien in Nederland, resulteert dit in een reductie van 0,44 tot 0,66 Mton CO_2-equivalenten per jaar. Grotere reducties zijn mogelijk wanneer voederadditieven worden gevoerd aan andere herkauwers, of wanneer efficiëntere additieven worden ontwikkeld.

Kosteneffectiviteit en persistentie zijn niet getest in dit huidige 'proof of principle' project. Er is meer onderzoek nodig om de optimale combinatie van voederadditieven te vinden. Dat moet resulteren in een kostenefficiënte reductie van methaan gedurende lange periodes, zonder nadelige gevolgen voor het vetzuurpatroon in de melk.

2b. TREFWOORDEN

Methaanreductie, melkvee, proof of principle, voederadditieven, vetzuren, melkureum, protozoa, genetisch profileren en kwantificeren, pensfermentatie

3. INTRODUCTION

3.1 Problem statement

While carbon dioxide receives the most attention as a factor in global warming, there are other gases to consider, including methane. In an effort to combat global warming, reducing methane emissions is an attractive target. Firstly, methane has a global warming potential 21 times that of carbon dioxide (IPCC, 2001). Secondly, methane is broken down quite rapidly in the atmosphere; within 9-15 years (FAO, 2006). Therefore a fall in methane emission would quickly result in a reduction in atmospheric greenhouse gas concentration.

Methane production in the digestive tract of ruminants, called enteric fermentation, is one of the major sources of global methane emissions. According to the recent FAO report 'Livestock's Long Shadow', enteric methane emissions amount to almost 86 million tonnes of methane each year (FAO, 2006). With an extra 17.5 million tonnes of methane produced from manure, livestock are responsible for 37% of anthropogenic methane (FAO, 2006). The total share of livestock in CO_2-emissions is 9%.

3.2 Methane reducing strategies

General strategies to reduce methane emission from ruminants are well-known: increase the level of production, and hence spread the relatively fixed emission of methane over more units of milk and meat, decrease the use of slowly-digestible fibrous feeds or reduce the number of non-productive days per animal (e.g. increase longevity of dairy cows to reduce the number of non-productive days per lifetime). A complementary strategy is to influence rumen fermentation to reduce methane emissions through the use of feed additives. Conceptually, a number of approaches might be considered. One approach is to target rumen protozoa, which live in the rumen in a symbiotic relationship with archaea, which actually produce the methane. Several populations of archaea live associated with protozoa (Ushida *et al.,* 1997; see Figure 3.1). A number of authors have estimated the contribution of protozoa-associated archaea to total methane production. Methane production accounted for 12% of gross energy intake in beef cattle fed a barley-based diet (Whitelaw *et al*., 1984). This figure was only 6-8% in protozoa-free animals. Finlay *et al.* (1994) estimated that endosymbiotic archaea may generate 37% of rumen methane emissions, while Newbold *et al.* (1995) concluded that archaea accounted for 25% of methane emissions in sheep.

Figure 3.1: Archaea living symbiotically with protozoa

Methane production is a major sink for hydrogen produced during anaerobic fermentation, and removal of this hydrogen is essential to maintain an efficient rumen fermentation. Therefore, another approach to reduce methane emission is to find alternative sinks for that hydrogen (Moss and Newbold, 2001; Newbold *et al*., 2005).

Dietary strategies to reduce methane by eliminating, or inhibiting, protozoa were reviewed by Hegarty (1999) and Boadi *et al.* (2004). These include an increase in the proportion of grain in the diet, the use of specific fatty acids such as lauric- (C12:0), myristic- (C14:0) or linolenic acid (C18:3), detergents with high concentrations of trace minerals such as Cu and Zn and various natural compounds such as saponins.

3.3 Functions of protozoa

Protozoa perform many functions that are beneficial to the productivity of the host animal (summarised by Williams and Coleman, 1988). They contribute directly and indirectly to fibre digestion and facilitate cellullolytic bacteria by scavenging deleterious traces of oxygen from the rumen environment. Some genera of protozoa help prevent low rumen pH, which inhibits fibre digestion and microbial growth and can adversely affect the host. Because of these many and divergent effects, *in vitro* studies that focused solely on methane production must be complemented by whole animal studies in which the net effects of defaunation can be quantified. Until now, most studies have used sheep, goats or beef cattle as experimental models and effects of defaunation on the productivity of high-yielding dairy cows fed intensive diets are not well known. See Eugène *et al.* (2004) for an overview on defaunation.

3.4 Measuring techniques

Research on methane production and protozoa, single or together, has been restricted by technical limitations. This is particularly true for large ruminants such as dairy cows. The classical 'gold standard' technique for measuring methane and its contribution to the energy balance of the cow is to house the animal in a climate respiration chamber in which all gas emissions can be collected and analysed. Such facilities are relatively few in number and expensive to run.

Classically, rumen protozoa are classified and quantified by microscopic techniques using samples of rumen fluid obtained by intubation or via a rumen cannula (Dehority, 1984). However, these methods are laborious and prone to error. The major limitation of this method lies in the infeasibility of tracking spatial and temporal variations in protozoa within the rumen. In addition, Firkins and Yu (2006) state that the classical technique includes the incorrect assumption that protozoal numbers are indicative of total protozoal activity as it ignores the large range in mass between different protozoal species (15 to 250µm in length and 10 to 200 µm in width, Dehority (2003)). Newer genomic techniques such as Microbial Community Profiling and Characterisation (MCPC) analysis and Quantitative Polymerase Chain Reaction (Q-PCR) offer the prospect of a more efficient and accurate quantification and profiling of ruminal protozoa. However, these techniques have not yet been developed for protozoa and limited work has been done to calibrate genomic quantitative techniques against classical microscopy counts (Skillman *et al*., 2006).

3.5 Objectives

Recognizing the twin challenges of reducing methane production through defaunation and quantifying changes in both protozoa and methane, and in view of the limited published information on production responses to defaunation in lactating dairy cows, the objectives of this project were:

1. To show that methane production from dairy cows can be significantly reduced using feed additives that are known to influence the size of the rumen protozoa population.
2. To develop and evaluate alternative methods for detecting differences in the size and composition of the protozoal population and in methane production.
3. To monitor effects of defaunation on milk yield, milk composition and composition of the protozoal population.

At the request of ROB-Klimaat, these ambitious objectives were downscaled to give proof of principle first and to focus in a subsequent project on cost-effective feed additives with a persistent effect. The experiments described in this report are therefore meant to 'prove the principles' that protozoal population size and methane were amenable to manipulation through the use of feed additives and that these effects could be detected by available research methods. Therefore, feed additives were chosen that have been successful either *in vitro* or in other animal models in order to increase the probability of creating a difference in protozoa and methane production.

3.6 Partners and third parties in the project

The project was carried out in cooperation with Dr. Van Haeringen Laboratorium BV (VHL), situated in Wageningen, the Netherlands. VHL was mainly responsible for developing genetic methods to quantify and profile rumen protozoa. Third parties in this project were Wageningen University (WUR), for carrying out trials with climate respiration chambers, and Animal Sciences Group (ASG), for methane measurements at Provimi's experimental farm De Viersprong. The relationship between the different elements of the project is shown in Figure 3.2.

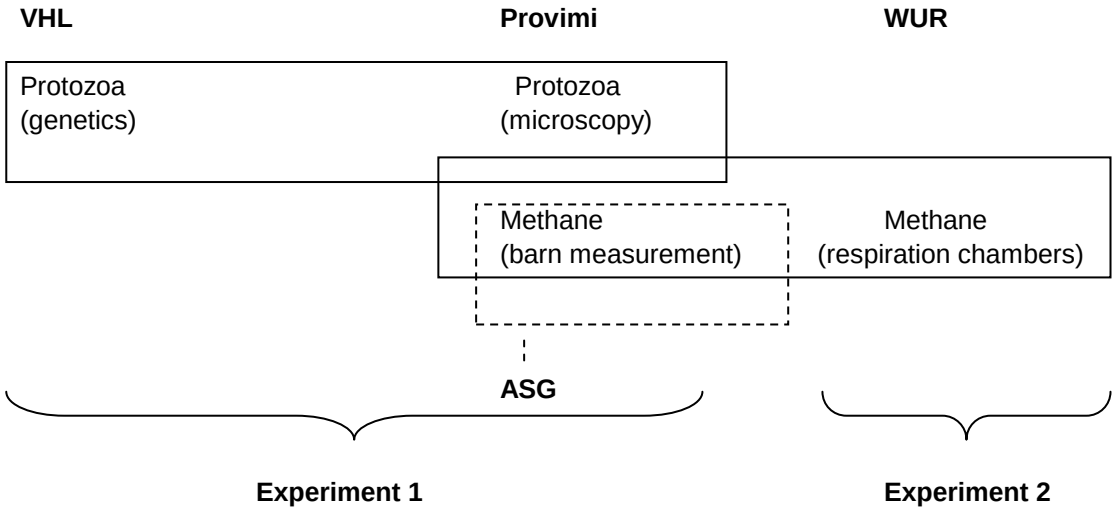

Figure 3.2: Structure of the total project. Experiment 1 included barn measurements of methane at De Viersprong and developing genetic methods to quantify and profile protozoa. Methane measurements in climate respiration chambers were carried out in Experiment 2.

4. MATERIALS AND METHODS

Two experiments were carried out: a first experiment at Provimi's research farm De Viersprong and a second experiment at the Department of Animal Sciences of WUR. In this chapter, the materials and methods of both experiments are described.

4.1 Experiment 1: De Viersprong

4.1.1 Experimental design and treatments

An experimental treatment was tested against a control treatment in a 2 x 2 Latin Square in which columns were periods of 28 days and rows were sequences of both treatments ("control-defaunator") and ("defaunator-control"). Each of both periods consisted of 28 days (see Figure 4.1).

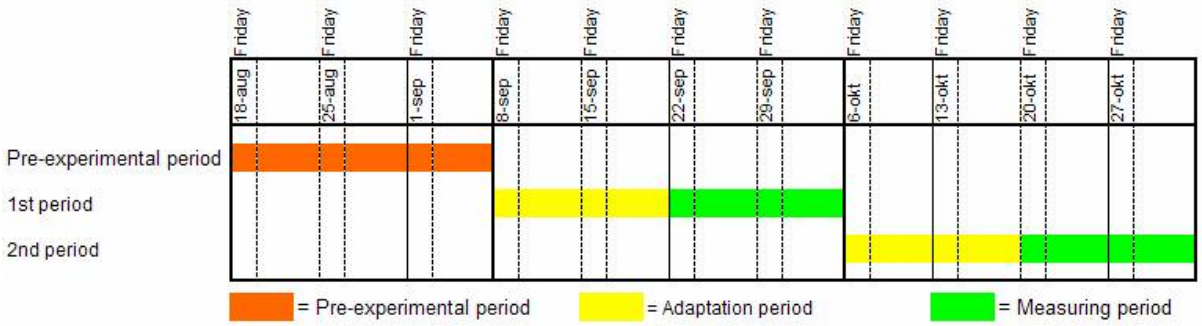

Figure 4.1: Time schedule experiment De Viersprong

Cows were paired according to stage of lactation and milk yield. One cow of each pair was allocated at random to each row of the Latin Square. To allow methane output to be measured, the dairy unit at De Viersprong was divided into two equal halves, with cows allocated to "control-defaunator" housed on one side of the barn and cows allocated to "defaunator-control" housed on the other side. Allocation of cows to pairs took place 3 weeks before the start of the experiment. During this pre-experimental period all cows were offered the control ration *ad libitum*. The combination of additives in the defaunating treatment was chosen based on previous experience and after a review of literature (see Table 4.1). It was intended to give a high chance of producing a detectable reduction in methane production per unit of dry matter intake (DMI) or milk output, and does not represent a cost-effective strategy for possible immediate commercial application.

Table 4.1: Experimental rations

Component (% DM weight)	Control	Defaunator
Maize silage (2005)	15.5	15.4
Grass silage (first cut 2006)	37.1	36.8
Concentrate DT 0615	42.0	41.7
Straw (wheat)	1.74	1.72
Hyprofat	3.2	-
Lauric acid	-	0.4
Myristic acid	-	1.2
Linseed oil	-	1.6
Calcium carbonate (Limestone)	0.52	-
Calcium fumarate	-	0.8
Vitamin E	-	0.38

Both treatments were provided as separate total mixed rations (TMR). Rations were offered to the cows once a day at the same time and in the same order. Rations were offered for *ad libitum* intake (approximately 10% feed refusals) from day 1 to day 10 of each period. From day 11 to day 28 feed intake was restricted as follows: for each pair the average DMI for each cow for days 3 to 10 was

calculated, the lowest DMI per pair was selected and 95% of this amount was fed to both cows within a pair. Cows had *ad libitum* access to clean water throughout the experiment.

4.1.2 Experimental animals and housing

Experiment 1 involved ten rumen fistulated Holstein-Friesian dairy cows and two non-fistulated Holstein-Friesian dairy cows. Four cows were in fourth lactation, three cows in third lactation, three cows in second lactation and two cows in first lactation. Average days in milk at the start of the experiment was 216 days (range 98 to 382d, SD = 93), average DMI was 21.7 kg cow^{-1} day^{-1} (range 13.4 to 25.7 kg, SD = 3.6) and average milk production was 36.5 kg cow^{-1} day^{-1} (range 23.0 to 49.9 kg, SD = 8.0). The cows were housed in a tie-stall barn divided into two to allow methane measurements (see Figures 4.2 and 4.3). When no methane measurements were carried out, cows were exercised in a sanded paddock for up to 2h day^{-1}. Manure from each half of the barn was collected in a separate pit. To minimize methane production from manure, the pits were emptied every four weeks.

Figure 4.2: The two equal halves of the experimental barn at De Viersprong with equipment to measure methane.

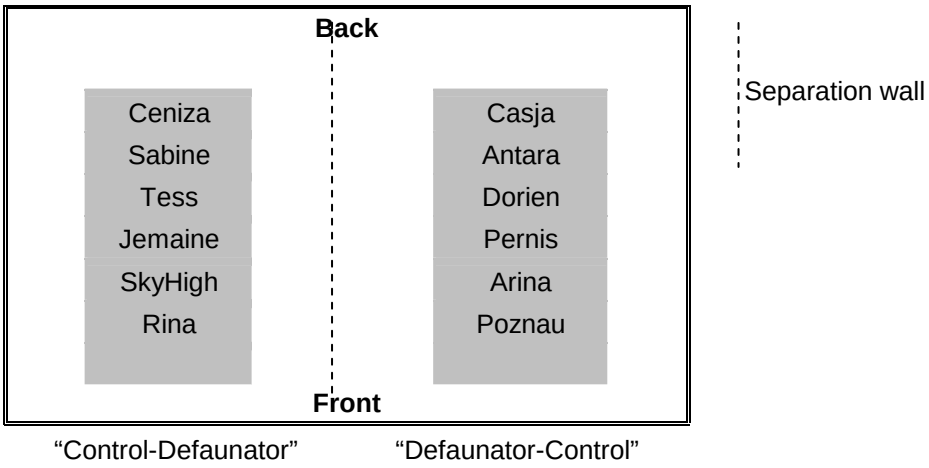

Figure 4.3: Cow placement in dairy unit at De Viersprong

4.1.3 Milk

Milk yield was measured daily with Tru Testers™. Milk composition of fat, protein, somatic cell count (SCC), urea and lactose was measured on day 6, 13, 19, 20, 26 and 27. Milk was analysed for fatty acid profile on day 6 and 21.

4.1.4 Methane

Methane production on both sides of the barn was measured separately on days 14 to 21 and days 21 to 28 of each period. Climate conditions, ventilation rate and methane concentrations were measured to determine methane emissions. Ventilation rates were averaged per 10 minutes and coupled with methane concentration data. Missing values were not extrapolated from other data points. Data around feeding times or opening of the doors for other purposes were filtered out.

4.1.5 Rumen function

On day 0, 5, 21 and 28 of each period at 6:30 h samples were taken from the centre of the rumen. Rumen pH was determined for each sample. Samples were strained and further prepared according to the method of Dehority (1993) to be able to count protozoa. The same person counted protozoa in all samples. Both strained and unstrained samples were sent to VHL for genetic quantification and profiling of rumen protozoa.

Cows in pair 1, 2, 3 and 4 were fitted with indwelling pH meters from day 18 till day 20. Rumen pH was measured continuously in that period. On day 5, 21 and 28 rumen samples were taken in the centre of the rumen and were analysed for VFA and NH3 (University of Ghent, Belgium).

4.1.6 **Genetic quantification and profiling of rumen protozoa**

The methods used by VHL to quantify the rumen protozoa and to determine the rumen protozoa composition are described in the next paragraph.

DNA isolation

A DNA isolation method based on Diatomaceous Earth (DE) extraction has been previously developed by VHL for effective isolation of bacterial DNA from a variety of sources including faeces and intestinal contents. The DE method is based on specific binding of the DNA to a solid matrix (DE), followed by a number of wash steps to remove contaminants. This method was chosen as the basis for the development of a method to isolate protozoal DNA from rumen samples.

Microbial Community Profiling and Characterisation (MCPC) analysis

MCPC was used to monitor the diversity of the protozoal flora. Fluorescent dye labelled primers that selectively amplify part of the protozoal 18S gene were designed and used in a PCR. The 18S gene of a large number of different protozoa can be amplified in a single PCR reaction. The length of the resulting product may vary and depends on the strain. However, the majority of the products have the same or nearly the same length for different strains. To allow better discrimination between the different protozoa strains, the PCR reaction products are digested with one or two restriction enzymes. Next, the resulting DNA fragments are size-separated by capillary electrophoresis. The digestion of the PCR products will result in a much larger variation of fragment length. The length of the reaction products is indicative for a limited number of protozoa species (see Figure 4.4); the length of the final reaction products can be deduced from the sequence of 18S rRNA genes of the individual strains. The availability of the 18S rRNA gene sequences of the protozoa strains present in the rumen is therefore critical for both the design and the analysis of the protozoa MCPC. The ultimate goal would be to find a unique fragment for each species.

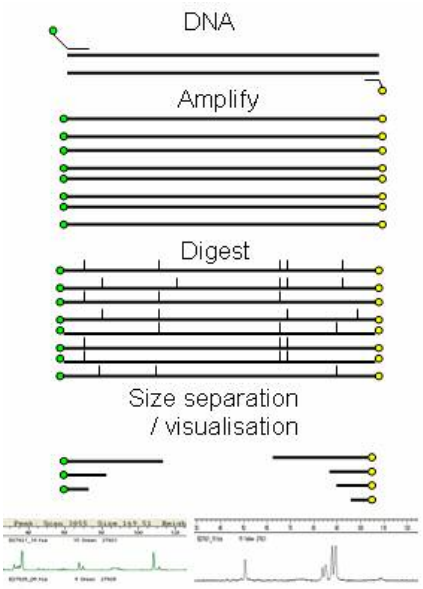

Figure 4.4: The MCPC technique; DNA is amplified by PCR using two fluorescent primers that specifically amplify protozoal DNA. After digestion with a restriction enzyme the fragments are size separated and the labelled fragments are visualised using an ABI prism 3100.

Quantitative PCR (Q-PCR)

Q-PCR technology is widely used for DNA based quantification of micro-organisms, since amplification of the target sequence by PCR allows for greater sensitivity of detection than otherwise could be achieved. Since there are many different targets (different protozoa strains) in the samples, a universal fluorescent reporter molecule, SYBR® Green, was chosen. In a Q-PCR reaction the amount of amplified product is linked to fluorescent intensity. The SYBR® Green binds to double stranded DNA and is 1000-fold more fluorescent in the bound state than in the unbound state. As PCR amplification progresses, the amount of double-stranded DNA increases. Consequently, the fluorescent signal will also increase (see Figure 4.5). Each PCR cycle the fluorescent signal increases and after a number of PCR cycles the fluorescent signal rises above the detection threshold value. The first cycle at which the instrument can distinguish the generated fluorescence above the ambient background signal, is called Ct or threshold cycle. With a calibration line, this Ct value can be used to calculate the concentration of the target DNA in the sample. The Ct value for a specific sample becomes lower if the amount of initial DNA template in the sample is higher. From the Ct values the relative amount of protozoa can be calculated using a calibration line. Such a calibration line can be produced using samples for which the number of protozoa has been established by other methods.

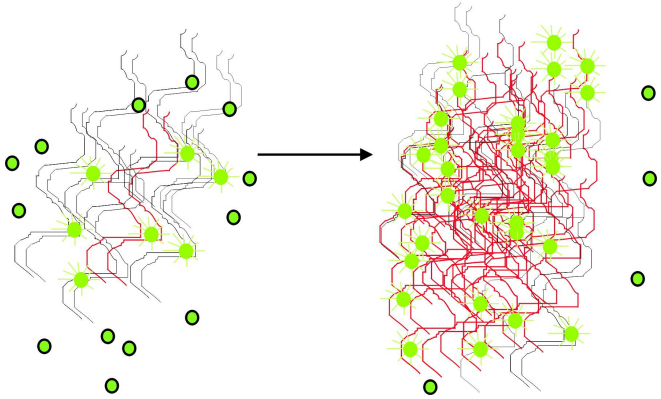

Figure 4.5: Gene X (red) is amplified in the presence of SYBR® Green. SYBR® Green binds to the double stranded DNA and is 1000-fold more fluorescent in the bound state than in the unbound state.

4.2 Experiment 2: Wageningen University

4.2.1 Experimental design and treatments

The second experiment was carried out at the experimental facilities of Wageningen University. The experiment was divided into five periods of three weeks. The first two weeks of each period were an adaptation period at experimental farm De Ossekampen and the third week was a measuring period at the climatic respiration chambers of De Haar to measure methane production. Between the measuring periods at De Haar, one day for cleaning and preparing the chambers was included. In each period, four cows were used for the experiment. The periods succeeded as depicted in Figure 4.6. As a maximum, three periods were running at the same time. Treatments were similar to the treatments described in the experiment at De Viersprong (see paragraph 4.1.1). Also feeding strategy and restriction of feed intake were similar to the first experiment.

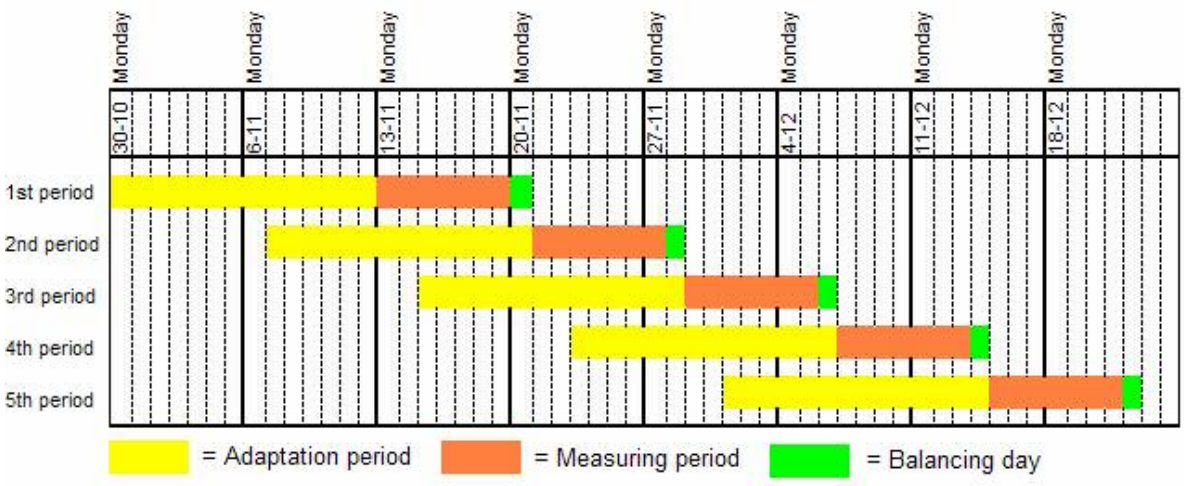

Figure 4.6: Time schedule experiment Wageningen University

4.2.2 Experimental animals and housing

Twenty non-fistulated, lactating Holstein-Friesian dairy cows were selected and divided into ten pairs of two similar cows. The animals were selected on parity, lactation stage and actual fat and protein corrected milk production (FPCM). Two cows were in fifth lactation, two cows in third lactation, ten cows in second lactation and six cows in first lactation. Average days in milk when the trial started was 176 days (range = 81 to 325d, SD = 76). FPCM was 32.8 kg cow^{-1} day^{-1} (range 22.9 to 38.1 kg, SD = 4.9). For each of the five experimental periods, two pairs of similar cows were used. One cow of each pair was assigned to the control treatment, the other cow to the defaunating treatment. The assignment of cows to treatments within pairs occurred at random.

The adaptation period allowed the cows to adapt to the treatments and housing conditions. During the adaptation period, the cows were housed in a tie-stall barn. In the measuring week, both cows receiving the same treatment were housed in one of two identical climate respiration chambers, with a content of $80m^3$. Within the chamber, cows were able to see each other. Temperature was maintained at 16˚C and relative humidity was kept at 70%. An under pressure inside the chambers of 50 mmHg was established to avoid leaking of air. The manure pits inside the respiration chambers were emptied every week. The ethical committee for animals of Wageningen University approved the experiment.

4.2.3 Milk

During the adaptation periods, milk yield was measured daily by using Tru-Testers™. During measuring weeks, the milk produced was weighed on a balance. On day 19 and 20 in the measuring period, milk samples were collected twice a day to measure contents of fat, protein, lactose and urea and somatic cell counts (SCC). The average milk composition of day 19 and 20 was calculated and assumed to be constant during the whole measuring period. For calculating daily milk-, fat-, and

protein yields in the measuring periods, the average daily milk yield of seven days preceding the adaptation period were included as a covariate. All cows received the same diet during that period. Since no data was available about milk composition of the individual cows directly before the beginning of the experiment, it was assumed that the composition was the same on average for all cows on both treatments. For calculating daily fat and protein yield in the measuring period, only milk yield of the pre-experimental period was included as a covariable.

4.2.4 Methane

Gas exchange was measured in 9 minute intervals by measuring the exchange of CH_4, oxygen (O_2) and CO_2 as described by Verstegen *et al*. (1987) in the climate respiration chambers (see Figure 4.7). Cows were weighed before and after each measuring period.

Figure 4.7: Outside and inside view of a climate respiration chambers at Wageningen University.

5. THE PROJECT RESULTS

5.1 Experiment 1: De Viersprong

5.1.1 Protozoa

Average protozoa numbers were higher for cows on the defaunating treatment at day 0. This difference was mainly created by one cow. After introducing the defaunating treatment to the cows in the first period, a rapid decrease in protozoa was noticed (see Figures 5.1 and 5.2). Protozoa numbers for the cows receiving the defaunating treatment remained lower than the control cows in this period. On day 5 in the second period, a rapid decrease of protozoa numbers was observed in cows receiving the defaunating treatment, similar to the first period. The protozoa numbers of the cows receiving the control diet in the second period did not recover as quickly as expected. Although protozoa numbers were lower in period 2 for the cows receiving the defaunating treatment, the difference in number of protozoa was smaller in period 2 than in period 1. One cow was excluded from Latin square analyses due to illness.

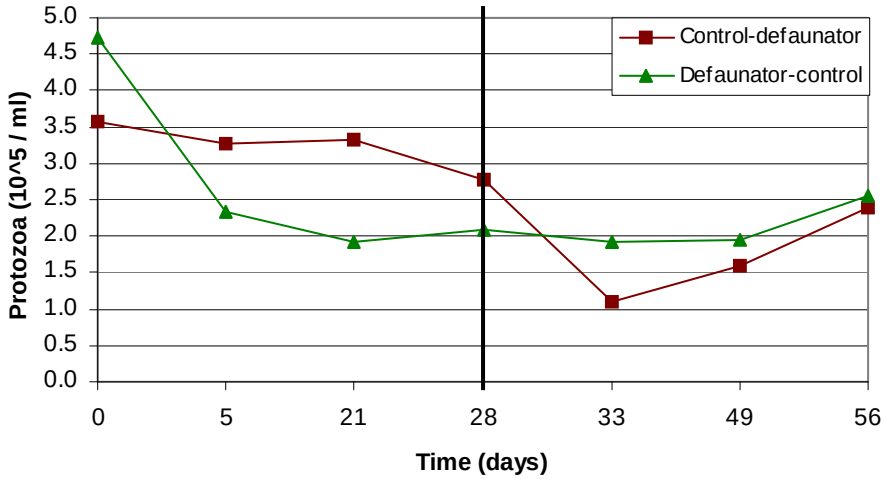

Figure 5.1: Protozoa counts with use of the traditional counting method

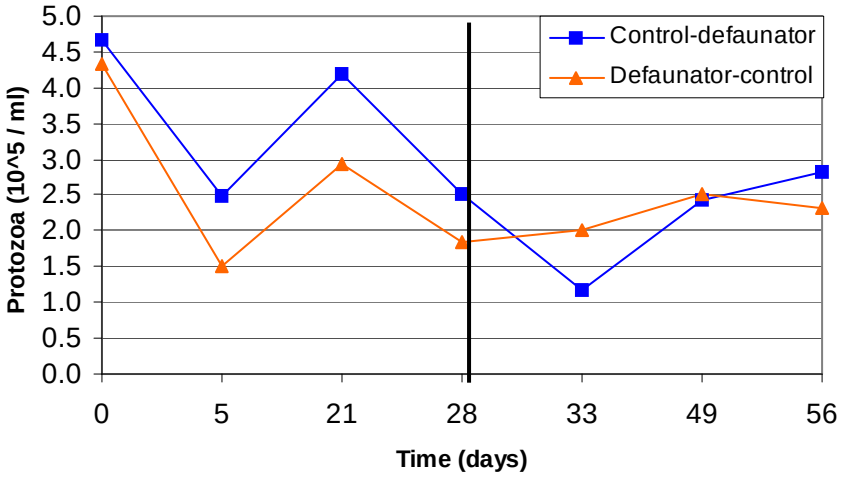

Figure 5.2: Estimated protozoa numbers with the use of Q-PCR

With the traditional method, a reduction of approximately 33% was found in protozoa numbers on day 5 and 21, when the defaunating treatment was fed (see Table 5.1). With genetic quantification carried

out by VHL, a reduction of 37% was found. Due to large variation in the data, differences in protozoa numbers are only significant on day 5. On day 28, a non-significant reduction of 15% was found. Overall protozoa numbers were significantly reduced with 28% by feeding the defaunating treatment.

Table 5.1: Results of manual and genetic protozoa counts analysed as a Latin square

		Control	Defaunator	s.e.d.	P	Def/Control
All data	Manual	2.63	1.91	0.187	0.01	0.72
	Genetic	2.66	2.12	0.115	0.00	0.80
Day 5	Manual	2.60	1.73	0.295	0.02	0.67
	Genetic	2.25	1.42	0.181	0.00	0.63
Day 21	Manual	2.64	1.75	0.532	0.13	0.66
	Genetic	3.35	2.71	0.360	0.12	0.81
Day 28	Manual	2.66	2.25	0.275	0.17	0.85
	Genetic	2.40	2.24	0.328	0.64	0.93

The number of Holotrich protozoa counted with the traditional method was a small fraction of the total number of protozoa. The reduction of Holotrichs for cows receiving the defaunating treatment was bigger than the reduction of all protozoa. In all counts there was a significant reduction in the number of Holotrich protozoa in the cows with the defaunating treatment. Over all counts this reduction was 62% (see Table 5.2).

Table 5.2: Results manual Holotrich protozoa counts, analysed as a Latin square

Holotrichs	Control	Defaunator	s.e.d.	P-value	Def / control
Absolute					
All	4292	1631	249.9	0.00	0.38
Day 5	3320	1266	404.6	0.00	0.38
Day 21	5748	1409	796.4	0.00	0.25
Day 28	3809	1853	400.4	0.00	0.49
% of total					
All	1.73	0.87	0.0	0.00	0.50
Day 5	1.23	1.09	0.4	0.73	0.89
Day 21	2.42	0.83	0.3	0.00	0.34
Day 28	1.53	0.70	0.2	0.00	0.46

When Holotrich protozoa are expressed as percentage of total number of protozoa, the percentage of Holotrich protozoa is lower when the defaunator was fed to the cows. On day 21, 28 and overall this percentage was significantly lower compared to the control.

5.1.2 Methane production at "De Viersprong"

Variation between treatments

Daily methane emissions for days 21-28 of each period were on average 15% lower across both experimental periods when cows received the defaunating treatment. An average methane emission of 835 g cow^{-1} day^{-1} was observed for the control treatment, whereas the defaunating treatment resulted in an average methane emission of 706 g cow^{-1} day^{-1}. Reductions of methane emission were bigger in period 1 compared with period 2 (19.5% and 8.4% respectively). Relative methane emissions were 41.8 and 35.5 g kg DMI^{-1} for the control and defaunating treatment respectively, and 25.5 and 20.8 g kg $milk^{-1}$ for both respective treatments (see Table 5.3).

Table 5.3: Absolute and relative methane emissions of the experiment carried out at "De Viersprong"

		Control	Defaunator	Def / control
CH_4 emission	(g cow^{-1} day^{-1})	835	706	0.85
CH_4 emission	(g kg DMI^{-1})	41.8	35.5	0.85
CH_4 emission	(% of GEI)	12.3	10.4	0.85
CH_4 emission	(g kg $milk^{-1}$)	25.5	20.8	0.82
CH_4 emission	(g $FPCM^{-1}$)	24.4	22.1	0.91

Because each section of the barn constituted one experimental unit, it is difficult to substantiate the statistical significance of these results. Data for the daily difference in methane emissions have been analysed by WUR Animal Sciences Group using a two-sided one-sample t-test. This assumes that individual days represent independent observations (when they are clearly correlated) and this procedure increases the risk of Type 1 error (i.e. identifying a difference as significant when it is not). Results suggest a high probability that the difference between treatments was not due to chance.

Variation within days

There was considerable variation in methane output within each day. For example, peak emissions equivalent up to a rate of about 400g/cow/h were found. Feed intake pattern within the day was not recorded, but peak methane output generally occurred directly after feeding. Further details concerning methane measurements are given in the report received from the contractor ASG.

5.1.3 Rumen function

The average rumen pH in the measuring periods was 6.1. Minimum pH was 5.4 and maximum pH was 6.7. No difference in average rumen pH was observed between treatments. On days 21 and 28 there was a significantly lower total VFA concentration for the cows on the defaunating treatment. This is caused by a reduction in acetate and butyrate concentrations, which indicates a reduced fibre digestibility. Only on day 5 there was a significant increase in the absolute propionate level. On all 3 measuring days, there was a significant reduction in the proportion of acetate and a significant increase in the proportion of propionate, when the defaunating treatment was fed. A lower ratio between acetate and propionate indicates a higher availability of energy for milk production.

Rumen ammonia concentrations were significantly lower on days 21 and 28 for the defaunating treatment compared with the control treatment. This observation is in line with observations by others (Eugène *et al*., 2004), who reported a lower rumen ammonia concentration when protozoa numbers were reduced. Within treatments, rumen ammonia levels were however higher at days 21 and 28 than at day 5. Cows were fed restricted during these days, which might have caused a shortage in rapidly fermentable carbohydrates and therefore increased the breakdown of protein into ammonia by the bacteria.

5.1.4 Cow performance

In Table 5.4, results for feed intake and milk output can be found. Due to restricted feed intake during methane measurements no difference in DMI was found between treatments. Water intake was also not significantly different between treatments. Due to inclusion of linseed oil at the defaunating treatment, a significant reduction in milk fat percentage was found. Therefore fat and protein corrected milk is also significantly lower for the defaunating treatment. Milk urea levels were also significant lower for the cows that received the defaunator.

Table 5.4: Cow performance from day 22 to 28 analysed as a Latin square

		Control	Defaunator	s.e.d.	P
Dry matter intake	(kg * d^{-1})	20.0	19.9	0.30	0.82
Water intake	(l * d^{-1})	91.8	92.4	2.25	0.80
Milk yield	(kg * d^{-1})	32.8	33.9	0.85	0.26
Fat	%	4.40	3.52	0.184	0.00
Protein	%	3.51	3.50	0.097	0.87
Lactose	%	4.39	4.40	0.077	0.92
FPCM*	(kg * d^{-1})	34.2	31.9	0.97	0.04
Fat yield	(kg * d^{-1})	1.41	1.17	0.056	0.00
Protein yield	(kg * d^{-1})	1.14	1.16	0.035	0.55
Urea	(mg * dl^{-1})	25	22	1.4	0.05
SCC	(1000 cells * ml^{-1})	57	63	7.4	0.47

The control diet was high in palmitic acid (C16:0); therefore milk fat of the cows on the control treatment contained significantly more palmitic acid compared with cows on the defaunating treatment (see Table 5.5). Milk of the cows on the defaunating treatment had significant higher lauric (C12:0) and myristic acid (C14:0) levels. The defaunating diet also contained linseed oil, which is high in linolenic acid (C18:3), but no difference in C18:3 levels in milk was observed. This was because C18:3 is completely biohydrogenated in the rumen (Bauman and Griinari, 2003). As a result, the end product (C18:0) of the biohydrogenation pathway is significantly increased by the defaunating treatment. Also intermediates of biohydrogenation like the two CLA isomers and C18:1 t10 and t11 are significantly increased by the defaunating diet (see Table 5.5).

*Table 5.5: Milk fatty acid profiles (g * 100 g fatty $acid^{-1}$) on day 6 and 21 for both treatments*

	Day 6				Day 21			
Fatty acid	Control	Defaunator	s.e.d.	P	Control	Defaunator	s.e.d.	P
C12:0	2.29	3.51	0.094	<0.001	2.07	3.11	0.103	<0.001
C14:0	9.09	15.70	0.279	<0.001	8.43	15.17	0.264	<0.001
C16:0	36.69	24.72	0.417	<0.001	36.69	23.54	0.500	<0.001
C18:0	8.05	8.97	0.322	0.02	7.49	8.38	0.287	0.01
C18:3	0.46	0.46	0.037	0.856	0.46	0.51	0.022	0.09
C18:1 t10+t11	0.93	1.50	0.282	0.073	1.27	2.53	0.506	0.04

5.1.5 MCPC

In the public databases (GENBANK), only a small number of full-length protozoal 18S sequences is available. Prof. C.J. Newbold (University of Wales Aberystwyth) kindly provided a number of currently unpublished protozoal 18S sequences. Eight 18S sequences from different species were derived from GENBANK and 26 additional full-length sequences from different species were obtained from Prof. C.J. Newbold. Based on these sequences and based on literature, a combination of specific primers and restrictions enzymes was developed, designated “Protozoa-set 1”. Protozoa species within one group cannot be discriminated by MCPC using this specific primer-set.

The MCPC profiles of the rumen samples that were collected by Provimi during the course of the first experiment were analysed using Primerset 1. The profiles showed clear differences between individual animals. Next to the differences between individual animals, there were also some differences between samples from control and the treated group. One combination of peaks was found in most of the samples from the control group, whereas it was absent from the majority of the samples from the defaunated group. To allow better identification of the species present in the rumen samples, a second combination of primers and enzymes has been developed, designated “Primerset 2”. An example of

an MCPC profile with Primerset 2 is given in Figure 5.3. Other combinations of specific primers and enzymes might result in a better discrimination of individual strains, however 18S rRNA sequence information from a larger number of strains is a prerequisite to design these primersets.

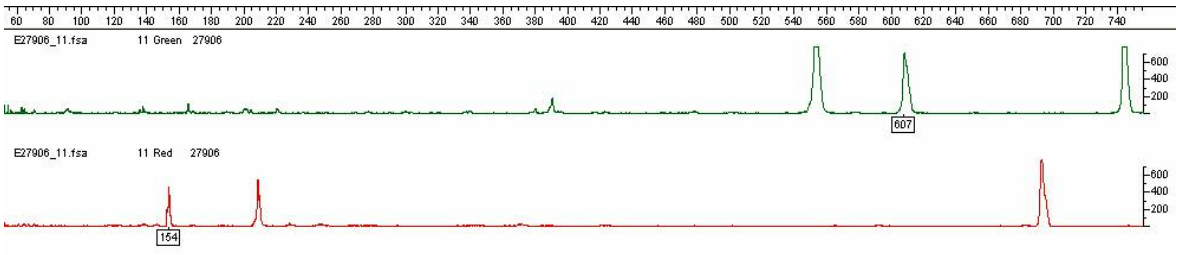

Figure 5.3: Example of an MCPC profile. The peaks generated by the species Isotricha are indicated with a label below the peak

5.2 Experiment 2: Wageningen University

5.2.1 Methane production

Based on observations in all periods, the average methane emission in gram per cow per day was 10.1% lower for cows receiving the defaunating treatment compared with the control treatment. Total methane emissions and methane emissions expressed as feed input or milk output are given in Table 5.6. On average, 148 observations with 9-minute intervals were included per respiration chamber per day for calculating methane emissions.

Table 5.6: Absolute and relative methane emissions of the experiment carried out at WUR

		Control	Defaunator	s.e.d.	P	Def / control
CH_4 emission	(g * cow^{-1} * day^{-1})	362.0	325.5	9.56	0.019	0.90
CH_4 emission	(g * kg DMI^{-1})	21.72	19.76	0.378	0.007	0.91
CH_4 emission	(% of GEI)	6.36	5.79	0.111	0.007	0.91
CH_4 emission	(g * kg $milk^{-1}$)	12.82	12.76	0.598	0.931	1.00
CH_4 emission	(g * $FPCM^{-1}$)	11.96	12.51	0.563	0.384	1.05

For calculating gross energy intake (GEI), the energy content of both treatments was assumed to be 19 MJ per kg DM in every period. Methane has an energy content of 55.65 MJ per kg (IPCC, 2001). No difference in methane emission per kg milk or kg FPCM was observed between both treatments (P = 0.931 and 0.384 for actual milk and FPCM production, respectively). Methane emissions were numerically lower for every single period compared with the control treatment. Differences within periods ranged from a 2.9 to 15.6% lower methane emission per cow for the defaunating treatment compared with the control treatment. The loss of 6.36% of gross energy intake as methane found for the control treatment is slightly high compared with values found under current Dutch circumstances. ROB-Klimaat assumes an average value of 5.90% loss. The higher value found in the current experiment might be explained by the restricted intake, which enhances the rate of fermentation on the one hand and lowers GEI on the other hand.

Variation between periods

There was a significant period effect (P = 0.049) for total CH_4 emission per cow per day. Since climate, feed and housing conditions in the respiration chambers were the same for all periods, the variation in difference in methane emission between control and defaunating treatments is likely to be caused by a cow effect. Since pairs of cows with different parities, lactation stages and production levels were randomly assigned to a period and no crossover design was used, this cannot be

confirmed. No period effect was found for methane emission per cow expressed as DMI and expressed as GEI (P = 0.742 for both parameters). No period effect was found for methane emission per cow expressed as milk yield (P = 0.012). No covariate was included when methane emissions were compared with actual milk yield and FPCM. Only actual observed figures were taken into account.

5.2.2 Cow performance

Based on the dry matter content of each individual feed component, it was assumed that the average dry matter content of the TMR was 60% for both treatments. As aimed for, daily feed intake did not differ significantly (P = 0.82) between treatments (see Table 5.7). Although the amount of feed offered to the cows was restricted to 95% of the lowest average *ad libitum* intake of one of both paired cows, there was a minor numerical difference in daily dry matter intake. This was caused by slightly more feed refusals with the defaunating treatment compared with the control treatment of 2.5 and 1.2% per cow per day on average, respectively.

There was no significant difference in daily milk yield per cow between treatments (P = 0.70). Milk fat content was significantly lower for the defaunating treatment (P = 0.06). There was no significant difference in protein and lactose content (P = 0.47 and 0.23, respectively). Although some minor differences were observed, the number of observations (n = 10) was too low to detect significant differences. Urea content decreased from 22 to 17 mg 100 ml^{-1} (P = 0.02) when the defaunating treatment was fed. This suggests reduced recycling of rumen bacterial protein by protozoa. FPCM production was significantly lower for cows fed the defaunating treatment (P = 0.02). Pre-experimental milk production was included as a covariable. No period effect was observed (P = 0.147). A lower milk fat content mainly caused the lower FPCM production, likely caused by linseed oil and calcium fumarate in the defaunating treatment. This also explains the lower daily milk fat yield (P = <0.01). There was no significant difference in daily protein yield between both treatments (P = 0.71).

Table 5.7: Animal performance of the cows in experiment 2

		Control	Defaunator	S.E.D.	P-value
DMI	(kg d^{-1})	16.69	16.47	0.93	0.82
Milk yield	(kg d^{-1})	27.8	27.2	1.23	0.70
Fat	(%)	4.63	4.10	0.24	0.06
Protein	(%)	3.27	3.35	0.09	0.47
Lactose	(%)	4.61	4.53	0.06	0.23
Urea	(mg $100ml^{-1}$)	22	17	1.70	0.02
FPCM	(kg d^{-1})	29.4	27.4	0.89	0.02
Fat yield	(kg d^{-1})	1.264	1.106	0.052	<0.01
Protein yield	(kg d^{-1})	0.896	0.901	0.025	0.71
SCC	(×1000 cells)	123	208	119	0.49

Actual dry matter intake per cow per day was plotted against methane production for both treatments separately (see Figure 5.4).

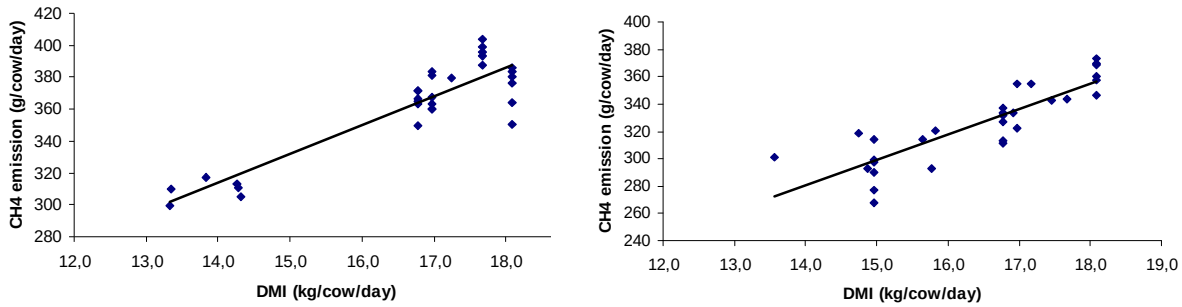

Figure 5.4: Methane emission in relation to DMI for the control treatment (left) and the defaunating treatment (right).

For the defaunating treatment, a strong correlation between feed intake and methane emission per day was found (R^2 = 0,741). For this treatment, methane emission per cow per day could be calculated as 18.4 + 18.71 * DMI. This equation was calculated with values for DMI between 13.6 and 18.1 kg and CH4 emission between 374.5 and 521.6 g cow^{-1} day^{-1}. For the control treatment, the correlation between DMI and CH_4 emission was even stronger (R^2 = 0,812) and the equation found was 61.86 + 18.01 * DMI. A weak correlation was found between FPCM and methane production for the control treatment (R^2 = 0.43) and no correlation for the defaunating treatment (R^2 = 0.05). Since the cows were fed restricted, no correlation was found between DMI and FPCM (R^2 < 0.02 for both treatments).

5.3 Environmental aspects

In the experiment carried out in the climate respiration chambers, it was found that dairy cows on average emit around 360 gram methane per cow per day. Literature records emissions up to 500 gram per cow per day (Johnson *et* al., 1994; Huis in 't Veld and Monteny, 2003; Kinsman, 1995; Kebreab *et al.*, 2006; Tamminga *et al.*, 2007), largely depending on feeding level, type of diet and level of production. The results of the barn measurements at De Viersprong are above this range and are therefore not included in calculating environmental benefits. Both experiments have however proven that short-term reductions of 10-15% of methane are possible when a combination of feed additives is fed. Assuming an average daily methane emission of 400 gram per cow and a reduction of 10-15%, emissions can be reduced by 307-460 kg CO_2-equivalents per cow per year with feed additives. Applying additives to the entire Dutch dairy herd is potentially possible; the total methane emission from the 1.43 million dairy cows in the Netherlands (CBS, 2005) could therefore be lowered by 0.44-0.66 Mton CO_2-equivalents per year. The reduction will even be larger if young stock and beef animals are also taken into account. Since production and distribution of additives is possible within the existing infrastructure in the Netherlands, no substantial extra greenhouse gasses will be emitted.

5.4 Economical aspects

The current project was designed to give a high chance of producing a detectable reduction in methane production, and does not represent a cost-effective strategy for possible immediate commercial application. Now that proof was given that methane emission can be mitigated with feed additives, the next step is to develop cost-effective additives that give persistent results. A group of Master students from WUR calculated for Provimi that net revenues per cow per year increase with € 9.10 when cows produce 150 kilograms of milk per year extra due to a more efficient rumen function. Costs for extra additives of € 0.10 per cow per day are included and a system without milk quota and without derogation is assumed. This is chosen since the quota system in the Netherlands will most likely be abolished in the midterm. If derogation applies and the cattle manure contains less N because of higher milk protein production, further substantial financial benefits may be obtained.

These figures indicate that a reduction in enteric methane emission by feeding additives can be cost-neutral or even financially profitable.
Currently, there is no mechanism to reward reductions in enteric methane production, although such schemes may be developed in future as 'Clean Development Mechanisms' or 'Joint Implementation projects' under the Kyoto Protocol. Future incorporation of mitigation of enteric methane into this framework will only be possible if the reduction in methane can be monitored, measured and shown to be consistent and sustainable. As FAO (2006) states, 'simply enhancing awareness will not lead to widespread adoption.' Therefore, at present, the first criterion for a feed additive that reduces enteric methane is that it must also be cost-effective for the producer, those involved in the distribution chain and the farmer.

5.5 Non-technical points of interest

EC Regulation 1831/2003 governs use of feed additives in the EU. This includes provision for a class of zootechnical feed additives that 'favourably affect the environment'. Successful registration requires a dossier demonstrating safety (to workers, farmers, animals, consumers and the environment), characterisation and methods of control (i.e. ability to quantify the additive through its chain of use) and efficacy. Costs of preparing dossiers for submission to the EC or FDA can be high. Some potential anti-methanogenic feed additives can be discarded after a relatively simple preliminary screening exercise, if major toxicology or other safety concerns are identified. It is particularly pertinent to note that protozoa, like mammalian cells, are eukaryotic: substances specifically active against protozoa may therefore pose risks for the cells of the host animal.

The saving in methane was achieved at the expense of a reduction in product quality, with a significant increase in the concentration of milk fatty acids that unfavourably affect health characteristics of the milk. This is a failure to meet the safety criterion for human health. Lauric and myristic acid caused this increase in these specific fatty acids and these additives are therefore not applicable in practice.

6. EXTENSIVE SUMMARY WITH DISCUSSION

The major objectives of this project were to reduce methane production from dairy cows through defaunation and to quantify changes in rumen protozoa populations.
Provimi and VHL achieved to successfully reduce rumen protozoa numbers with 28% on average and methane production from dairy cows with 10-15% using feed additives. To detect differences in the size and composition of the protozoal population, VHL achieved to develop new genetic methods. However, refinement and validation of these methods is necessary.
Effects of defaunation on dry matter intake, milk yield and milk composition were monitored but the experimental periods were short to obtain differences. Milk yield was not affected. Milk fat content was reduced with 0.53-0.88 percent points, likely caused by the linseed oil in the defaunating treatment.

The project described in this report were meant to 'prove the principles' that protozoal population size and methane emission are susceptible to manipulation through the use of feed additives and that these effects could be detected by available research methods. Results and discussion points are more extensively described below.

6.1 Protozoa and methane production

In the first experiment, an overall reduction in protozoal population size of 28% was associated with a 13.9% reduction in methane emission. The percentage of methane emissions associated with protozoa has been variously estimated as 42%, 37%, 25%, 30-45% and 15% by Whitelaw *et al.* (1984), Finlay *et al.* (1994), Newbold *et al.* (1995), Ushida *et al*. (1997) and Machmüller *et al.* (2003), respectively. There are several possible explanations for these differences, including methodological issues concerning the measurement of the protozoa population and/or methane emissions (see sections 4.3 and 4.4), animal species (none of the published experiments cited above used dairy cows) and diet (a larger effect of defaunation on methane emission has been postulated with high-grain diets, due to higher production of hydrogen by protozoa (Hegarty, 1999)). However, the most probable explanation is that the experimental treatments used in these experiments influenced methanogenesis directly, as well as indirectly through an effect on protozoa.

Strategies to reduce methane production inevitably beg the question: what will happen to the hydrogen that will still be produced by the anaerobic fermentation, but which can no longer be disposed of as methane. Hino and Asanuma (2003) stated that '...the direct inhibition of methanogens may be detrimental to overall rumen fermentation... unless other hydrogen sinks are provided'. This was the main logic behind the inclusion of calcium fumarate. Fumarate was chosen to complement antiprotozoal / antimethanogenic effects of linseed oil, lauric acid and myristic acid, rather than as an attempt to reduce protozoa.

6.2 Animal performance

To prevent that DMI differed between the defaunating and control treatment, it was restricted during the periods that methane was measured. Therefore dry matter intake cannot be used as a variable to explain the observed differences in methane emissions. It is however known from literature, that level of feed intake might influence CH_4 emission. An increase in feeding level increases passage rate of the feed through the rumen and intestines, and as a result, the time of exposure to fermentation is decreased. Since methane production is directly related to fermentation in the rumen, an increased feeding level will result in a decreased methane production per unit of GEI (Moss *et al*., 2001). Inversely, restriction of feed intake might increase the production rate of methane production, expressed as units of GEI.

Major parameter to measure animal performance output in dairy cows, daily milk yield, was not significantly affected by the defaunating treatment in both experiments conducted for this study. The composition of the milk from the animals on the defaunating treatment was changed. Observed significant differences were a reduction in milk fat percentage and a reduction in milk urea levels. The levels of rumen ammonia influence levels of milk urea. In the experiment at De Viersprong a significant reduction in rumen ammonia was found for the cows receiving the defaunating treatment.

Due to the restriction some cows will have had a shortage of rumen fermentable carbohydrates as energy source for microbial yield. In that case amino acids will be used as an energy source and this will have caused an increase of rumen ammonia concentration. From the literature also improved nitrogen efficiency was expected (Eugène et al. 2004).

6.3 Methods of estimating protozoa

The defaunating treatment successfully created a reduction in the size of the protozoal population, as estimated by classical, microscopic counting. Numbers of protozoa were numerically lower at all observed days, but only significantly at day 5. This positive association was also found using the method of Q-PCR, developed by VHL. The statistics were however hampered by the limited number of data points for the time span of the experiment. A possible error in the measurements at one specific point in time can have a major impact on the final results. Since the number of protozoa or the attachment of protozoa to feed particles in the rumen can vary within a day, between days or at different sites inside the rumen (Martin *et al*., 1999), sampling rumen contents for protozoa quantification more frequently will result in a more reliable estimation of the number and dynamics of protozoa in the rumen. This is however not possible in practice using the classical method of counting protozoa with a microscope (Dehority, 1993), primarily because the large amount of labour involved. Besides, making a profile and quantifying protozoal species in a rumen sample by counting with a microscope requires extra skills of the person counting and is prone to error. In contrast, Q-PCR allows to process large numbers of samples with relatively little labour and with a potential high accuracy. This emphasises the importance of using new techniques to quantify and profile protozoa.

Since there are no pure cultures from protozoa available to VHL at the moment, a correction factor based on the microscopic counts of the samples provided by Provimi was calculated. For this way of calculating a correction factor it is required that the profile of different protozoa of the individual samples is fairly similar, especially when comparing individual samples. The MCPC profiles however show that this assumption is not true. In practice this means that the numbers of protozoa in individual samples cannot yet be accurately determined. However, when the results of all samples are combined and analysed as a Latin square, the outcomes of the Q-PCR counts are remarkably similar to the results obtained by microscopic counting. This implies a large potential for the newly developed technique.

6.4 Methods of estimating methane production by dairy cows

One of the objectives of this project was to compare two different methods for measuring methane emissions: a method based on measuring methane output from two identical barns housing groups of cows (experiment 1) and the classical approach using climate respiration chambers (experiment 2). Both methods successfully detected a difference between treatments. However, barn measurements yielded a higher absolute estimate of methane than measurements with the respiration chambers, regardless of the units of expression.

Considerable efforts were made to make feeding conditions as similar as possible, with the same forages, concentrates and feed supplements mixed using the same equipment. The two major differences were the used cows and the method of methane measurement. Cows in experiment 1 had a higher feed intake and yielded more milk than those in experiment 2. This leads to the expectation of more methane per day in experiment 1. Because of reduced digestibility due to a higher feeding level, less methane per unit of DMI was expected as well. In fact, methane per unit of DMI was considerably higher in experiment 1 than experiment 2. Therefore, the difference in absolute methane measurements between the two experiments is considered to be caused by the method of measurement, rather than differences between the two groups of cows used.

Both methods of measurement can be evaluated by comparing our results with published estimates and with results of methane prediction models. Values reported in experiment 1 are in excess of most values reported in the literature, regardless of the method used (Figure 6.1)

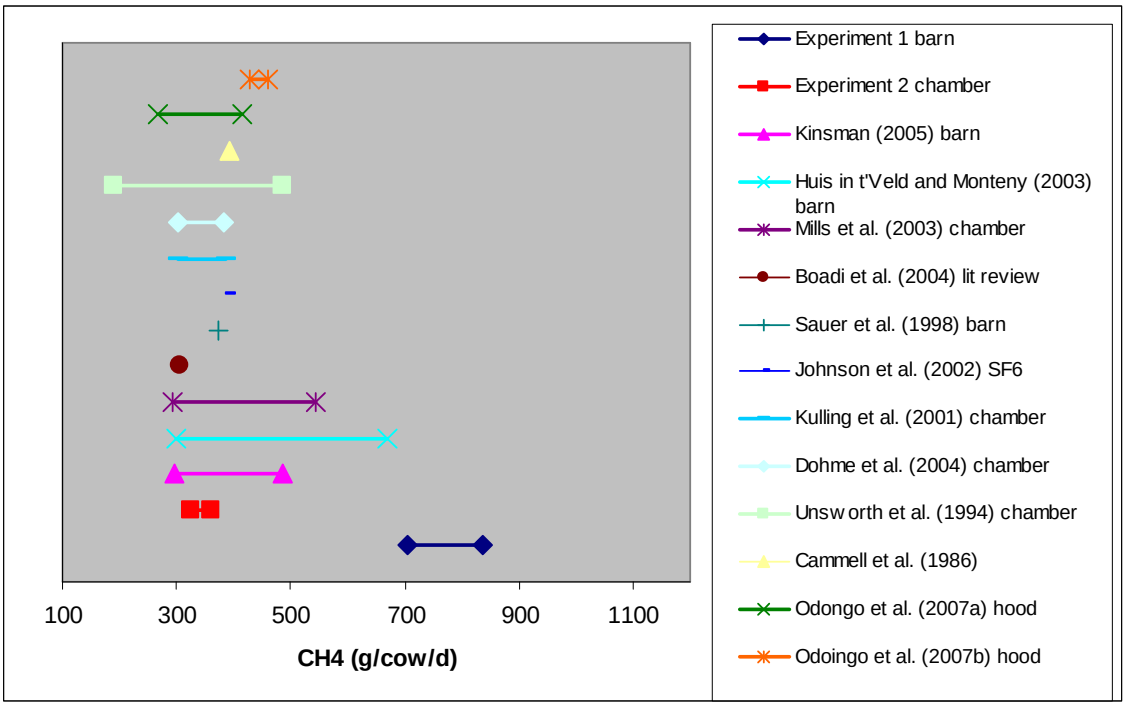

Figure 6.1: Absolute methane emissions from lactating dairy cows in gram per cow per day.

This overview of literature clearly suggests that results obtained in experiment 1 are, in absolute terms, anomalous. Methane measurements in experiment 2 are towards the lower end of the range reported in the literature, but highly consistent with other measurements, most of which were also obtained in respiration chambers. Kebreab *et al.* (2006) reviewed different methods for measuring methane output and suggested that the stress of keeping cows in chambers, away from their normal physical and social environment, might depress DMI and lead to some underestimation of methane production. However, this seems highly unlikely to account for the large difference between experiments 1 and 2, since DMI in the respiration chambers was restricted and equal to the DMI in the period preceding the measuring period.

Both systems to measure methane have some specific advantages and disadvantages. Climatic conditions inside respiration chambers can be maintained between specific ranges. It is known that the climatic environment has a large influence on production traits of animals (Verstegen *et al*., 1987). However, specialised knowledge and experience is necessary to operate the system. Besides, it is undesirable to house animals inside calorimeters for longer periods of time, which excludes the possibility to measure methane during several months or even longer. Data from the second experiment showed that results from less than 7 days (i.e. 3, 4, 5 or 6 days in the respiration chambers) are highly consistent with results obtained from a complete week.
Farm-based measurements at De Viersprong proved to be practical with the current barn configuration. It is possible to house large groups of animals and to permanently store manure inside the experimental unit. This is in line with situations on commercial dairy farms. In other studies (Kinsman *et al*., 1995), CH_4 was directly measured on a large group of animals, which avoided the need to extrapolate results to a large number of animals and therefore the observed results are likely to better represent reality. The fact that methane production is highly variable between animals (Johnson and Johnson, 1995) supports this thought.

7. CONCLUSIONS

The strategy of selected additives was generally successful in both experiments: reductions in methane and protozoa (experiment 1 only) were measurable (and significant where statistical analysis was possible) and falls in dry matter intake and milk yield were largely avoided. If control of enteric methane production – responsible for 31% of anthropogenic methane (FAO, 2006) – is ever to be incorporated into mechanisms for controlling climate change, research is needed to develop rapid, reliable systems for monitoring, measuring or precisely predicting the reductions in methane emissions that a feed additive really achieves.

Levels of methane emission measured during barn measurements in experiment 1 were higher than values observed with respiration chambers in experiment 2 and in literature. Barn-measurements are at the moment not accurate enough to estimate absolute levels of methane emissions. Relative differences are similar with results from respiration chambers. Respiration chambers at WUR are preferred in future work to develop and test feed additives for dairy cows which aim for a reduction in methane emission. When more test facilities are needed, for example to test interactions between additives, barn measurements at De Viersprong are possible.

Recent developments in genetic techniques provide more accurate quantification of protozoa and give information about protozoa species and protozoa activity. At the start of this experiment, VHL had no experience in quantifying and qualifying rumen protozoa. Eight months later, results indicate that there is a correlation between traditional quantification and genetic quantification, but that correlation is relatively weak and more research is necessary. Focus in future work should be calibration with pure cultures and further comparison of results with microscopic quantification. Therefore, it is recommended to use both techniques in future experiments until the genetic techniques are mature enough to use solely.

In conclusion, both methods employed in this project were able to detect effects of a feed supplement on methane production. Both may be suitable for use in further evaluations of candidate feed additives. However, there was a large discrepancy between the two methods in the average estimates of methane emissions. The farm-based method used in experiment 1 gave results that are inconsistent with reports in the literature. Pending further investigation, this method cannot be recommended if the objective is to evaluate the methane mitigation value of a feed additive in absolute terms. Results from experiments carried out in respiration chambers for short time periods and with a diet of known quality and quantity, obtained by careful monitoring of various parameters, are usually extrapolated to estimate overall ruminant CH_4 emissions. However, dairy cows placed in respiration chambers for relatively short periods of time cannot readily respond to differences in feeds and management practices. Short-term trials in respiration chambers or in barns are, therefore, unsuitable for estimating long-term CH_4 emission from cattle. More extensive trials for long periods of time are necessary to detect sustainable effects on animal performance and methane emission.

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