Aanmeldingsformulier voor proeven met gewervelde dieren.

Secretariaat DEC
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Aanvrager: [redacted]
Afdeling:

Titel dierproef: Indicator for methane emission in dairy cattle: grass silage versus maize silage

Aanmeldcode / Protocol: 2012086.a
Stadia van de proef:

28-09-2012	Aangemeld	[redacted]
19-10-2012	Wijzigen	Secretaris van de DEC
19-10-2012	Gekopieerd	[redacted]

Is deze proef wetenschappelijk getoetst en goedgekeurd? Ja
Toelichting:
Yes, in association with a PhD procedure of [redacted]. The project has been approved by the financial sponsors ([redacted]). Furthermore, the research committee of the [redacted] has discussed the project.

1.a. Met dit onderzoek te beantwoorden concrete vraag:
F. Wetenschappelijke vraag m.b.t. andere wetensch. vraag
Methane is produced during microbial fermentation of feed in the rumen and hind gut of ruminants (i.e. enteric methane). During anaerobic conditions occurring in the rumen, the fermentation of organic matter produces volatile fatty acids (VFA), hydrogen (H2), carbon dioxide (CO2) and microbial biomass. Methanogenic archaea utilize this hydrogen as an energy source and reduce CO2 to methane. The produced methane is predominately released to the environment through breath and eructation. Methane (CH4) is one of the most important greenhouse gases (GHG) and a significant contributor to global warming. In the dairy cattle chain, methane represents more than 50% of all the GHG produced. In addition, methane also represents an energy loss for the animal, which is assumed to vary between 2 and 12% of gross energy intake (Johnson and Johnson, 1995). Therefore, it is desired to reduce the methane emission from dairy cattle, in order to reduce the ecological footprint of milk production, and improve feed efficiency and animal productivity. The current experiment is designed to observe the effect of replacing grass silage (no starch) with maize silage (starch-rich) on methane emission from lactating dairy cows, with the ultimate goal to develop an indicator for methane emission that can be measured in milk of individual cows.

Enteric methane is produced in the gastrointestinal tract of ruminants, predominantly within the rumen (87%) and to a small extent (13%) in the large intestine (Murray et al., 1976). The rumen is an anaerobic environment in which microbiota digest feed components, including plant materials which are indigestible to all monogastric animals. Different microbial species (i.e. bacteria, protozoa and fungi) are involved in the conversion of feed material to methane in the rumen, with the final step carried out by methanogenic bacteria (McAllister et al., 1996). Digestive microorganisms hydrolyze proteins, starch, lipids and plant cell wall (i.e. carbohydrates) into amino acids and sugars. These products are then fermented to volatile fatty acids (VFA), H2 and CO2 by the digestive microorganisms. The major VFA are acetate, propionate

and butyrate. The produced VFA are absorbed through the rumen wall of the ruminants and supply them with most of the energy required for maintenance and production. The major producers of H2 are the organisms which produce acetate and butyrate (Van Soest, 1982), while the formation of propionate is a competitive pathway for hydrogen use in the rumen (Boadi et al., 2004).
Although H2 is a major end product of fermentation, it does not accumulate in the rumen. It is used as substrate by methanogenic archeae to reduce CO2 to methane (McAllister and Newbold, 2008; Ellis et al., 2008). The process of formation of methane (i.e. CO2 + 4H2 --> CH4 + 2H2O) is thermodynamically favorable to methanogens to generate metabolic energy in the form of adenosine triphosphate (ATP) that is subsequently utilized by these microorganisms for their maintenance and growth (Ellis et al., 2008). The production of methane contributes to the efficiency of the system, because it avoids increases in the partial pressure of H2 levels that might inhibit metabolism of rumen microorganisms (McAllister and Newbold, 2008; Morgavi et al., 2010). Thus, methanogenesis is essential for an optimal performance of the rumen, because it avoids H2 accumulation (Martin et al., 2010). The produced methane is predominately released to the environment through breath and eructation. Although CH4 production benefits many microbes in the rumen, methane also represents an energy loss to the animal since CH4 has no nutritional value to the animal (Boadi et al., 2004) . About 6% of the total gross energy consumed by the animal is converted to CH4 in the rumen (Johnson and Johnson, 1995). This energy loss can reach up to 12% depending upon the quality of the diet (O'Mara, 2004).

The rate of CH4 produced from enteric fermentation in dairy cows depends greatly on the level of feed intake, the quantity of energy consumed, and dietary composition, in particular the nature of carbohydrates such as starch and its degradation rate. There have been several dietary strategies proposed to reduce the production of CH4 from dairy cattle (Grainder and Beauchemin, 2011). Nutritional strategies to decrease the production of methane include the use of diets rich in starch and low in fiber, since the fermentation of starch (as compared to fiber fermentation) increases the production of propionic acid and decreases the production of acetic acid, and increased ratios of propionic to acetic acid are associated with reduced methane levels. An example of such a strategy is the replacement of fiber-rich grass silage with starch-rich maize silage. However, the scientific evidence for this particular replacement strategy is scarce. Recently, Staerfl et al. (2012) investigated this strategy, but the maize silage used had a net energy content some 10% lower than that of the grass silage, which in many countries (including the Netherlands) is highly uncommon as maize silage nearly always has a higher net energy content than grass silage.

The ability to measure (directly or indirectly) methane emission by individual cows is essential for increasing our understanding of factors that contribute to variation in methane emission and thereby for interventions leading to its reduction. Climate respiration chambers are the golden standard for measuring methane emission. However, this technique is very expensive and therefore not suited for large scale measurement or as indicator at farm level. Mathematic models may allow prediction of CH4 production from cattle without extensive experiments, however, the accuracy of these models is low (Ellis et al., 2010). Thus, it is of great importance to develop a simple method to estimate CH4 production in cattle. The aim of this experiment is to evaluate the production of methane in dairy cattle upon replacing grass silage with maize silage, with the longer-term goal to develop an indicator for methane emission that can be measured in milk of individual cows. Grass (silage) and maize silage are the most common feed ingredients in dairy cattle diets. In the Netherlands for example, on average some 30% of the diet of dairy cattle is grass silage, and 25% is maize silage. In this experiment, the hypothesis is that replacement of grass silage with maize silage will decrease the production of methane. The indicator will be used to build further on a prediction model recently proposed by Dijkstra et al. (2011) which is exclusively based on the fatty acid composition of the milk. It is hypothesized that the addition of other metabolites in this prediction model will enhance its predictive power and thus leads to a better indicator for methane emission in milk.

This experiment can be considered novel and differs from recent methane trials in [redacted] for two reasons. Firstly, the scientific evidence worldwide for the nutritional strategy to decrease the production of methane by replacing fibre-rich grass silage with starch-rich maize silage is scarce. This experiment targets to gain more scientific evidence for this replacement strategy. Secondly, the longer term goal of this experiment is to develop an indicator for methane emission that can be measured in milk, for which data are required on feeds commonly used in practice.

Second objective:
Within the setup of the methane experiment, a rapid method to assess the nutritional status of dairy cattle will be developed. The method includes measurement of isotopic enrichment in metabolic end-products in breath (CO2) and milk (fat, protein and lactose) after intravenous administration of isotopically labelled nutrients.

In general, the partitioning of nutrients over anabolic and catabolic pathways is strongly affected by the nutritional status of a dairy cow. However, accurate but simple diagnostic tests for assessing the nutritional status of dairy cows are currently not available. Labelling nutrients with stable isotopes (e.g. 13C, 15N) may be a suitable technique to trace utilization of nutrients and hence provide a proxy for the nutritional status. It is expected that appearance of an injected bolus of an isotopically labelled nutrient in milk components depends on the balance between nutrient requirements (to synthesize milk lactose, fat and protein) and nutrient availability (from diet). Therefore, we hypothesize that the recovery of a labelled nutrient in milk components increases when this nutrient limits milk production, indicating a more efficient utilization for milk synthesis. Similarly, non-limiting nutrients will probably be oxidized to a larger extent and are therefore increasingly recovered as CO2 in breath. A proof of this principle could be obtained from an experiment with a large contrasts in nutrient supply, i.e. glucogenic vs. lipogenic nutrients. The present experiment, in which grass silage is replaced by maize silage, will result in a shift from supply of lipogenic nutrients to glucogenic nutrients. Grass silage is expected to result (after fermentation) mainly in acetic acid, which is lipogenic, whereas maize silage will produce more propionic acid (glucogenic) and some of the starch will escape rumen fermentation and will be hydrolysed in the small intestine to glucose and absorebd as glucose in the blood. Hence, the present experiment will result in a contrast in supply of lipogenic versus glucogenic nutrients.

For intravenous administration of 13C-glucose , it is expected that lactating dairy cows receiving a lipogenic diet will use a larger proportion of available glucose for milk production purposes than cows receiving a glucogenic diet. After injection of [U-13C]glucose, the recovery of isotope in milk lactose is therefore expected to be higher for cows receiving a lipogenic diet. Measuring also isotopic enrichment of the protein and fat fractions of milk may provide information about the use of glucose for other (milk production) purposes. It is known from literature that increased glucose availability may have positive effects on milk lactose and milk protein production (see e.g. Lapierre et al., 2010, Lemosquet et al., 2010, Rigout et al., 2002, Rius et al., 2010, Rulquin et al., 2004). In addition, after intravenous administration of 13C-acetate, as [1-13C] sodium acetate, the recovery of isotope in the milk fat fraction is expected to be higher for cows receiving the lipogenic diet, because milk fat production is usually lower in cows fed glucogenic rations (Van Knegsel et al., 2007). Moreover, assuming that a difference in the nitrogen (N) content of the diet may result in differences in protein metabolism and that such differences can be detected by itntravenous infusion of 15N-glycine, it is expected that intravenous infusion of 15N-glycine could be used as a means to investigate a difference in the nitrogen (N) content of the diet.

1.b. Het uiteindelijk doel (Maatschappelijke en wetenschappelijke relevantie):
Doel:
Methane (CH4) has a global warming potential of 25 carbon dioxide equivalents (Forster et al., 2007). By signing the Kyoto protocol, the EU agreed to have reduced greenhouse gas emissions by at least 20 % by 2020 compared to emission levels in 1990. It is estimated that over half of the CH4 flux into the atmosphere is anthropogenic (IPCC, 2001), comprising 320 million tonnes per year, of which half of the amount is caused by agriculture (Aardenne et al., 2001; Maas et al., 2010; Smith et al. 2007). Methane is the single most important greenhouse gas produced on dairy farms. Besides the effect on global warming, methane emission from cattle represents an energy loss of 2-12% of gross energy intake (Johnson and Ward, 1996).
Finding feeding strategies to reduce methane emission, supports achieving the targets for the Kyoto protocol. Besides, reduction in methane emission will reduce energy losses, which may increase feed

efficiency. The ability to measure (directly or indirectly) methane emission by individual cows is essential for increasing our understanding of factors that contribute to variation in methane emission and thereby for interventions leading to its reduction. Thus, it is of great importance to develop a simple method (i.e. indicator in the milk) to estimate CH4 production in individual cows and dairy herds.

A second aim, within the setup of the methane experiment, is to develop a method for rapid assessment of nutritional status of dairy cattle. The method includes measurement of isotopic enrichment in metabolic end-products in breath (CO2) and milk (fat, protein and lactose) after intravenous administration of isotopically labelled nutrients. Ultimately this method could be used to determine which nutrient is limiting for milk production.

1.c. Lekensamenvatting:

Een indicator voor methaanproductie bij melkkoeien: kuilgras versus snijmais.
Een Nederlandse melkkoe produceert dagelijks gemiddeld 350 gram methaan. Het gas wordt voornamelijk in de pens van de koe geproduceerd bij de voedselvertering. Een kwart van methaangas in onze atmosfeer is afkomstig van melkkoeien. Voor de verduurzaming van de melkproductie in Nederland is het van het grootste belang om de uitstoot van broeikasgassen, in het bijzonder methaan, te verminderen.
Tijdens dit project wordt een indicator ontwikkeld in melk om methaan gas uitstoot bij individuele koeien te meten. Met deze te ontwikkelen indicator in melk zal de methaanuitstoot niet alleen bij individuele koeien kunnen worden gemeten maar ook op kudde niveau. Voor melkveehouders is deze indicator een middel om methaangasuitstoot op hun boerderijen te monitoren en te sturen. Op deze manier zal het project een waardevolle bijdrage leveren aan de verdere verduurzaming van de primaire sector.
Om de indicator te ontwikkelen, worden melkkoeien gebruikt. Aangezien het uiteindelijke doel van dit project het verminderen van methaan uitstoot bij melkkoeien is, wordt tijdens dit onderzoek ook gebruik gemaaktvan melkkoeien. Er worden in totaal 28 melkkoeien gebruikt, verdeeld over 4 groepen. Elke groep zal een ander rantsoen krijgen: (1) 100% kuilgras, (2) 67% kuilgras met 33% snijmais, (3) 33% kuilgras en 67% snijmais, en (4) 100% snijmais. Deze rantsoenen variëren in de hoeveelheid vezels en zetmeel, met de gedachte om verschillen in methaan uitstoot te creëren. Onderzoek met andere voedermiddelen heeft namelijk aangetoond dat een rantsoen rijk aan vezels resulteert in een hogere methaan uitstoot ten opzichte van een rantsoen rijk aan zetmeel. Kuilgras en snijmais zijn de meest gebruikte voedermiddelen in de Nederlandse melkveehouderij, maar de specifieke kennis over methaanproduktie bij vervanging van kuilgras door snijmais (of omgekeerd) is vrijwel afwezig.
De koeien worden gedurende 12 dagen in een grupstal gehouden voor de adaptatieperiode. Tijdens deze periode raken de koeien geadapteerd aan het nieuwe rantsoen en aan het aangebonden staan. Na deze 12 dagen, worden de koeien overgeplaatst naar de klimaatkamers. Ze worden individueel gehuisvest in de klimaatkamers, maar door de aanwezigheid van ramen kunnen ze elkaar zien en horen. In deze klimaatkamers blijven de koeien hetzelfde rantsoen ontvangen en staan ze aangebonden. Ze zullen bijna 5 dagen in deze klimaatkamers staan, zodat we de methaan uitstoot kunnen meten. Elke dag wordt tijdens het ochtend en avond melken, melkmonsters genomen. Na deze 5 dagen, worden de melkkoeien weer teruggebracht naar de stal.
Naderhand zal de melk geanalyseerd worden op samenstelling. Het is de bedoeling dat de samenstelling van de melk in relatie gebracht kan worden met de hoeveelheid methaan uitstoot. Er zal uiteindelijk, aan de hand van de relatie, een indicator in melk ontwikkeld worden om methaan gas uitstoot bij koeien te meten.

Binnen de opzet van het hiervoor beschreven onderzoek kan nog een tweede onderzoeksvraag worden beantwoord. In voedingsonderzoek, ook bij melkvee, wordt in toenemende mate gebruik gemaakt van labeling met stabiele isotopen. Dergelijke studies bieden de mogelijkheid het gebruik van individuele nutriënten te volgen en worden dan ook voornamelijk ingezet bij kwantitatieve studies naar de effecten van voeding op productie. Het voorgestelde experiment is gebaseerd op de hypothese dat de manier waarop een melkkoe haar metabolieten gebruikt afhankelijk is van haar voedingsstatus. Er kan dus een relatie worden verwacht tussen de voedingsstatus van een koe en de recovery van een geïnjecteerde dosis van een met isotopen gelabelde metaboliet in de melk. Een dergelijke relatie kan, zonder kwantitatief te zijn, zeker informatief zijn over de voedingsstatus van de koe. Deze techniek zou in de

praktijk op koppelniveau kunnen worden toegepast.

2. Gepland vanaf: 03-11-2012 **tot** 21-12-2012

3. Diersoort: runderen ; **Totaal aantal:** 34

4.a. Nadere aanduiding gebruikte dieren:
28 lactating dairy cows (4 treatments, 7 cows per treatment), of which 12 cows will be rumen fistulated (i.e., only cows who already have a fistula; no new surgery is required). Since the experiment is a randomised block design with 4 treatments, always blocks of 4 animals of similar characteristics are considered.
In addition, 4 reserve animals will be assigned to a reserve block. These reserve animals will only be used if the other cows would become unsuitable for use in the experiment.

Moreover, 2 cows will be used to test the intravenous infusion technique. The intravenous infusion technique will be tested first on these two animals, in order to refine the technique and to minimize the stress induced for the animals in the running experiment.

4.b. Motivatie waarom is gekozen voor deze diersoort:
The aim is to reduce methane emission from dairy cows, thus these are the target animals. Fistulated cows are needed for measurements of rumen volatile fatty acid (VFA) concentration and pH. Also, the standard in vitro technique will be applied, in which rumen fluid of individual cows is used as inoculum to study fermentation in vitro.

4.c. Toelichting voor het aantal gebruikte dieren:
During this experiment, the experimental unit will be one individual lactating dairy cow. There will be 7 cows assigned to each dietary treatment (i.e. 4 treatments, see following section). Thus, this results in 28 cows in total. This number of cows per treatment is the minimal number that allows sufficient statistical power and is based on the variation in methane emission found in previous studies with a similar experimental set-up. The recent studies of [redacted] in the [redacted] have shown that a number of 10 cows per experimental treatment is sufficient to detect ($P<0.05$) treatment effect of approximately 10%. However, [redacted] used a different experimental unit. The 10 lactating cows were paired and the experimental unit was 2 cows per respiration chamber. This results in 5 experimental units. In this type of experimental setting, a lower number of cows will not allow a methane difference to be significant. For the present experiment, the cows will be housed individually in a respiration chamber instead of in pairs. Using 5 experimental units, as done by [redacted], is not sufficient because using pairs of cows per chamber as an experimental unit gives less statistical variation than using only 1 cow per chamber as an experimental unit. Therefore, it is estimated that 6 individual cows per treatment should give a similar statistical power compared with the 5 units (i.e. 2 cows per chamber) per treatment. However, chamber techniques differ worldwide (e.g., accuracy depends on frequency of gas sampling). Therefore, 7 cows per treatment will be required. Based on various rumen models, and depending on the actual nutritional quality of the grass and the maize silage, the predicted difference in methane production between grass silage and maize silage diets is some 10-15%.

Of the 28 cows, 12 cows will be rumen fistulated (i.e. only cows who already have a fistula). Fistulated cows are needed to sample rumen fluid for determination of pH and volatile fatty acid (VFA) concentration, , and as inoculum for the gas production technique. The aim is to use 3 fistulated cows per treatment. This number is based upon a study with a block-design with 3 fistulated cows per treatment by Abrahamse et al. (2009), showing that significant pH differences of 0.1 units could be detected. In addition, differences in the ratio between non-glucogenic and glucogenic VFAs (and therefore the ratio between VFAs that release hydrogen and VFA that act as hydrogen sinks; the balance of which is strongly related to methane production) of 0.1 were detected. It is therefore expected that using 3 fistulated cows per treatment will result in finding differences of approximately the same level.

Moreover, 2 cows will be used to practice the intravenous infusion technique. The intravenous infusion technique will be practiced first on these two animals, in order to refine the technique and to minimize the

stress induced for the animals in the running experiment.

4.d. Herkomst: A. van gereg. fok/toeleveringsbedrijf in Nederland
Toelichting:
Lactating dairy cows of experimental accommodations [redacted]) and [redacted].

In total, 34 cows will be selected from [redacted], of which 12 fistulated cows. Only cows that have been fistulated for previous purposes will be used; no new surgery will be done for this experiment. As much as possible cows of [redacted] will be chosen, provided that cows match in parity, lactation stage and milk production per group of 4 animals (since there are 4 treatments).

5.a. Accommodatie: [redacted]
Accommodation is in the dairy cattle tie-stall and climate respiration chambers at [redacted].

The lactating dairy cows for this experiment will be selected from [redacted]) and [redacted]. The cows from [redacted] will be transported to [redacted], placed together in a group at [redacted] after arrival and receive a pre-adaptation period of at least 5 days. The lactating cows from [redacted] will be housed separately in the free barn from the already present animals, to avoid unrest due to arrival of new animals. During this period, they will receive the basic ration fed at [redacted]. It is expected that this pre-adaptation period of 5 days is sufficient, for two reasons. Firstly, these 5 days will give the cows from [redacted] a sufficient amount of time to recover from their journey to [redacted]. In addition, >80% of the micro flora in the rumen is adapted to a newly introduced ration after 5 to 7 days (Boeckaert et al., 2008). However, a pre-adaptation of 7 days (i.e. complete adaptation to the basic ration fed at [redacted]) is not needed since it is not the experimental diet yet. This period can also be considered as a synchronization period, during which all subjects (i.e. both from [redacted] and [redacted]) get accustomed to the same total mixed ration diet. Animals will be group-fed in this period and feed intake will not be monitored.

During the first 12 days during the experimental period, the cows are housed in the tie-stalls. The period in the tie-stalls is considered to be the adaptation period in which the dairy cows can adapt to the experimental diet and get accustomed to being tied down. A previous study of Van Zijderveld et al. (2011) showed that an adaptation period of 12 days is sufficient. Methane production in respiration chambers seems rather constant after entering the respiration chambers on day 13, and there is no clear day-effect which would have been expected if adaptation have been incomplete. The tie-stalls will be illuminated by daylight and TL-lamps which will be switched on between 5:30 a.m. and 21:30 p.m..
After the adaptation period in the tie stalls, the measuring period begins by transporting the 4 subjects to the calorimetric respiration chambers. During this measuring period, the cows will be fed the very same restricted ration as before. The subjects will be moved to the respiration chambers on day 13 at 15:00h and will be removed from the chambers on day 17 at 9:00h. In these respiration chambers, the TL-lamps will be switched on between 5:30 a.m. and 21:30 p.m. At night, a small lamp will illuminate the chambers. The relative humidity in the chambers will be set at 70% and the temperature at 16 C. The methane production will be determined during 3 days of the measuring period (i.e. 2nd-4th day of the measuring period). Van Zijderveld et al. (2011) has applied this procedure before. Cows will be housed individually in a respiration chamber (i.e. 1 cow per chamber). The individually housed cows are able to see each other through windows and are capable of hearing the sounds another cow makes.

5.b. Huisvesting & Verzorging:
Cows are fed and milked twice daily. Drinking water is supplied at libitum. During the adaptation period and in the respiration chambers, feed intake and milk production will be measured twice daily. This provides the opportunity to recognize health issues. All subjects will be monitored on a daily basis for clear signs of difficult of refusing to get up, eat, or any other remarkably reason, and if any of this occurs and

other health problems a veterinarian will be called immediately. In general, cows will be withdrawn from the experiment when a situation occurs that affects the characteristics to be measured. In case of suspicion of health issues, experts (i.e., the caretaker of the animals and [redacted], article 14 expert on laboratory animals) will be consulted, after which it will be decided if veterinary treatment should be obtained. When antibiotics have to be supplied, or in case of health issues or medicine supplies which might interfere with metabolism and feed intake, the cow will be withdrawn from the study.

5.c. Voeding:
This experiment has a completely randomized block design in order to examine the effect of starch level in the diet on methane emission in dairy cattle. The cows will receive 1 of the 4 experimental diets. The diets will consist of grass silage and / or maize silage in order to create different fiber and starch levels in the diet. The four diets are (% of roughage compared to the total the total amount of roughage in the diet): 100% grass silage (GS100), 67% grass silage and 33% maize silage (GSMS67:33), 33% grass silage and 67% maize silage (GSMS33:67) and 100% maize silage (MS100).

The cows will be milked and fed twice daily (06.00 and 16.00 hours) and have ad libitum access to clean drinking water. All cows will receive their experimental diet as a total mixed ration. The forage:concentrate ratio will be 80:20 based on dry matter content. The proportion of concentrate in the diet will be kept low in order to create large differences in nutrient composition between the experimental diets, but concentrates need to be included in the ration to meet the requirements for maintenance and lactation. The composition of the concentrate will be the same for all four treatments. The composition of the concentrates will be chosen carefully, in order to meet the minimal OEB (rumen degradable protein balance) and DVE (intestinal available protein) requirements according to Dutch standards for dairy cattle (Centraal Veevoer Bureau). In addition, the concentrate will contain minerals and nutrients (premix).

The experimental diets will be fed individually and ad libitum during the first 7 days of the adaptation period in the tie stalls. From day 8 till 17 (i.e. last 5 days adaptation period and measuring period) the feed intake will be restricted per block to 95% of the ad libitum feed intake of the subject consuming the lowest amount of feed during day 5 till 8 within a block (Van Zijderveld et al., 2011). Since feed intake is a major determinant of methane production, restriction of the feed intake is required. The feed intake will be restricted to have identical dry matter intake for cows in the same block as much as possible throughout the whole experiment, provided that the cow with the highest feed intake in the block is never restricted to less than 82% of the ad libitum feed intake. It is expected that the 4 cows within a block will have approximately the same feed intake, due to the fact that they have been grouped into the blocks according to lactation stage, parity, milk production, fat- and protein levels in the milk. However, even a small difference in milk production can be accompanied with a large difference in feed intake (i.e. cows that differ of 2.2. kg milk production, differ approximately 1 kg of dry matter feed intake when fed ad libitum). Thus, restricted feeding is needed, because the variation in methane production will be unacceptable due to the variation in feed intake level between the subjects when fed ad libitum. This could potentially make it more difficult to find statistical valid conclusions. Feeding restrictedly will lead to a more similar feed intake within blocks and the reduced variation will help to detect smaller differences between treatments.

When animals consume lower amounts of feed than they are used to, less nutrients are available for production. We expect milk production to adjust to the feed intake level. Previous studies in [redacted] of [redacted] have demonstrated that this method does not lead to problems. Staff of [redacted] and researchers of [redacted] that will be involved in the trial, are experienced with this method. To prevent problems with negative energy balance as much as possible, we will not use cows that have only been in lactation for 8 weeks or less.

6.a. Proefschema / proefbehandelingen:
This experiment has a completely randomized block design in order to examine the effect of starch level in the diet on methane emission in dairy cattle.

For this experiment, a total of 28 lactating dairy cows will be selected and used. The 28 lactating dairy

cows will be grouped into 7 blocks of 4 cows according to lactation stage, parity, milk production, fat- and protein levels in the milk and presence of a fistula. By doing this, 7 blocks are made, each with relative similarly cows. Therefore, it is expected that the dry matter feed intake will be quite similar for all 4 animals of one block. Within blocks, animals will be randomly assigned to one of the four experimental diets, resulting that all treatments occur within a block. In addition, 1 reserve block of 4 reserve cows will be made. These reserve cows will only be used if other cows would become unsuitable for use in the experiment.

The cows will receive 1 of the 4 experimental diets (forage:concentrate ratio of 8:2). The diets will consist of grass silage and / or maize silage in order to create different starch level in the diet. The four diets are (% of roughage compared to the total the total amount of roughage in the diet):

1. 100% grass silage (GS100)
2. 67% grass silage and 33% maize silage (GSMS67:33)
3. 33% grass silage and 67% maize silage (GSMS33:67)
4. 100% maize silage (MS100).

Feed:
Dry matter content will be measured daily and the feed intake will be calculated based on the dry matter content. Feed will be supplied twice daily (at 6.00 and 16.00) as total mixed ration, consisting of forage and concentrate in the ratio 8:2. Each ration will fulfil requirements and will not cause any feed-related health issues such as acidosis. The proportion of concentrate in the diet will be kept low in order to create large differences in nutrient composition between the experimental diets, but concentrates need to be included in the ration to meet the requirements for maintenance and lactation. The composition of the concentrate will be the same for all four treatments. The composition of the concentrates will be chosen carefully, in order to meet the minimal OEB (rumen degradable protein balance) and DVE (intestinal available protein) requirements according to Dutch standards for dairy cattle (Centraal Veevoer Bureau). In addition, the concentrate will contain minerals and nutrients (premix).
In the respiration chambers and the last few days of adaptation, feed is supplied restrictedly (95% of the lowest intake of the 4 animals of a block). The feed restriction is required to reduce variation in feed intake between animals, since feed intake in itself is a major determinant of methane production. Animals will have ad libitum access to drinking water in free barn, tie stall and in respiration chambers.

Experimental set-up:
The cows from [redacted] will be transported to [redacted], placed together in a group at [redacted] after arrival and receive a pre-adaptation period of at least 5 days. The lactating cows from [redacted] will be housed separately in the free barn from the already present animals, to avoid unrest due to arrival of new animals. After at least 5 days, the cows are moved to the tie-stalls. In every block, the 4 animals will be moved to the tie-stalls. The period in the tie-stalls is considered to be the adaptation period in which the dairy cows can adapt to the experimental diet and get accustomed to being tied down. A previous study of Van Zijderveld et al. (2011) showed that an adaptation period of 12 days is sufficient. In addition, during this adaptation period the feed intake can be controlled and measured.
During the first 7 days feed is supplied ad libitum. After 7 days, feed supply is restricted. This procedure has been used in a number of experiments before (Van Zijderveld et al., 2011) and is needed since feed intake is a major determinant of methane production. During the last 2 days of the adaptation period, samples of rumen fluid will be taken from fistulated cows to measure volatile fatty acid concentration and pH levels. Rumen samples will be taken once before morning feeding and at 7 time points after morning feeding; 1, 2, 3, 4, 6, 8 and 10h after feeding. Similarly, part of the rumen sample taken just before morning feeding is used as inoculum for the gasproduction technique at the Animal Nutrition Group.
After the adaptation period in the tie stalls, the measuring period begins by transporting the 4 subjects to the respiration chambers. During this measuring period, the cows will be fed the very same restricted ration as before. The subjects will be moved to the respiration chambers on day 13 at 15:00h and will be removed from the chambers on day 17 at 9:00h. Cows will be housed individually in a respiration chamber (i.e. 1 cow per chamber). The individually housed cows are able to see each other through windows and are capable of hearing the sounds another cow makes.
The methane production will be determined during 3 full days of the measuring period (i.e. starting at 09.00h on day 14 until 09.00h on day 17). Methane emission is expressed per day. Van Zijderveld et al.

(2011) has applied this procedure before. If measurements would only be done for 2 days, this could affect the accuracy of the methane measurements (e.g. when something disturbs the data on one day, like an error in the measurement equipment). Also for measuring the energy balance, which has been done many times in Wageningen, a measurement period of 3 days has been shown to give reliable data. Methane emission, O2 consumption, CO2 production, urine plus faeces production and milk production will be registered. The ration components and the manure collected over the whole period in the chambers will be analyzed for amongst others organic matter, cellulose, hemicellulose, starch and crude fat. The milk samples will be analysed on composition, fatty acid profile, fat, protein, lactose and urea levels. After the measurements in the respiration chambers, most cows are transported back to the free barn of [redacted]. Cows from [redacted] are transported back all at once after the last group of cows has been in the respiration chambers. At the end of each experimental period, the respiration chambers have to be cleaned completely using as little cleaning water as possible. All urine, faeces and cleaning water will be collected in a large tank. This will be mixed for homogeneity and sampled for dry matter, ash, nitrogen, crude fat, neutral detergent fiber and gross energy determination

The isotope experiment will be carried out within the previously explained experimental setup. The experimental procedure per cow will be as follows:
Infusion grade isotopes will be dissolved in sterile water. Solutions will be prepared in collaboration with the pharmacy of [redacted] and administered to the animals as a sterile nutrient solution.
On day 2 in the respiration chambers, after morning milking and feeding around 6am, a labeled nutrient solution (13C-glucose and 15N-glycine) will be injected in the jugular vein. 4 g 15N-glycine and 2 g of 13C-glucose will be dissolved in a volume of 500 ml sterile water. Before injection, a catheter (Type: Intraflon i.v. 12G 80mm) will be placed into the jugular vein to make sure that the entire dose is injected in the blood stream. To facilitate catheter insertion, a small incision in the skin will be made. After isotope administration the catheter will be flushed with saline and subsequently removed. The skin will be closed with suturing staples. The bottle used for injection of the isotope solution will be weighed before and after injection to determine the infused volume. On day 4 in the respiration chambers, 9 g of 13C-NaAcetate will be infused in a volume of 500 ml sterile water following the same procedure as for infusion of isotopically labeled glucose and glycine. The amounts of isotopically labelled nutrients have been chosen in such a way to detect sufficient amounts of isotope in milk by combustion-IRMS.
Duplicate samples (200ml) of the morning milking of day 2 and 4 in the respiration chambers will be taken for background measurement of isotopic enrichment. Isotopic enrichment in breath will be measured at 9 minute intervals. After infusion of isotopically labelled material, milk from the 3 subsequent milking (duplicate sample of 200ml per milking) will be collected. The third milking after administration of 13C-acetate will be collected outside the chambers, but after a similar time interval as for 13C-glucose. For each milking, a representative subsample of the milk will be analysed for milk composition(i.e. fat, protein and lactose) and a second subsample will be fractionated. Fat will be extracted by centrifugation. The skimmed milk will be used to extract protein using ultrafiltration of skimmed milk. The lactose fraction is left in the permeate of the ultrafiltration procedure. After freeze drying, the different milk fractions will be analysed for isotopic enrichment using combustion IRMS. During this procedure enrichment with 15N and 13C can be measured simultaneously.

6.b. Mate van ongerief: B. Gering/Matig

6.c. Waaruit bestaat het ongerief en hoe bent u tot uw inschatting van de mate van ongerief gekomen?
- Housing the cows tied during the adaptation period and in the respiration chambers
- Opening/closing the fistula for collecting rumen fluid
- Individual housing of animals in chambers. The effect of individual housing on animals is minimised by allowing animals to see each other through the windows in the chambers and when animals make a sound, the other cows will hear that as well.
- During the period that the animals will be housed in the respiration chambers, an intravenous catheter

will be inserted twice for injection of a solution isotopically labelled nutrients into the bloodstream.

The discomfort has been estimated based on the above and based on previous experiments within [redacted] chambers with dairy cattle.

7. Welke maatregelen heeft u getroffen om het ongerief tot een minimum te beperken?
Anesthesie: A. Niet toegepast (geen aanleiding).
Pijnbestrijding: A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.

Re-use of previously fistulated animals.
Placing animals from [redacted] in a separate group after arrival at [redacted].
Housing animals in the respiration chambers in which they can see each other through windows.
Practicing the intravenous infusion technique under instruction of an experienced veterinarian. To do this, two cows from the [redacted] herd will be used. Also for the first infusions, adequate supervision will take place.

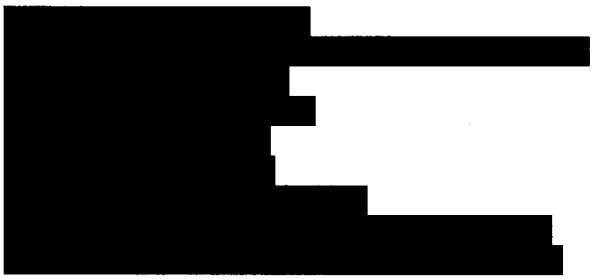
8. Toestand van dieren na einde van de proef: Het dier is na de proef in leven gelaten.
Toelichting:
Cows will return to the accommodation after the experiment [redacted]

9. Welke alternatieven (vervanging, verfijning, vermindering) zijn voor de beschreven experimenten overwogen en waarom zijn deze verworpen?
Replacement:
In vitro analyses of some previous studies have been shown not to be representative for in vivo measurements. It is also difficult to represent processes like pH, VFA absorption rate and passage rate in vitro; such processes are expected to have a major impact on methane. Therefore this study should be performed in vivo with the target animal (dairy cow) itself. The measurement of pH and of VFA concentration and type, major elements associated with methane production, also has to be performed in the target animal (fistulated animals) as the buffered in vitro systems do not allow such effects to be evaluated.

Refinement:
Social isolation will be prevented by housing cows in individual respiration chambers with windows in the walls; the sounds of the animals will be heard by other animals as the walls of the chambers are quite thin. In the adaptation area they will be housed in larger numbers.
Transport of cattle from [redacted] will be transported for some 80 km distance and will be housed separately in the free barn from the already present animals, to avoid unrest due to arrival of new animals; from their special, separate part of the free barn, they will move after a minimal of 5 days to the tie-stall.
Of the fistulated animals, only animals that already have a fistula will be used. To make optimal use of the available fistulated cows, cows of [redacted] will be used. Therefore no new surgery is required.

Reduction:
We will use the lowest number of animals possible to obtain the desired statistical accuracy (see part 4c). By doing some measurements repeatedly in the same animals (pH and VFA measurements) we try to obtain as many data as possible from one animal. In addition, we will also use data from previous experiments (i.e. [redacted]) in order to find a robust indicator for methane emission with the use of a minimal number of cows.
Moreover, answering multiple research questions within the same experimental setup will further contribute to reduction as it is not needed to setup a separate experiment.

10. Namen van direct betrokkenen bij de dierproef (artikel 9- en 12-functionarissen):
[redacted]



Tabel registratiecode opties voor aanvraag 2012086.a (K14):

1	2	3	4	5	6	7	8	9	10	11	12	13
1	**45**	**1**	**34**	**37**	**1**	**1**	**01**	**01**	**1**	**1**	**2**	**3**

Aanmeldingsformulier voor proeven met gewervelde dieren.

Secretariaat DEC
[redacted]

Aanvrager: [redacted]
Afdeling:

Titel dierproef: Indicator for methane emission in dairy cattle: grass silage versus maize silage

Aanmeldcode / Protocol: 2012086.b
Stadia van de proef:

19-10-2012	Aangemeld	[redacted]
25-10-2012	Wijzigen	Secretaris van de DEC
25-10-2012	Gekopieerd	[redacted]

Is deze proef wetenschappelijk getoetst en goedgekeurd? Ja
Toelichting:
Yes, in association with a PhD procedure of [redacted]. The project has been approved by the financial sponsors ([redacted]). Furthermore, the research committee of the [redacted] has discussed the project.

1.a. Met dit onderzoek te beantwoorden concrete vraag:
. Wetenschappelijke vraag m.b.t.wetensch. vraag
Methane is produced during microbial fermentation of feed in the gastrointestinal tract of ruminants, predominantly within the rumen (87%) and to a small extent (13%) in the large intestine (Murray et al., 1976). The rumen is an anaerobic environment in which microbiota digest feed components. Different microbial species (i.e. bacteria, protozoa and fungi) are involved in the conversion of feed material to methane in the rumen, with the final step carried out by methanogenic archeae (McAllister et al., 1996). Methanogenic archeae reduce CO2 to methane (McAllister and Newbold, 2008; Ellis et al., 2008). The process of formation of methane (i.e. CO2 + 4H2 --> CH4 + 2H2O) by methanogens generates metabolic energy for their maintenance and growth (Ellis et al., 2008).
The produced methane is predominately released to the environment through breath and eructation. Methane (CH4) is one of the most important greenhouse gases (GHG) and a significant contributor to global warming. In the dairy cattle chain, methane represents more than 50% of all the GHG produced. In addition, methane also represents an energy loss to the animal since CH4 has no nutritional value to the animal (Boadi et al., 2004) . About 6% of the total gross energy consumed by the animal is converted to CH4 in the rumen (Johnson and Johnson, 1995). This energy loss can reach up to 12% depending upon the quality of the diet (O'Mara, 2004). Therefore, it is desired to reduce the methane emission from dairy cattle, in order to reduce the ecological footprint of milk production, and improve feed efficiency and animal productivity.
The rate of CH4 produced from enteric fermentation in dairy cows depends greatly on the level of feed intake, the quantity of energy consumed, and dietary composition, in particular the nature of carbohydrates such as starch and its degradation rate. There have been several dietary strategies proposed to reduce the production of CH4 from dairy cattle (Grainger and Beauchemin, 2011), including the use of diets rich in starch and low in fiber, since the fermentation of starch (as compared to fiber fermentation) increases the ratios of propionic to acetic acid, which are associated with reduced methane

levels. An example of such a strategy is the replacement of fiber-rich grass silage with starch-rich maize silage. However, the scientific evidence for this particular replacement strategy is scarce. Recently, Staerfl et al. (2012) investigated this strategy, but the maize silage used had a net energy content some 10% lower than that of the grass silage, which in many countries (including the Netherlands) is highly uncommon as maize silage nearly always has a higher net energy content than grass silage.

The ability to measure methane emission by cows is essential for increasing our understanding of factors that contribute to variation in methane emission and thereby for interventions leading to its reduction. Climate respiration chambers are the golden standard for measuring methane emission. However, this technique is very expensive and therefore not suited for large scale measurement or as indicator at farm level. Mathematic models may allow prediction of CH4 production from cattle without extensive experiments, however, the accuracy of these models is low (Ellis et al., 2010). Thus, it is of great importance to develop a simple method to estimate CH4 production in cattle.
The current experiment is designed to observe the effect of replacing grass silage (no starch) with maize silage (starch-rich) on methane emission from lactating dairy cows, with the ultimate goal to develop an indicator for methane emission that can be measured in milk of individual cows. The hypothesis is that replacement of grass silage with maize silage will decrease the production of methane. The indicator will be used to build further on a prediction model recently proposed by Dijkstra et al. (2011) which is exclusively based on the fatty acid composition of the milk. It is hypothesized that the addition of other metabolites in this prediction model will enhance its predictive power and thus leads to a better indicator for methane emission in milk.
This experiment can be considered novel and differs from recent methane trials in [redacted] for two reasons. Firstly, the scientific evidence worldwide for the nutritional strategy to decrease the production of methane by replacing fibre-rich grass silage with starch-rich maize silage is scarce. This experiment targets to gain more scientific evidence for this replacement strategy. Secondly, the longer term goal of this experiment is to develop an indicator for methane emission that can be measured in milk, for which data are required on feeds commonly used in practice. Grass (silage) and maize silage are the most common feed ingredients in dairy cattle diets. In the Netherlands for example, on average some 30% of the diet of dairy cattle is grass silage, and 25% is maize silage.

Second objective:
Within the setup of the methane experiment, a rapid method to assess the nutritional status of dairy cattle will be developed. The method includes measurement of isotopic enrichment in metabolic end-products in breath (CO2) and milk (fat, protein and lactose) after intravenous administration of isotopically labelled nutrients.

In general, the partitioning of nutrients over anabolic and catabolic pathways is strongly affected by the nutritional status of a dairy cow. However, accurate but simple diagnostic tests for assessing the nutritional status of dairy cows are currently not available. Labelling nutrients with stable isotopes (e.g. 13C, 15N) may be a suitable technique to trace utilization of nutrients and hence provide a proxy for the nutritional status. It is expected that appearance of an injected bolus of an isotopically labelled nutrient in milk components depends on the balance between nutrient requirements (to synthesize milk lactose, fat and protein) and nutrient availability (from diet). Therefore, we hypothesize that the recovery of a labelled nutrient in milk components increases when this nutrient limits milk production, indicating a more efficient utilization for milk synthesis. Similarly, non-limiting nutrients will probably be oxidized to a larger extent and are therefore increasingly recovered as CO2 in breath. A proof of this principle could be obtained from an experiment with a large contrasts in nutrient supply, i.e. glucogenic vs. lipogenic nutrients. The present experiment, in which grass silage is replaced by maize silage, will result in a shift from supply of lipogenic nutrients to glucogenic nutrients. Grass silage is expected to result (after fermentation) mainly in acetic acid, which is lipogenic, whereas maize silage will produce more propionic acid (glucogenic) and some of the starch will escape rumen fermentation and will be hydrolysed in the small intestine to glucose and absorbed as glucose in the blood. Hence, the present experiment will result in a contrast in supply of lipogenic versus glucogenic nutrients.

For intravenous administration of 13C-glucose , it is expected that lactating dairy cows receiving a

lipogenic diet will use a larger proportion of available glucose for milk production purposes than cows receiving a glucogenic diet. After injection of [U-13C]glucose, the recovery of isotope in milk lactose is therefore expected to be higher for cows receiving a lipogenic diet. Measuring also isotopic enrichment of the protein and fat fractions of milk may provide information about the use of glucose for other (milk production) purposes. It is known from literature that increased glucose availability may have positive effects on milk lactose and milk protein production (see e.g. Lapierre et al., 2010, Lemosquet et al., 2010, Rigout et al., 2002, Rius et al., 2010, Rulquin et al., 2004). In addition, after intravenous administration of 13C-acetate, as [1-13C] sodium acetate, the recovery of isotope in the milk fat fraction is expected to be higher for cows receiving the lipogenic diet, because milk fat production is usually lower in cows fed glucogenic rations (Van Knegsel et al., 2007). Moreover, assuming that a difference in the nitrogen (N) content of the diet may result in differences in protein metabolism and that such differences can be detected by itntravenous infusion of 15N-glycine, it is expected that intravenous infusion of 15N-glycine could be used as a means to investigate a difference in the nitrogen (N) content of the diet.

1.b. Het uiteindelijk doel (Maatschappelijke en wetenschappelijke relevantie):
Methane (CH4) has a global warming potential of 25 carbon dioxide equivalents (Forster et al., 2007). By signing the Kyoto protocol, the EU agreed to have reduced greenhouse gas emissions by at least 20 % by 2020 compared to emission levels in 1990. Methane is the single most important greenhouse gas produced on dairy farms. Besides the effect on global warming, methane emission from cattle represents an energy loss of 2-12% of gross energy intake (Johnson and Ward, 1996). Finding feeding strategies to reduce methane emission, supports achieving the targets for the Kyoto protocol. Besides, reduction in methane emission will reduce energy losses, which may increase feed efficiency.
With this experiment we aim to gain more scientific evidence for the nutritional strategy to decrease the methane production by replacing grass silage with maize silage. Our ultimate goal is to put this nutritional strategy into practice on dairy farms in The Netherlands and develop a simple method (i.e. indicator in the milk) to estimate CH4 production in individual cows and dairy herds, in order to increase our understanding of factors that contribute to variation in methane emission and thereby for interventions leading to its reduction.

A second aim, within the setup of the methane experiment, is to develop a method for rapid assessment of nutritional status of dairy cattle. The method includes measurement of isotopic enrichment in metabolic end-products in breath (CO2) and milk (fat, protein and lactose) after intravenous administration of isotopically labelled nutrients. Ultimately this method could be used to determine which nutrient is limiting for milk production.

1.c. Lekensamenvatting:
Een indicator voor methaanproductie bij melkkoeien: kuilgras versus snijmais.
Een Nederlandse melkkoe produceert dagelijks gemiddeld 350 gram methaan. Het gas wordt voornamelijk in de pens van de koe geproduceerd bij de voedselvertering. Een kwart van methaangas in onze atmosfeer is afkomstig van melkkoeien. Voor de verduurzaming van de melkproductie in Nederland is het van het grootste belang om de uitstoot van broeikasgassen, in het bijzonder methaan, te verminderen.
Tijdens dit project wordt een indicator ontwikkeld in melk om methaan gas uitstoot bij individuele koeien te meten. Met deze te ontwikkelen indicator in melk zal de methaanuitstoot niet alleen bij individuele koeien kunnen worden gemeten maar ook op kudde niveau. Voor melkveehouders is deze indicator een middel om methaangasuitstoot op hun boerderijen te monitoren en te sturen. Op deze manier zal het project een waardevolle bijdrage leveren aan de verdere verduurzaming van de primaire sector.
Om de indicator te ontwikkelen, worden melkkoeien gebruikt. Aangezien het uiteindelijke doel van dit project het verminderen van methaan uitstoot bij melkkoeien is, wordt tijdens dit onderzoek ook gebruik gemaaktvan melkkoeien. Er worden in totaal 28 melkkoeien gebruikt, verdeeld over 4 groepen. Elke groep zal een ander rantsoen krijgen: (1) 100% kuilgras, (2) 67% kuilgras met 33% snijmais, (3) 33% kuilgras en

67% snijmais, en (4) 100% snijmais. Deze rantsoenen variëren in de hoeveelheid vezels en zetmeel, met de gedachte om verschillen in methaan uitstoot te creëren. Onderzoek met andere voedermiddelen heeft namelijk aangetoond dat een rantsoen rijk aan vezels resulteert in een hogere methaan uitstoot ten opzichte van een rantsoen rijk aan zetmeel. Kuilgras en snijmais zijn de meest gebruikte voedermiddelen in de Nederlandse melkveehouderij, maar de specifieke kennis over methaanproduktie bij vervanging van kuilgras door snijmais (of omgekeerd) is vrijwel afwezig.
De koeien worden gedurende 12 dagen in een grupstal gehouden voor de adaptatieperiode. Tijdens deze periode raken de koeien geadapteerd aan het nieuwe rantsoen en aan het aangebonden staan. Na deze 12 dagen, worden de koeien overgeplaatst naar de klimaatkamers. Ze worden individueel gehuisvest in de klimaatkamers, maar door de aanwezigheid van ramen kunnen ze elkaar zien en horen. In deze klimaatkamers blijven de koeien hetzelfde rantsoen ontvangen en staan ze aangebonden. Ze zullen bijna 5 dagen in deze klimaatkamers staan, zodat we de methaan uitstoot kunnen meten. Elke dag wordt tijdens het ochtend en avond melken, melkmonsters genomen. Na deze 5 dagen, worden de melkkoeien weer teruggebracht naar de stal.
Naderhand zal de melk geanalyseerd worden op samenstelling. Het is de bedoeling dat de samenstelling van de melk in relatie gebracht kan worden met de hoeveelheid methaan uitstoot. Er zal uiteindelijk, aan de hand van de relatie, een indicator in melk ontwikkeld worden om methaan gas uitstoot bij koeien te meten.

Binnen de opzet van het hiervoor beschreven onderzoek kan nog een tweede onderzoeksvraag worden beantwoord. In voedingsonderzoek, ook bij melkvee, wordt in toenemende mate gebruik gemaakt van labeling met stabiele isotopen. Dergelijke studies bieden de mogelijkheid het gebruik van individuele nutriënten te volgen en worden dan ook voornamelijk ingezet bij kwantitatieve studies naar de effecten van voeding op productie. Het voorgestelde experiment is gebaseerd op de hypothese dat de manier waarop een melkkoe haar metabolieten gebruikt afhankelijk is van haar voedingsstatus. Er kan dus een relatie worden verwacht tussen de voedingsstatus van een koe en de recovery van een geïnjecteerde dosis van een met isotopen gelabelde metaboliet in de melk. Een dergelijke relatie kan, zonder kwantitatief te zijn, zeker informatief zijn over de voedingsstatus van de koe. Deze techniek zou in de praktijk op koppelniveau kunnen worden toegepast.

2. Gepland vanaf: 03-11-2012 **tot** 21-12-2012

3. Specificatie diergroepen:

Cows with rumen fistela (already have a fistula, no operation required)	12	runderen	Cows which are rumen fistutaled
Cows with no rumen fistela	22	runderen	Cows which are not rumen fistulated.

4.a. Nadere aanduiding gebruikte dieren:
28 lactating dairy cows (4 treatments, 7 cows per treatment), of which 12 cows will be rumen fistulated (i.e., only cows who already have a fistula; no new surgery is required). The 28 lactating dairy cows will be grouped into 7 blocks of 4 cows according to lactation stage, parity, milk production, fat- and protein levels in the milk and presence of a fistula. By doing this, 7 blocks are made, each with relative similarly cows. Therefore, it is expected that the dry matter feed intake will be quite similar for all 4 animals of one block. Within blocks, animals will be randomly assigned to one of the four experimental diets, resulting that all treatments occur within a block. In addition, 1 reserve block of 4 reserve cows will be made. These reserve cows will only be used if other cows would become unsuitable for use in the experiment.

Moreover, 2 cows will be used to test the intravenous infusion technique. The intravenous infusion technique will be tested first on these two animals, in order to refine the technique and to minimize the stress induced for the animals in the running experiment.

4.b. Motivatie waarom is gekozen voor deze diersoort:
The aim is to reduce methane emission from dairy cows, thus these are the target animals. Fistulated

cows are needed for measurements of rumen volatile fatty acid (VFA) concentration and pH. Also, the standard in vitro technique will be applied, in which rumen fluid of individual cows is used as inoculum to study fermentation in vitro.

4.c. Toelichting voor het aantal gebruikte dieren:
During this experiment, the experimental unit will be one individual lactating dairy cow. There will be 7 cows assigned to each dietary treatment (i.e. 4 treatments, see following section). Thus, this results in 28 cows in total. This number of cows per treatment is the minimal number that allows sufficient statistical power and is based on the variation in methane emission found in previous studies with a similar experimental set-up. The recent studies of [redacted]) in [redacted] have shown that a number of 10 cows per experimental treatment is sufficient to detect ($P<0.05$) treatment effect of approximately 10%. However, [redacted] used a different experimental unit. The 10 lactating cows were paired and the experimental unit was 2 cows per respiration chamber. This results in 5 experimental units. In this type of experimental setting, a lower number of cows will not allow a methane difference to be significant. For the present experiment, the cows will be housed individually in a respiration chamber instead of in pairs. Using 5 experimental units, as done by [redacted], is not sufficient because using pairs of cows per chamber as an experimental unit gives less statistical variation than using only 1 cow per chamber as an experimental unit. Therefore, it is estimated that 6 individual cows per treatment should give a similar statistical power compared with the 5 units (i.e. 2 cows per chamber) per treatment. However, chamber techniques differ worldwide (e.g., accuracy depends on frequency of gas sampling). Therefore, 7 cows per treatment will be required. Based on various rumen models, and depending on the actual nutritional quality of the grass and the maize silage, the predicted difference in methane production between grass silage and maize silage diets is some 10-15%.

Of the 28 cows, 12 cows will be rumen fistulated (i.e. only cows who already have a fistula). Fistulated cows are needed to sample rumen fluid for determination of pH and volatile fatty acid (VFA) concentration, and as inoculum for the gas production technique. The aim is to use 3 fistulated cows per treatment. This number is based upon a study with a block-design with 3 fistulated cows per treatment by Abrahamse et al. (2009), showing that significant pH differences of 0.1 units could be detected. In addition, differences in the ratio between non-glucogenic and glucogenic VFAs (and therefore the ratio between VFAs that release hydrogen and VFA that act as hydrogen sinks; the balance of which is strongly related to methane production) of 0.1 were detected. It is therefore expected that using 3 fistulated cows per treatment will result in finding differences of approximately the same level.

Moreover, 2 cows will be used to practice the intravenous infusion technique. The intravenous infusion technique will be practiced first on these two animals, in order to refine the technique and to minimize the stress induced for the animals in the running experiment.

4.d. Herkomst:

Cows with rumen fistela	A. van gereg. fok/toeleveringsbedrijf in Nederland
Cows with no rumen fistela	A. van gereg. fok/toeleveringsbedrijf in Nederland

Toelichting:
Lactating dairy cows of experimental accommodations [redacted]) and [redacted].

In total, 34 cows will be selected from [redacted], of which 12 fistulated cows. Only cows that have been fistulated for previous purposes will be used; no new surgery will be done for this experiment. As much as possible cows of [redacted] will be chosen, provided that cows match in parity, lactation stage and milk production per group of 4 animals (since there are 4 treatments).

5.a. Accommodatie: [redacted]
Accommodation is in the dairy cattle tie-stall and climate respiration chambers at [redacted].

The lactating dairy cows for this experiment will be selected from [redacted] and [redacted]. The cows from [redacted] will be transported to [redacted], placed together in a group at [redacted] after arrival and receive a pre-adaptation period of at least 5 days. The lactating cows from [redacted] will be housed separately in the free barn from the already present animals, to avoid unrest due to arrival of new animals. During this period, they will receive the basic ration fed at [redacted].
During the first 12 days during the experimental period, the cows are housed in the tie-stalls. The period in the tie-stalls is considered to be the adaptation period in which the dairy cows can adapt to the experimental diet and get accustomed to being tied down. A previous study of Van Zijderveld et al. (2011) showed that an adaptation period of 12 days is sufficient. Methane production in respiration chambers seems rather constant after entering the respiration chambers on day 13, and there is no clear day-effect which would have been expected if adaptation have been incomplete. The tie-stalls will be illuminated by daylight and TL-lamps which will be switched on between 5:30 a.m. and 21:30 p.m..
After the adaptation period in the tie stalls, the measuring period begins by transporting the 4 cows to the calorimetric respiration chambers. During this measuring period, the cows will be fed the very same restricted ration as before. The cows will be moved to the respiration chambers on day 13 at 15:00h and will be removed from the chambers on day 17 at 9:00h. In these respiration chambers, the TL-lamps will be switched on between 5:30 a.m. and 21:30 p.m. At night, a small lamp will illuminate the chambers. The relative humidity in the chambers will be set at 70% and the temperature at 16 C. The methane production will be determined during 3 days of the measuring period (i.e. 2nd-4th day of the measuring period). Van Zijderveld et al. (2011) has applied this procedure before. Cows will be housed individually in a respiration chamber (i.e. 1 cow per chamber). The individually housed cows are able to see each other through windows and are capable of hearing the sounds another cow makes.

5.b. Huisvesting & Verzorging:
Cows are fed and milked twice daily. Drinking water is supplied at libitum. During the adaptation period and in the respiration chambers, feed intake and milk production will be measured twice daily. This provides the opportunity to recognize health issues. All subjects will be monitored on a daily basis for clear signs of difficult of refusing to get up, eat, or any other remarkably reason, and if any of this occurs and other health problems a veterinarian will be called immediately. In general, cows will be withdrawn from the experiment when a situation occurs that affects the characteristics to be measured. In case of suspicion of health issues, experts (i.e., the caretaker of the animals and [redacted], article 14 expert on laboratory animals) will be consulted, after which it will be decided if veterinary treatment should be obtained. When antibiotics have to be supplied, or in case of health issues or medicine supplies which might interfere with metabolism and feed intake, the cow will be withdrawn from the study.

As mentioned before, the cows from [redacted] will receive a pre-adaptation period of at least 5 days. It is expected that this pre-adaptation period of 5 days is sufficient, for two reasons. Firstly, these 5 days will give the cows from [redacted] a sufficient amount of time to recover from their transport to [redacted]. In addition, >80% of the micro flora in the rumen is adapted to a newly introduced ration after 5 to 7 days (Boeckaert et al., 2008). Complete adaptation to the [redacted] ration is not required, because the pre-adaptation phase is immediately followed by a 12 day adaptation phase, in which all cows will become adapted to the experimental diets. After 12 days, we expect that all cows are adapted to the experimental diets and have developed a stabile rumen micro flora. The five-day pre-adaptation period functions mainly as a synchronization period, during which all cows (i.e. both from [redacted] and [redacted]) get accustomed to the same total mixed ration and it will prevent that the cows from [redacted] will have an extreme change of diets in a short time period. All cows will be group-fed in this period and feed intake will not be monitored.

5.c. Voeding:
This experiment has a completely randomized block design in order to examine the effect of starch level in the diet on methane emission in dairy cattle. The cows will receive 1 of the 4 experimental diets. The diets

will consist of grass silage and / or maize silage in order to create different fiber and starch levels in the diet.
The four diets are (% of roughage compared to the total the total amount of roughage in the diet):
1. 100% grass silage (GS100)
2. 67% grass silage and 33% maize silage (GSMS67:33)
3. 33% grass silage and 67% maize silage (GSMS33:67)
4. 100% maize silage (MS100).

These four different diets are included for several reasons. Firstly, we want to find a linear relation between the nutritional replacing strategy and the production of methane. If we would only use the two extreme diets (i.e. 100% grass silage and 100% maize silage), we can only assume a linear relation. Therefore, the two diets in between (i.e. diet 2 and 4, a combination of grass silage and maize silage) are needed to be able to find a linear relation. Secondly, we want to find an indicator for methane in the milk. For this we need diets commonly used in practice. As mentioned before, grass silage and maize silage are the most common feed ingredients in dairy cattle diets. In the Netherlands for example, on average some 30% of the diet of dairy cattle is grass silage, and 25% is maize silage.
The four diets are not isocaloric. We want to measure methane production in relation to the amount of feed intake. Since feed intake is a major determinant of methane production, we decided that restriction of the feed intake is required (see restriction method below). Therefore, all cows within the same block will have a rather similar dry matter intake, resulting in a similar feed intake between the different diets. This has to come with the compromise that the four diets are not isocaloric.

The cows will be milked and fed twice daily (06.00 and 16.00 hours) and have ad libitum access to clean drinking water. All cows will receive their experimental diet as a total mixed ration. The forage:concentrate ratio will be 80:20 based on dry matter content. Each ration will fulfil requirements and will not cause any feed-related health issues such as acidosis. The proportion of concentrate in the diet will be kept low in order to create large differences in nutrient composition between the experimental diets, but concentrates need to be included in the ration to meet the requirements for maintenance and lactation. The composition of the concentrate will be the same for all four treatments. The composition of the concentrates will be chosen carefully, in order to meet the minimal OEB (rumen degradable protein balance) and DVE (intestinal available protein) requirements according to Dutch standards for dairy cattle (Centraal Veevoer Bureau). In addition, the concentrate will contain minerals and nutrients (premix).

The experimental diets will be fed individually and ad libitum during the first 7 days of the adaptation period in the tie stalls. From day 8 till 17 (i.e. last 5 days adaptation period and measuring period) the feed intake will be restricted per block to 95% of the ad libitum feed intake of the cow consuming the lowest amount of feed during day 5 till 8 within a block (Van Zijderveld et al., 2011). The feed restriction is required to reduce variation in feed intake between animals, since feed intake in itself is a major determinant of methane production. As mentioned before, by grouping cows into groups, we assume that the dry matter feed intake will be quite similar for all 4 animals of one block. However, even a small difference in milk production can be accompanied with a large difference in feed intake (i.e. cows that differ of 2.2. kg milk production, differ approximately 1 kg of dry matter feed intake when fed ad libitum). Thus, restricted feeding is needed, because the variation in methane production will be unacceptable due to the variation in feed intake level between the cows when fed ad libitum. This could potentially make it more difficult to find statistical valid conclusions. Feeding restrictedly will lead to a more similar feed intake within blocks and the reduced variation will help to detect smaller differences between treatments.
The feed intake will be restricted to have identical dry matter intake for cows in the same block as much as possible throughout the whole experiment, provided that the cow with the highest feed intake in the block is never restricted to less than 82% of the ad libitum feed intake.

When animals consume lower amounts of feed than they are used to, less nutrients are available for production. We expect milk production to adjust to the feed intake level. Previous studies in [redacted] have demonstrated that this method does not lead to problems. Staff of [redacted] and researchers of [redacted] that will be involved in the trial, are experienced with this method. To prevent problems with negative energy balance as much as possible, we will not use cows that have only been in lactation for 8 weeks or less.

6.a. Proefschema / proefbehandelingen:
This experiment has a completely randomized block design in order to examine the effect of starch level in the diet on methane emission in dairy cattle. As mentioned previously, 28 cows will be grouped into 7 blocks of 4. Within each block, cows will be randomly assigned to the four different diets. These four diets differ in starch level and consists of grass silage and/or maize silage (see section 5c).

Experimental set-up:
The cows from [redacted] will be transported to [redacted] placed together in a group at [redacted] after arrival and receive a pre-adaptation period of at least 5 days. The lactating cows from [redacted] will be housed separately in the free barn from the already present animals, to avoid unrest due to arrival of new animals. After at least 5 days, the cows are moved to the tie-stalls. In every block, the 4 animals will be moved to the tie-stalls. The period in the tie-stalls is considered to be the adaptation period in which the dairy cows can adapt to the experimental diet and get accustomed to being tied down. A previous study of Van Zijderveld et al. (2011) showed that an adaptation period of 12 days is sufficient. In addition, during this adaptation period the feed intake can be controlled and measured.
During the first 7 days feed is supplied ad libitum. After 7 days, feed supply is restricted. This procedure has been used in a number of experiments before (Van Zijderveld et al., 2011) and is needed since feed intake is a major determinant of methane production. During the last 2 days of the adaptation period, samples of rumen fluid will be taken from fistulated cows to measure volatile fatty acid concentration and pH levels. Rumen samples will be taken once before morning feeding and at 6 time points after morning feeding; 1, 2, 3, 4, 6, and 8h after feeding.
In addition, we want to define and establish mixed population reflecting the metabolic map in the rumen. For this, we will take 3 rumen samples from fistulated cows. On day -1 (i.e. 1 day before start adaptation period in tie stalls), day 10 and day 17 we will take both rumen fluid and rumen fiber fraction samples at 3 hours after morning feeding.
After the adaptation period in the tie stalls, the measuring period begins by transporting the 4 cows to the respiration chambers. During this measuring period, the cows will be fed the very same restricted ration as before. The cows will be moved to the respiration chambers on day 13 at 15:00h and will be removed from the chambers on day 17 at 9:00h. Cows will be housed individually in a respiration chamber (i.e. 1 cow per chamber). The individually housed cows are able to see each other through windows and are capable of hearing the sounds another cow makes.
The methane production will be determined during 3 full days of the measuring period (i.e. starting at 09.00h on day 14 until 09.00h on day 17). Methane emission is expressed per day. Van Zijderveld et al. (2011) has applied this procedure before. If measurements would only be done for 2 days, this could affect the accuracy of the methane measurements (e.g. when something disturbs the data on one day, like an error in the measurement equipment). Also for measuring the energy balance, which has been done many times in Wageningen, a measurement period of 3 days has been shown to give reliable data.
Methane emission, O2 consumption, CO2 production, urine plus faeces production and milk production will be registered. The ration components and the manure collected over the whole period in the chambers will be analyzed for amongst others organic matter, cellulose, hemicellulose, starch and crude fat. The milk samples will be analysed on composition, fatty acid profile, fat, protein, lactose and urea levels.
After the measurements in the respiration chambers, most cows are transported back to the free barn of [redacted]. Cows from [redacted] are transported back all at once after the last group of cows has been in the respiration chambers. At the end of each experimental period, the respiration chambers have to be cleaned completely using as little cleaning water as possible. All urine, faeces and cleaning water will be collected in a large tank. This will be mixed for homogeneity and sampled for dry matter, ash, nitrogen, crude fat, neutral detergent fiber and gross energy determination

The isotope experiment will be carried out within the previously explained experimental setup. The experimental procedure per cow will be as follows:
Infusion grade isotopes will be dissolved in sterile water. Solutions will be prepared in collaboration with the pharmacy of [redacted] and administered to the animals as a sterile nutrient solution.
On day 2 in the respiration chambers, directly after morning milking around 6am, a labeled nutrient solution (13C-glucose and 15N-glycine) will be injected in the jugular vein. 4 g 15N-glycine and 2 g of

13C-glucose will be dissolved in a volume of 500 ml sterile water. Before injection, a catheter (Type: Intraflon i.v. 12G 80mm) will be placed into the jugular vein to make sure that the entire dose is injected in the blood stream. To facilitate catheter insertion, a small incision in the skin will be made. After isotope administration the catheter will be flushed with saline and subsequently removed. The skin will be closed with suturing staples. Based on advice from an experienced veterinarian, it was decided that no local anaesthetics will be administered before making a small incision (5-10mm) in the skin. However, after making a small incision in the skin, an anaesthetic in the form of lidocaine spray will be applied on the skin, to minimize discomfort during catheterization and application of the suturing staples. Most likely, the discomfort to the cows caused by puncturing the skin for the injection (of the anaesthetic), will be similar to the discomfort caused by making the actual incision. Moreover, infiltration with lidocaine results in a depot above the jugular vein, which may complicate insertion of the catheter. It is also possible to accidentally puncture the jugular vein while injecting the anaesthetic. The latter may cause a hematoma which would again complicate insertion of the catheter.
The bottle used for injection of the isotope solution will be weighed before and after injection to determine the infused volume. On day 4 in the respiration chambers, 9 g of 13C-NaAcetate will be infused in a volume of 500 ml sterile water following the same procedure as for infusion of isotopically labeled glucose and glycine. The amounts of isotopically labelled nutrients have been chosen in such a way to detect sufficient amounts of isotope in milk by combustion-IRMS.
Duplicate samples of the morning milking of day 2 and 4 in the respiration chambers will be taken for background measurement of isotopic enrichment. Isotopic enrichment in breath will be measured at 9 minute intervals. After infusion of isotopically labelled material, milk from the 3 subsequent milkings (duplicate sample per milking) will be collected. The third milking after administration of 13C-acetate will be collected outside the chambers, but after a similar time interval as for 13C-glucose. For each milking, a representative subsample of the milk will be analysed for milk composition(i.e. fat, protein and lactose) and a second subsample will be fractionated. After freeze drying, the different milk fractions will be analysed for isotopic enrichment using combustion IRMS. During this procedure enrichment with 15N and 13C can be measured simultaneously.

6.b. Mate van ongerief:

Cows with rumen fistela C. Matig
Cows with no rumen fistela B. Gering/Matig

6.c. Waaruit bestaat het ongerief en hoe bent u tot uw inschatting van de mate van ongerief gekomen?

- Housing the cows tied during the adaptation period and in the respiration chambers
- Opening/closing the fistula for collecting rumen fluid (only applicable for the cows with a rumen fistula)
- Individual housing of animals in chambers.
- During the period that the animals will be housed in the respiration chambers, an intravenous catheter will be inserted twice for injection of a solution isotopically labelled nutrients into the bloodstream.

The discomfort has been estimated based on the above and based on previous experiments within [redacted] chambers with dairy cattle.

7. Welke maatregelen heeft u getroffen om het ongerief tot een minimum te beperken?

Anesthesie:

Cows with rumen fistela D. Is wel toegepast.
Cows with no rumen fistela D. Is wel toegepast.

Pijnbestrijding:

Cows with rumen fistela A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.
Cows with no rumen fistela A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.

Re-use of previously fistulated animals (only applicable for the cows with a rumen fistula).
Placing animals from [redacted] in a separate group after arrival at [redacted].
The effect of individual housing on animals is minimised by allowing animals to see each other through the windows in the chambers and when animals make a sound, the other cows will hear that as well.
Practicing the intravenous infusion technique under instruction of an experienced veterinarian. To do this, two cows from the [redacted] herd will be used. Also for the first infusions, adequate supervision will take place.

8. Toestand van dieren na einde van de proef:

Cows with rumen fistela Het dier is na de proef in leven gelaten.
Cows with no rumen fistela Het dier is na de proef in leven gelaten.

Toelichting:

Cows will return to the accommodation after the experiment [redacted]

9. Welke alternatieven (vervanging, verfijning, vermindering) zijn voor de beschreven experimenten overwogen en waarom zijn deze verworpen?

Replacement:
In vitro analyses of some previous studies have been shown not to be representative for in vivo measurements. It is also difficult to represent processes like pH, VFA absorption rate and passage rate in vitro; such processes are expected to have a major impact on methane. Therefore this study should be performed in vivo with the target animal (dairy cow) itself. The measurement of pH and of VFA concentration and type, major elements associated with methane production, also has to be performed in the target animal (fistulated animals) as the buffered in vitro systems do not allow such effects to be evaluated.

Refinement:
Social isolation will be prevented by housing cows in individual respiration chambers with windows in the walls; the sounds of the animals will be heard by other animals as the walls of the chambers are quite thin. In the adaptation area they will be housed in larger numbers.
Transport of cattle from [redacted] will be transported for some 80 km distance and will be housed separately in the free barn from the already present animals, to avoid unrest due to arrival of new animals; from their special, separate part of the free barn, they will move after a minimal of 5 days to the tie-stall.
Of the fistulated animals, only animals that already have a fistula will be used. To make optimal use of the available fistulated cows, cows of [redacted] will be used. Therefore no new surgery is required.

Reduction:
We will use the lowest number of animals possible to obtain the desired statistical accuracy (see part 4c). By doing some measurements repeatedly in the same animals (pH and VFA measurements) we try to obtain as many data as possible from one animal. In addition, we will also use data from previous experiments ([redacted]) in order to find a robust indicator for methane emission with the use of a minimal number of cows.
Moreover, answering multiple research questions within the same experimental setup will further contribute to reduction as it is not needed to setup a separate experiment.

10. Namen van direct betrokkenen bij de dierproef (artikel 9- en 12-functionarissen):

[redacted]

[redacted]

Tabel registratiecode opties voor aanvraag 2012086.b (K14):

	1	2	3	4	5	6	7	8	9	10	11	12	13
					37	**1**	**1**	**01**					
Cows with rumen fistela	**1**	**45**	**1**	**12**					**01**	**4**	**1**	**3**	**3**
Cows with no rumen fistela	**1**	**45**	**1**	**22**					**01**	**4**	**1**	**2**	**3**

Aanmeldingsformulier voor proeven met gewervelde dieren.

Secretariaat DEC
[redacted]

Aanvrager: [redacted]
Afdeling:

Titel dierproef: Indicator for methane emission in dairy cattle: grass silage versus maize silage

Aanmeldcode / Protocol: 2012086.c
Stadia van de proef:

25-10-2012	Aangemeld	[redacted]
01-11-2012	Positief advies na behandeling DEC	Secretaris van de DEC
10-12-2012	Wijzigen	[redacted]
10-12-2012	Gekopieerd	

Is deze proef wetenschappelijk getoetst en goedgekeurd? Ja
Toelichting:
Yes, in association with a PhD procedure of [redacted]. The project has been approved by the financial sponsors ([redacted]). Furthermore, the research committee of the [redacted] has discussed the project.

1.a. Met dit onderzoek te beantwoorden concrete vraag:
. Wetenschappelijke vraag m.b.t.wetensch. vraag
Methane is produced during microbial fermentation of feed in the gastrointestinal tract of ruminants, predominantly within the rumen (87%) and to a small extent (13%) in the large intestine (Murray et al., 1976). The rumen is an anaerobic environment in which microbiota digest feed components. Different microbial species (i.e. bacteria, protozoa and fungi) are involved in the conversion of feed material to methane in the rumen, with the final step carried out by methanogenic archeae (McAllister et al., 1996). Methanogenic archeae reduce CO2 to methane (McAllister and Newbold, 2008; Ellis et al., 2008). The process of formation of methane (i.e. CO2 + 4H2 --> CH4 + 2H2O) by methanogens generates metabolic energy for their maintenance and growth (Ellis et al., 2008).
The produced methane is predominately released to the environment through breath and eructation. Methane (CH4) is one of the most important greenhouse gases (GHG) and a significant contributor to global warming. In the dairy cattle chain, methane represents more than 50% of all the GHG produced. In addition, methane also represents an energy loss to the animal since CH4 has no nutritional value to the animal (Boadi et al., 2004) . About 6% of the total gross energy consumed by the animal is converted to CH4 in the rumen (Johnson and Johnson, 1995). This energy loss can reach up to 12% depending upon the quality of the diet (O'Mara, 2004). Therefore, it is desired to reduce the methane emission from dairy cattle, in order to reduce the ecological footprint of milk production, and improve feed efficiency and animal productivity.
The rate of CH4 produced from enteric fermentation in dairy cows depends greatly on the level of feed intake, the quantity of energy consumed, and dietary composition, in particular the nature of carbohydrates such as starch and its degradation rate. There have been several dietary strategies proposed to reduce the production of CH4 from dairy cattle (Grainger and Beauchemin, 2011), including the use of diets rich in starch and low in fiber, since the fermentation of starch (as compared to fiber

fermentation) increases the ratios of propionic to acetic acid, which are associated with reduced methane levels. An example of such a strategy is the replacement of fiber-rich grass silage with starch-rich maize silage. However, the scientific evidence for this particular replacement strategy is scarce. Recently, Staerfl et al. (2012) investigated this strategy, but the maize silage used had a net energy content some 10% lower than that of the grass silage, which in many countries (including the Netherlands) is highly uncommon as maize silage nearly always has a higher net energy content than grass silage.

The ability to measure methane emission by cows is essential for increasing our understanding of factors that contribute to variation in methane emission and thereby for interventions leading to its reduction. Climate respiration chambers are the golden standard for measuring methane emission. However, this technique is very expensive and therefore not suited for large scale measurement or as indicator at farm level. Mathematic models may allow prediction of CH4 production from cattle without extensive experiments, however, the accuracy of these models is low (Ellis et al., 2010). Thus, it is of great importance to develop a simple method to estimate CH4 production in cattle.
The current experiment is designed to observe the effect of replacing grass silage (no starch) with maize silage (starch-rich) on methane emission from lactating dairy cows, with the ultimate goal to develop an indicator for methane emission that can be measured in milk of individual cows. The hypothesis is that replacement of grass silage with maize silage will decrease the production of methane. The indicator will be used to build further on a prediction model recently proposed by Dijkstra et al. (2011) which is exclusively based on the fatty acid composition of the milk. It is hypothesized that the addition of other metabolites in this prediction model will enhance its predictive power and thus leads to a better indicator for methane emission in milk.
This experiment can be considered novel and differs from recent methane trials in [redacted] for two reasons. Firstly, the scientific evidence worldwide for the nutritional strategy to decrease the production of methane by replacing fibre-rich grass silage with starch-rich maize silage is scarce. This experiment targets to gain more scientific evidence for this replacement strategy. Secondly, the longer term goal of this experiment is to develop an indicator for methane emission that can be measured in milk, for which data are required on feeds commonly used in practice. Grass (silage) and maize silage are the most common feed ingredients in dairy cattle diets. In the Netherlands for example, on average some 30% of the diet of dairy cattle is grass silage, and 25% is maize silage.

Second objective:
Within the setup of the methane experiment, a rapid method to assess the nutritional status of dairy cattle will be developed. The method includes measurement of isotopic enrichment in metabolic end-products in breath (CO2) and milk (fat, protein and lactose) after intravenous administration of isotopically labelled nutrients.

In general, the partitioning of nutrients over anabolic and catabolic pathways is strongly affected by the nutritional status of a dairy cow. However, accurate but simple diagnostic tests for assessing the nutritional status of dairy cows are currently not available. Labelling nutrients with stable isotopes (e.g. 13C, 15N) may be a suitable technique to trace utilization of nutrients and hence provide a proxy for the nutritional status. It is expected that appearance of an injected bolus of an isotopically labelled nutrient in milk components depends on the balance between nutrient requirements (to synthesize milk lactose, fat and protein) and nutrient availability (from diet). Therefore, we hypothesize that the recovery of a labelled nutrient in milk components increases when this nutrient limits milk production, indicating a more efficient utilization for milk synthesis. Similarly, non-limiting nutrients will probably be oxidized to a larger extent and are therefore increasingly recovered as CO2 in breath. A proof of this principle could be obtained from an experiment with a large contrasts in nutrient supply, i.e. glucogenic vs. lipogenic nutrients. The present experiment, in which grass silage is replaced by maize silage, will result in a shift from supply of lipogenic nutrients to glucogenic nutrients. Grass silage is expected to result (after fermentation) mainly in acetic acid, which is lipogenic, whereas maize silage will produce more propionic acid (glucogenic) and some of the starch will escape rumen fermentation and will be hydrolysed in the small intestine to glucose and absorbed as glucose in the blood. Hence, the present experiment will result in a contrast in supply of lipogenic versus glucogenic nutrients.

For intravenous administration of 13C-glucose , it is expected that lactating dairy cows receiving a lipogenic diet will use a larger proportion of available glucose for milk production purposes than cows receiving a glucogenic diet. After injection of [U-13C]glucose, the recovery of isotope in milk lactose is therefore expected to be higher for cows receiving a lipogenic diet. Measuring also isotopic enrichment of the protein and fat fractions of milk may provide information about the use of glucose for other (milk production) purposes. It is known from literature that increased glucose availability may have positive effects on milk lactose and milk protein production (see e.g. Lapierre et al., 2010, Lemosquet et al., 2010, Rigout et al., 2002, Rius et al., 2010, Rulquin et al., 2004). In addition, after intravenous administration of 13C-acetate, as [1-13C] sodium acetate, the recovery of isotope in the milk fat fraction is expected to be higher for cows receiving the lipogenic diet, because milk fat production is usually lower in cows fed glucogenic rations (Van Knegsel et al., 2007). Moreover, assuming that a difference in the nitrogen (N) content of the diet may result in differences in protein metabolism and that such differences can be detected by itntravenous infusion of 15N-glycine, it is expected that intravenous infusion of 15N-glycine could be used as a means to investigate a difference in the nitrogen (N) content of the diet.

1.b. Het uiteindelijk doel (Maatschappelijke en wetenschappelijke relevantie):
Methane (CH4) has a global warming potential of 25 carbon dioxide equivalents (Forster et al., 2007). By signing the Kyoto protocol, the EU agreed to have reduced greenhouse gas emissions by at least 20 % by 2020 compared to emission levels in 1990. Methane is the single most important greenhouse gas produced on dairy farms. Besides the effect on global warming, methane emission from cattle represents an energy loss of 2-12% of gross energy intake (Johnson and Ward, 1996). Finding feeding strategies to reduce methane emission, supports achieving the targets for the Kyoto protocol. Besides, reduction in methane emission will reduce energy losses, which may increase feed efficiency.
With this experiment we aim to gain more scientific evidence for the nutritional strategy to decrease the methane production by replacing grass silage with maize silage. Our ultimate goal is to put this nutritional strategy into practice on dairy farms in The Netherlands and develop a simple method (i.e. indicator in the milk) to estimate CH4 production in individual cows and dairy herds, in order to increase our understanding of factors that contribute to variation in methane emission and thereby for interventions leading to its reduction.

A second aim, within the setup of the methane experiment, is to develop a method for rapid assessment of nutritional status of dairy cattle. The method includes measurement of isotopic enrichment in metabolic end-products in breath (CO2) and milk (fat, protein and lactose) after intravenous administration of isotopically labelled nutrients. Ultimately this method could be used to determine which nutrient is limiting for milk production.

1.c. Lekensamenvatting:
Een indicator voor methaanproductie bij melkkoeien: kuilgras versus snijmais.
Een Nederlandse melkkoe produceert dagelijks gemiddeld 350 gram methaan. Het gas wordt voornamelijk in de pens van de koe geproduceerd bij de voedselvertering. Een kwart van methaangas in onze atmosfeer is afkomstig van melkkoeien. Voor de verduurzaming van de melkproductie in Nederland is het van het grootste belang om de uitstoot van broeikasgassen, in het bijzonder methaan, te verminderen.
Tijdens dit project wordt een indicator ontwikkeld in melk om methaan gas uitstoot bij individuele koeien te meten. Met deze te ontwikkelen indicator in melk zal de methaanuitstoot niet alleen bij individuele koeien kunnen worden gemeten maar ook op kudde niveau. Voor melkveehouders is deze indicator een middel om methaangasuitstoot op hun boerderijen te monitoren en te sturen. Op deze manier zal het project een waardevolle bijdrage leveren aan de verdere verduurzaming van de primaire sector.
Om de indicator te ontwikkelen, worden melkkoeien gebruikt. Aangezien het uiteindelijke doel van dit project het verminderen van methaan uitstoot bij melkkoeien is, wordt tijdens dit onderzoek ook gebruik gemaaktvan melkkoeien. Er worden in totaal 28 melkkoeien gebruikt, verdeeld over 4 groepen. Elke groep

zal een ander rantsoen krijgen: (1) 100% kuilgras, (2) 67% kuilgras met 33% snijmais, (3) 33% kuilgras en 67% snijmais, en (4) 100% snijmais. Deze rantsoenen variëren in de hoeveelheid vezels en zetmeel, met de gedachte om verschillen in methaan uitstoot te creëren. Onderzoek met andere voedermiddelen heeft namelijk aangetoond dat een rantsoen rijk aan vezels resulteert in een hogere methaan uitstoot ten opzichte van een rantsoen rijk aan zetmeel. Kuilgras en snijmais zijn de meest gebruikte voedermiddelen in de Nederlandse melkveehouderij, maar de specifieke kennis over methaanproduktie bij vervanging van kuilgras door snijmais (of omgekeerd) is vrijwel afwezig.
De koeien worden gedurende 12 dagen in een grupstal gehouden voor de adaptatieperiode. Tijdens deze periode raken de koeien geadapteerd aan het nieuwe rantsoen en aan het aangebonden staan. Na deze 12 dagen, worden de koeien overgeplaatst naar de klimaatkamers. Ze worden individueel gehuisvest in de klimaatkamers, maar door de aanwezigheid van ramen kunnen ze elkaar zien en horen. In deze klimaatkamers blijven de koeien hetzelfde rantsoen ontvangen en staan ze aangebonden. Ze zullen bijna 5 dagen in deze klimaatkamers staan, zodat we de methaan uitstoot kunnen meten. Elke dag wordt tijdens het ochtend en avond melken, melkmonsters genomen. Na deze 5 dagen, worden de melkkoeien weer teruggebracht naar de stal.
Naderhand zal de melk geanalyseerd worden op samenstelling. Het is de bedoeling dat de samenstelling van de melk in relatie gebracht kan worden met de hoeveelheid methaan uitstoot. Er zal uiteindelijk, aan de hand van de relatie, een indicator in melk ontwikkeld worden om methaan gas uitstoot bij koeien te meten.

Binnen de opzet van het hiervoor beschreven onderzoek kan nog een tweede onderzoeksvraag worden beantwoord. In voedingsonderzoek, ook bij melkvee, wordt in toenemende mate gebruik gemaakt van labeling met stabiele isotopen. Dergelijke studies bieden de mogelijkheid het gebruik van individuele nutriënten te volgen en worden dan ook voornamelijk ingezet bij kwantitatieve studies naar de effecten van voeding op productie. Het voorgestelde experiment is gebaseerd op de hypothese dat de manier waarop een melkkoe haar metabolieten gebruikt afhankelijk is van haar voedingsstatus. Er kan dus een relatie worden verwacht tussen de voedingsstatus van een koe en de recovery van een geïnjecteerde dosis van een met isotopen gelabelde metaboliet in de melk. Een dergelijke relatie kan, zonder kwantitatief te zijn, zeker informatief zijn over de voedingsstatus van de koe. Deze techniek zou in de praktijk op koppelniveau kunnen worden toegepast.

2. Gepland vanaf: 03-11-2012 **tot** 21-12-2012

3. Specificatie diergroepen:

Cows for experiment	32	runderen	All cows (with and without rumen fistula, inclusding the reserve cows) for the methane experiment
Cows for testing infusion technique	2	runderen	Cows used to test the intravenous infusion technique

4.a. Nadere aanduiding gebruikte dieren:
28 lactating dairy cows (4 treatments, 7 cows per treatment), of which 12 cows will be rumen fistulated (i.e., only cows who already have a fistula; no new surgery is required). The 28 lactating dairy cows will be grouped into 7 blocks of 4 cows according to lactation stage, parity, milk production, fat- and protein levels in the milk and presence of a fistula. By doing this, 7 blocks are made, each with relative similarly cows. Therefore, it is expected that the dry matter feed intake will be quite similar for all 4 animals of one block. Within blocks, animals will be randomly assigned to one of the four experimental diets, resulting that all treatments occur within a block. In addition, 1 reserve block of 4 reserve cows will be made. These reserve cows will only be used if other cows would become unsuitable for use in the experiment.

Moreover, 2 cows will be used to test the intravenous infusion technique. The intravenous infusion technique will be tested first on these two animals, in order to refine the technique and to minimize the stress induced for the animals in the running experiment.

4.b. Motivatie waarom is gekozen voor deze diersoort:

The aim is to reduce methane emission from dairy cows, thus these are the target animals. Fistulated cows are needed for measurements of rumen volatile fatty acid (VFA) concentration and pH. Also, the standard in vitro technique will be applied, in which rumen fluid of individual cows is used as inoculum to study fermentation in vitro.

4.c. Toelichting voor het aantal gebruikte dieren:
During this experiment, the experimental unit will be one individual lactating dairy cow. There will be 7 cows assigned to each dietary treatment (i.e. 4 treatments, see following section). Thus, this results in 28 cows in total. This number of cows per treatment is the minimal number that allows sufficient statistical power and is based on the variation in methane emission found in previous studies with a similar experimental set-up. The recent studies of [redacted]) in [redacted] have shown that a number of 10 cows per experimental treatment is sufficient to detect ($P<0.05$) treatment effect of approximately 10%. However, [redacted] used a different experimental unit. The 10 lactating cows were paired and the experimental unit was 2 cows per respiration chamber. This results in 5 experimental units. In this type of experimental setting, a lower number of cows will not allow a methane difference to be significant. For the present experiment, the cows will be housed individually in a respiration chamber instead of in pairs. Using 5 experimental units, as done by [redacted], is not sufficient because using pairs of cows per chamber as an experimental unit gives less statistical variation than using only 1 cow per chamber as an experimental unit. Therefore, it is estimated that 6 individual cows per treatment should give a similar statistical power compared with the 5 units (i.e. 2 cows per chamber) per treatment. However, chamber techniques differ worldwide (e.g., accuracy depends on frequency of gas sampling). Therefore, 7 cows per treatment will be required. Based on various rumen models, and depending on the actual nutritional quality of the grass and the maize silage, the predicted difference in methane production between grass silage and maize silage diets is some 10-15%.

Of the 28 cows, 12 cows will be rumen fistulated (i.e. only cows who already have a fistula). Fistulated cows are needed to sample rumen fluid for determination of pH and volatile fatty acid (VFA) concentration, and as inoculum for the gas production technique. The aim is to use 3 fistulated cows per treatment. This number is based upon a study with a block-design with 3 fistulated cows per treatment by Abrahamse et al. (2009), showing that significant pH differences of 0.1 units could be detected. In addition, differences in the ratio between non-glucogenic and glucogenic VFAs (and therefore the ratio between VFAs that release hydrogen and VFA that act as hydrogen sinks; the balance of which is strongly related to methane production) of 0.1 were detected. It is therefore expected that using 3 fistulated cows per treatment will result in finding differences of approximately the same level.

Moreover, 2 cows will be used to practice the intravenous infusion technique. The intravenous infusion technique will be practiced first on these two animals, in order to refine the technique and to minimize the stress induced for the animals in the running experiment.

4.d. Herkomst:
Cows for experiment A. van gereg. fok/toeleveringsbedrijf in Nederland
Cows for testing infusion technique A. van gereg. fok/toeleveringsbedrijf in Nederland
Toelichting:
Lactating dairy cows of experimental accommodations [redacted]) and [redacted].

In total, 34 cows will be selected from [redacted], of which 12 fistulated cows. Only cows that have been fistulated for previous purposes will be used; no new surgery will be done for this experiment. As much as possible cows of [redacted] will be chosen, provided that cows match in parity, lactation stage and milk production per group of 4 animals (since there are 4 treatments).

5.a. Accommodatie: [redacted]
Accommodation is in the dairy cattle tie-stall and climate respiration chambers at [redacted].

The lactating dairy cows for this experiment will be selected from [redacted] and [redacted]. The cows from [redacted] will be transported to [redacted], placed together in a group at [redacted] after arrival and receive a pre-adaptation period of at least 5 days. The lactating cows from [redacted] will be housed separately in the free barn from the already present animals, to avoid unrest due to arrival of new animals. During this period, they will receive the basic ration fed at [redacted].
During the first 12 days during then experimental period, the cows are housed in the tie-stalls. The period in the tie-stalls is considered to be the adaptation period in which the dairy cows can adapt to the experimental diet and get accustomed to being tied down. A previous study of Van Zijderveld et al. (2011) showed that an adaptation period of 12 days is sufficient. Methane production in respiration chambers seems rather constant after entering the respiration chambers on day 13, and there is no clear day-effect which would have been expected if adaptation have been incomplete. The tie-stalls will be illuminated by daylight and TL-lamps which will be switched on between 5:30 a.m. and 21:30 p.m..
After the adaptation period in the tie stalls, the measuring period begins by transporting the 4 cows to the calorimetric respiration chambers. During this measuring period, the cows will be fed the very same restricted ration as before. The cows will be moved to the respiration chambers on day 13 at 15:00h and will be removed from the chambers on day 17 at 9:00h. In these respiration chambers, the TL-lamps will be switched on between 5:30 a.m. and 21:30 p.m. At night, a small lamp will illuminate the chambers. The relative humidity in the chambers will be set at 70% and the temperature at 16 C. The methane production will be determined during 3 days of the measuring period (i.e. 2nd-4th day of the measuring period). Van Zijderveld et al. (2011) has applied this procedure before. Cows will be housed individually in a respiration chamber (i.e. 1 cow per chamber). The individually housed cows are able to see each other through windows and are capable of hearing the sounds another cow makes.

5.b. Huisvesting & Verzorging:
Cows are fed and milked twice daily. Drinking water is supplied at libitum. During the adaptation period and in the respiration chambers, feed intake and milk production will be measured twice daily. This provides the opportunity to recognize health issues. All subjects will be monitored on a daily basis for clear signs of difficult of refusing to get up, eat, or any other remarkably reason, and if any of this occurs and other health problems a veterinarian will be called immediately. In general, cows will be withdrawn from the experiment when a situation occurs that affects the characteristics to be measured. In case of suspicion of health issues, experts (i.e., the caretaker of the animals and [redacted], article 14 expert on laboratory animals) will be consulted, after which it will be decided if veterinary treatment should be obtained. When antibiotics have to be supplied, or in case of health issues or medicine supplies which might interfere with metabolism and feed intake, the cow will be withdrawn from the study.

As mentioned before, the cows from [redacted] will receive a pre-adaptation period of at least 5 days. It is expected that this pre-adaptation period of 5 days is sufficient, for two reasons. Firstly, these 5 days will give the cows from [redacted] a sufficient amount of time to recover from their transport to [redacted]. In addition, >80% of the micro flora in the rumen is adapted to a newly introduced ration after 5 to 7 days (Boeckaert et al., 2008). Complete adaptation to the [redacted] ration is not required, because the pre-adaptation phase is immediately followed by a 12 day adaptation phase, in which all cows will become adapted to the experimental diets. After 12 days, we expect that all cows are adapted to the experimental diets and have developed a stabile rumen micro flora. The five-day pre-adaptation period functions mainly as a synchronization period, during which all cows (i.e. both from [redacted] and [redacted]) get accustomed to the same total mixed ration and it will prevent that the cows from [redacted] will have an extreme change of diets in a short time period. All cows will be group-fed in this period and feed intake will not be monitored.

5.c. Voeding:
This experiment has a completely randomized block design in order to examine the effect of starch level in

the diet on methane emission in dairy cattle. The cows will receive 1 of the 4 experimental diets. The diets will consist of grass silage and / or maize silage in order to create different fiber and starch levels in the diet.
The four diets are (% of roughage compared to the total the total amount of roughage in the diet):
1. 100% grass silage (GS100)
2. 67% grass silage and 33% maize silage (GSMS67:33)
3. 33% grass silage and 67% maize silage (GSMS33:67)
4. 100% maize silage (MS100).

These four different diets are included for several reasons. Firstly, we want to find a linear relation between the nutritional replacing strategy and the production of methane. If we would only use the two extreme diets (i.e. 100% grass silage and 100% maize silage), we can only assume a linear relation. Therefore, the two diets in between (i.e. diet 2 and 4, a combination of grass silage and maize silage) are needed to be able to find a linear relation. Secondly, we want to find an indicator for methane in the milk. For this we need diets commonly used in practice. As mentioned before, grass silage and maize silage are the most common feed ingredients in dairy cattle diets. In the Netherlands for example, on average some 30% of the diet of dairy cattle is grass silage, and 25% is maize silage.
The four diets are not isocaloric. We want to measure methane production in relation to the amount of feed intake. Since feed intake is a major determinant of methane production, we decided that restriction of the feed intake is required (see restriction method below). Therefore, all cows within the same block will have a rather similar dry matter intake, resulting in a similar feed intake between the different diets. This has to come with the compromise that the four diets are not isocaloric.

The cows will be milked and fed twice daily (06.00 and 16.00 hours) and have ad libitum access to clean drinking water. All cows will receive their experimental diet as a total mixed ration. The forage:concentrate ratio will be 80:20 based on dry matter content. Each ration will fulfil requirements and will not cause any feed-related health issues such as acidosis. The proportion of concentrate in the diet will be kept low in order to create large differences in nutrient composition between the experimental diets, but concentrates need to be included in the ration to meet the requirements for maintenance and lactation. The composition of the concentrate will be the same for all four treatments. The composition of the concentrates will be chosen carefully, in order to meet the minimal OEB (rumen degradable protein balance) and DVE (intestinal available protein) requirements according to Dutch standards for dairy cattle (Centraal Veevoer Bureau). In addition, the concentrate will contain minerals and nutrients (premix).

The experimental diets will be fed individually and ad libitum during the first 7 days of the adaptation period in the tie stalls. From day 8 till 17 (i.e. last 5 days adaptation period and measuring period) the feed intake will be restricted per block to 95% of the ad libitum feed intake of the cow consuming the lowest amount of feed during day 5 till 8 within a block (Van Zijderveld et al., 2011). The feed restriction is required to reduce variation in feed intake between animals, since feed intake in itself is a major determinant of methane production. As mentioned before, by grouping cows into groups, we assume that the dry matter feed intake will be quite similar for all 4 animals of one block. However, even a small difference in milk production can be accompanied with a large difference in feed intake (i.e. cows that differ of 2.2. kg milk production, differ approximately 1 kg of dry matter feed intake when fed ad libitum). Thus, restricted feeding is needed, because the variation in methane production will be unacceptable due to the variation in feed intake level between the cows when fed ad libitum. This could potentially make it more difficult to find statistical valid conclusions. Feeding restrictedly will lead to a more similar feed intake within blocks and the reduced variation will help to detect smaller differences between treatments.
The feed intake will be restricted to have identical dry matter intake for cows in the same block as much as possible throughout the whole experiment, provided that the cow with the highest feed intake in the block is never restricted to less than 82% of the ad libitum feed intake.

When animals consume lower amounts of feed than they are used to, less nutrients are available for production. We expect milk production to adjust to the feed intake level. Previous studies in [redacted] have demonstrated that this method does not lead to problems. Staff of [redacted] and researchers of [redacted] that will be involved in the trial, are experienced with this method. To prevent problems with negative energy balance as much as possible, we will not use cows that have only been in lactation for 8 weeks or less.

6.a. Proefschema / proefbehandelingen:
This experiment has a completely randomized block design in order to examine the effect of starch level in the diet on methane emission in dairy cattle. As mentioned previously, 28 cows will be grouped into 7 blocks of 4. Within each block, cows will be randomly assigned to the four different diets. These four diets differ in starch level and consists of grass silage and/or maize silage (see section 5c).

Experimental set-up:
The cows from [redacted] will be transported to [redacted], placed together in a group at [redacted] after arrival and receive a pre-adaptation period of at least 5 days. The lactating cows from [redacted] will be housed separately in the free barn from the already present animals, to avoid unrest due to arrival of new animals. After at least 5 days, the cows are moved to the tie-stalls. In every block, the 4 animals will be moved to the tie-stalls. The period in the tie-stalls is considered to be the adaptation period in which the dairy cows can adapt to the experimental diet and get accustomed to being tied down. A previous study of Van Zijderveld et al. (2011) showed that an adaptation period of 12 days is sufficient. In addition, during this adaptation period the feed intake can be controlled and measured.
During the first 7 days feed is supplied ad libitum. After 7 days, feed supply is restricted. This procedure has been used in a number of experiments before (Van Zijderveld et al., 2011) and is needed since feed intake is a major determinant of methane production. During the last 2 days of the adaptation period, samples of rumen fluid will be taken from fistulated cows to measure volatile fatty acid concentration and pH levels. Rumen samples will be taken once before morning feeding and at 6 time points after morning feeding; 1, 2, 3, 4, 6, and 8h after feeding.
In addition, we want to define and establish mixed population reflecting the metabolic map in the rumen. For this, we will take 3 rumen samples from fistulated cows. On day -1 (i.e. 1 day before start adaptation period in tie stalls), day 10 and day 17 we will take both rumen fluid and rumen fiber fraction samples at 3 hours after morning feeding.
After the adaptation period in the tie stalls, the measuring period begins by transporting the 4 cows to the respiration chambers. During this measuring period, the cows will be fed the very same restricted ration as before. The cows will be moved to the respiration chambers on day 13 at 15:00h and will be removed from the chambers on day 17 at 9:00h. Cows will be housed individually in a respiration chamber (i.e. 1 cow per chamber). The individually housed cows are able to see each other through windows and are capable of hearing the sounds another cow makes.
The methane production will be determined during 3 full days of the measuring period (i.e. starting at 09.00h on day 14 until 09.00h on day 17). Methane emission is expressed per day. Van Zijderveld et al. (2011) has applied this procedure before. If measurements would only be done for 2 days, this could affect the accuracy of the methane measurements (e.g. when something disturbs the data on one day, like an error in the measurement equipment). Also for measuring the energy balance, which has been done many times in Wageningen, a measurement period of 3 days has been shown to give reliable data.
Methane emission, O2 consumption, CO2 production, urine plus faeces production and milk production will be registered. The ration components and the manure collected over the whole period in the chambers will be analyzed for amongst others organic matter, cellulose, hemicellulose, starch and crude fat. The milk samples will be analysed on composition, fatty acid profile, fat, protein, lactose and urea levels.
After the measurements in the respiration chambers, most cows are transported back to the free barn of [redacted]. Cows from [redacted] are transported back all at once after the last group of cows has been in the respiration chambers. At the end of each experimental period, the respiration chambers have to be cleaned completely using as little cleaning water as possible. All urine, faeces and cleaning water will be collected in a large tank. This will be mixed for homogeneity and sampled for dry matter, ash, nitrogen, crude fat, neutral detergent fiber and gross energy determination

The isotope experiment will be carried out within the previously explained experimental setup. The experimental procedure per cow will be as follows:
Infusion grade isotopes will be dissolved in sterile water. Solutions will be prepared in collaboration with the pharmacy of [redacted] and administered to the animals as a sterile nutrient solution.
On day 2 in the respiration chambers, directly after morning milking around 6am, a labeled nutrient

solution (13C-glucose and 15N-glycine) will be injected in the jugular vein. 4 g 15N-glycine and 2 g of 13C-glucose will be dissolved in a volume of 500 ml sterile water. Before injection, a catheter (Type: Intraflon i.v. 12G 80mm) will be placed into the jugular vein to make sure that the entire dose is injected in the blood stream. To facilitate catheter insertion, a small incision in the skin will be made. After isotope administration the catheter will be flushed with saline and subsequently removed. The skin will be closed with suturing staples. Based on advice from an experienced veterinarian, it was decided that no local anaesthetics will be administered before making a small incision (5-10mm) in the skin. However, after making a small incision in the skin, an anaesthetic in the form of lidocaine spray will be applied on the skin, to minimize discomfort during catheterization and application of the suturing staples. Most likely, the discomfort to the cows caused by puncturing the skin for the injection (of the anaesthetic), will be similar to the discomfort caused by making the actual incision. Moreover, infiltration with lidocaine results in a depot above the jugular vein, which may complicate insertion of the catheter. It is also possible to accidentally puncture the jugular vein while injecting the anaesthetic. The latter may cause a hematoma which would again complicate insertion of the catheter.
The bottle used for injection of the isotope solution will be weighed before and after injection to determine the infused volume. On day 4 in the respiration chambers, 9 g of 13C-NaAcetate will be infused in a volume of 500 ml sterile water following the same procedure as for infusion of isotopically labeled glucose and glycine. The amounts of isotopically labelled nutrients have been chosen in such a way to detect sufficient amounts of isotope in milk by combustion-IRMS.
Duplicate samples of the morning milking of day 2 and 4 in the respiration chambers will be taken for background measurement of isotopic enrichment. Isotopic enrichment in breath will be measured at 9 minute intervals. After infusion of isotopically labelled material, milk from the 3 subsequent milkings (duplicate sample per milking) will be collected. The third milking after administration of 13C-acetate will be collected outside the chambers, but after a similar time interval as for 13C-glucose. For each milking, a representative subsample of the milk will be analysed for milk composition(i.e. fat, protein and lactose) and a second subsample will be fractionated. After freeze drying, the different milk fractions will be analysed for isotopic enrichment using combustion IRMS. During this procedure enrichment with 15N and 13C can be measured simultaneously.

6.b. Mate van ongerief:

Cows for experiment B. Gering/Matig
Cows for testing infusion technique A. Gering

6.c. Waaruit bestaat het ongerief en hoe bent u tot uw inschatting van de mate van ongerief gekomen?

- Housing the cows tied during the adaptation period and in the respiration chambers
- Opening/closing the fistula for collecting rumen fluid (only applicable for the cows with a rumen fistula)
- Individual housing of animals in chambers.
- During the period that the animals will be housed in the respiration chambers, an intravenous catheter will be inserted twice for injection of a solution isotopically labelled nutrients into the bloodstream.

The discomfort has been estimated based on the above and based on previous experiments within [redacted] chambers with dairy cattle.

7. Welke maatregelen heeft u getroffen om het ongerief tot een minimum te beperken?

Anesthesie:

Cows for experiment D. Is wel toegepast.
Cows for testing infusion technique D. Is wel toegepast.

Pijnbestrijding:

Cows for experiment A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.
Cows for testing infusion technique A. Wordt niet toegepast omdat hiertoe geen aanleiding

bestaat.

Re-use of previously fistulated animals (only applicable for the cows with a rumen fistula).
Placing animals from [redacted] in a separate group after arrival at [redacted].
The effect of individual housing on animals is minimised by allowing animals to see each other through the windows in the chambers and when animals make a sound, the other cows will hear that as well.
Practicing the intravenous infusion technique under instruction of an experienced veterinarian. To do this, two cows from the [redacted] herd will be used. Also for the first infusions, adequate supervision will take place.
After making a small incision in the skin, an anaesthetic in the form of lidocaine spray will be applied on the skin, to minimize discomfort during catheterization and application of the suturing staples.

8. Toestand van dieren na einde van de proef:

Cows for experiment Het dier is na de proef in leven gelaten.
Cows for testing infusion technique Het dier is na de proef in leven gelaten.

Toelichting:
Cows will return to the accommodation after the experiment [redacted]

9. Welke alternatieven (vervanging, verfijning, vermindering) zijn voor de beschreven experimenten overwogen en waarom zijn deze verworpen?

Replacement:
In vitro analyses of some previous studies have been shown not to be representative for in vivo measurements. It is also difficult to represent processes like pH, VFA absorption rate and passage rate in vitro; such processes are expected to have a major impact on methane. Therefore this study should be performed in vivo with the target animal (dairy cow) itself. The measurement of pH and of VFA concentration and type, major elements associated with methane production, also has to be performed in the target animal (fistulated animals) as the buffered in vitro systems do not allow such effects to be evaluated.

Refinement:
Social isolation will be prevented by housing cows in individual respiration chambers with windows in the walls; the sounds of the animals will be heard by other animals as the walls of the chambers are quite thin. In the adaptation area they will be housed in larger numbers.
Transport of cattle from [redacted] will be transported for some 80 km distance and will be housed separately in the free barn from the already present animals, to avoid unrest due to arrival of new animals; from their special, separate part of the free barn, they will move after a minimal of 5 days to the tie-stall.
Of the fistulated animals, only animals that already have a fistula will be used. To make optimal use of the available fistulated cows, cows of [redacted] will be used. Therefore no new surgery is required.

Reduction:
We will use the lowest number of animals possible to obtain the desired statistical accuracy (see part 4c). By doing some measurements repeatedly in the same animals (pH and VFA measurements) we try to obtain as many data as possible from one animal. In addition, we will also use data from previous experiments (i.e. [redacted]) in order to find a robust indicator for methane emission with the use of a minimal number of cows.
Moreover, answering multiple research questions within the same experimental setup will further contribute to reduction as it is not needed to setup a separate experiment.

10. Namen van direct betrokkenen bij de dierproef (artikel 9- en 12-functionarissen):

[redacted]

[redacted]

Tabel registratiecode opties voor aanvraag 2012086.c (K14):

	1	2	3	4	5	6	7	8	9	10	11	12	13	
					37	**1**	**1**	**01**						
Cows for experiment	**1**	**45**	**1**	**32**					**01**	**4**	**1**	**2**	**3**	
Cows for testing infusion technique	**1**	**45**	**1**	**2**						**01**	**4**	**1**	**1**	**3**

Aanmeldingsformulier voor proeven met gewervelde dieren.

Secretariaat DEC
[redacted]

Aanvrager: [redacted]
Afdeling:

Titel dierproef: Indicator for methane emission in dairy cattle: grass silage versus maize silage

Aanmeldcode / Protocol: 2012086.d
Stadia van de proef:

10-12-2012	Aangemeld [redacted]
11-12-2012	Positief advies
14-01-2013	Welzijnsevaluatie aangemaakt [redacted]
14-01-2013	Welzijnsevaluatie aangemeld
16-01-2013	Welzijnsevaluatie goedgekeurd [redacted]

Is deze proef wetenschappelijk getoetst en goedgekeurd? Ja
Toelichting:
Yes, in association with a PhD procedure of [redacted]. The project has been approved by the financial sponsors ([redacted]n). Furthermore, the research committee of the [redacted] has discussed the project.

1.a. Met dit onderzoek te beantwoorden concrete vraag:
. Wetenschappelijke vraag m.b.t.wetensch. vraag
Methane is produced during microbial fermentation of feed in the gastrointestinal tract of ruminants, predominantly within the rumen (87%) and to a small extent (13%) in the large intestine (Murray et al., 1976). The rumen is an anaerobic environment in which microbiota digest feed components. Different microbial species (i.e. bacteria, protozoa and fungi) are involved in the conversion of feed material to methane in the rumen, with the final step carried out by methanogenic archeae (McAllister et al., 1996). Methanogenic archeae reduce CO2 to methane (McAllister and Newbold, 2008; Ellis et al., 2008). The process of formation of methane (i.e. CO2 + 4H2 --> CH4 + 2H2O) by methanogens generates metabolic energy for their maintenance and growth (Ellis et al., 2008).
The produced methane is predominately released to the environment through breath and eructation. Methane (CH4) is one of the most important greenhouse gases (GHG) and a significant contributor to global warming. In the dairy cattle chain, methane represents more than 50% of all the GHG produced. In addition, methane also represents an energy loss to the animal since CH4 has no nutritional value to the animal (Boadi et al., 2004) . About 6% of the total gross energy consumed by the animal is converted to CH4 in the rumen (Johnson and Johnson, 1995). This energy loss can reach up to 12% depending upon the quality of the diet (O'Mara, 2004). Therefore, it is desired to reduce the methane emission from dairy cattle, in order to reduce the ecological footprint of milk production, and improve feed efficiency and animal productivity.
The rate of CH4 produced from enteric fermentation in dairy cows depends greatly on the level of feed intake, the quantity of energy consumed, and dietary composition, in particular the nature of carbohydrates such as starch and its degradation rate. There have been several dietary strategies proposed to reduce the production of CH4 from dairy cattle (Grainger and Beauchemin, 2011), including

the use of diets rich in starch and low in fiber, since the fermentation of starch (as compared to fiber fermentation) increases the ratios of propionic to acetic acid, which are associated with reduced methane levels. An example of such a strategy is the replacement of fiber-rich grass silage with starch-rich maize silage. However, the scientific evidence for this particular replacement strategy is scarce. Recently, Staerfl et al. (2012) investigated this strategy, but the maize silage used had a net energy content some 10% lower than that of the grass silage, which in many countries (including the Netherlands) is highly uncommon as maize silage nearly always has a higher net energy content than grass silage.

The ability to measure methane emission by cows is essential for increasing our understanding of factors that contribute to variation in methane emission and thereby for interventions leading to its reduction. Climate respiration chambers are the golden standard for measuring methane emission. However, this technique is very expensive and therefore not suited for large scale measurement or as indicator at farm level. Mathematic models may allow prediction of CH4 production from cattle without extensive experiments, however, the accuracy of these models is low (Ellis et al., 2010). Thus, it is of great importance to develop a simple method to estimate CH4 production in cattle.
The current experiment is designed to observe the effect of replacing grass silage (no starch) with maize silage (starch-rich) on methane emission from lactating dairy cows, with the ultimate goal to develop an indicator for methane emission that can be measured in milk of individual cows. The hypothesis is that replacement of grass silage with maize silage will decrease the production of methane. The indicator will be used to build further on a prediction model recently proposed by Dijkstra et al. (2011) which is exclusively based on the fatty acid composition of the milk. It is hypothesized that the addition of other metabolites in this prediction model will enhance its predictive power and thus leads to a better indicator for methane emission in milk.
This experiment can be considered novel and differs from recent methane trials in [redacted] for two reasons. Firstly, the scientific evidence worldwide for the nutritional strategy to decrease the production of methane by replacing fibre-rich grass silage with starch-rich maize silage is scarce. This experiment targets to gain more scientific evidence for this replacement strategy. Secondly, the longer term goal of this experiment is to develop an indicator for methane emission that can be measured in milk, for which data are required on feeds commonly used in practice. Grass (silage) and maize silage are the most common feed ingredients in dairy cattle diets. In the Netherlands for example, on average some 30% of the diet of dairy cattle is grass silage, and 25% is maize silage.

Second objective:
Within the setup of the methane experiment, a rapid method to assess the nutritional status of dairy cattle will be developed. The method includes measurement of isotopic enrichment in metabolic end-products in breath (CO2) and milk (fat, protein and lactose) after intravenous administration of isotopically labelled nutrients.

In general, the partitioning of nutrients over anabolic and catabolic pathways is strongly affected by the nutritional status of a dairy cow. However, accurate but simple diagnostic tests for assessing the nutritional status of dairy cows are currently not available. Labelling nutrients with stable isotopes (e.g. 13C, 15N) may be a suitable technique to trace utilization of nutrients and hence provide a proxy for the nutritional status. It is expected that appearance of an injected bolus of an isotopically labelled nutrient in milk components depends on the balance between nutrient requirements (to synthesize milk lactose, fat and protein) and nutrient availability (from diet). Therefore, we hypothesize that the recovery of a labelled nutrient in milk components increases when this nutrient limits milk production, indicating a more efficient utilization for milk synthesis. Similarly, non-limiting nutrients will probably be oxidized to a larger extent and are therefore increasingly recovered as CO2 in breath. A proof of this principle could be obtained from an experiment with a large contrasts in nutrient supply, i.e. glucogenic vs. lipogenic nutrients. The present experiment, in which grass silage is replaced by maize silage, will result in a shift from supply of lipogenic nutrients to glucogenic nutrients. Grass silage is expected to result (after fermentation) mainly in acetic acid, which is lipogenic, whereas maize silage will produce more propionic acid (glucogenic) and some of the starch will escape rumen fermentation and will be hydrolysed in the small intestine to glucose and absorbed as glucose in the blood. Hence, the present experiment will result in a contrast in supply of lipogenic versus glucogenic nutrients.

For intravenous administration of 13C-glucose , it is expected that lactating dairy cows receiving a lipogenic diet will use a larger proportion of available glucose for milk production purposes than cows receiving a glucogenic diet. After injection of [U-13C]glucose, the recovery of isotope in milk lactose is therefore expected to be higher for cows receiving a lipogenic diet. Measuring also isotopic enrichment of the protein and fat fractions of milk may provide information about the use of glucose for other (milk production) purposes. It is known from literature that increased glucose availability may have positive effects on milk lactose and milk protein production (see e.g. Lapierre et al., 2010, Lemosquet et al., 2010, Rigout et al., 2002, Rius et al., 2010, Rulquin et al., 2004). In addition, after intravenous administration of 13C-acetate, as [1-13C] sodium acetate, the recovery of isotope in the milk fat fraction is expected to be higher for cows receiving the lipogenic diet, because milk fat production is usually lower in cows fed glucogenic rations (Van Knegsel et al., 2007). Moreover, assuming that a difference in the nitrogen (N) content of the diet may result in differences in protein metabolism and that such differences can be detected by itntravenous infusion of 15N-glycine, it is expected that intravenous infusion of 15N-glycine could be used as a means to investigate a difference in the nitrogen (N) content of the diet.

1.b. Het uiteindelijk doel (Maatschappelijke en wetenschappelijke relevantie):
Methane (CH4) has a global warming potential of 25 carbon dioxide equivalents (Forster et al., 2007). By signing the Kyoto protocol, the EU agreed to have reduced greenhouse gas emissions by at least 20 % by 2020 compared to emission levels in 1990. Methane is the single most important greenhouse gas produced on dairy farms. Besides the effect on global warming, methane emission from cattle represents an energy loss of 2-12% of gross energy intake (Johnson and Ward, 1996). Finding feeding strategies to reduce methane emission, supports achieving the targets for the Kyoto protocol. Besides, reduction in methane emission will reduce energy losses, which may increase feed efficiency.
With this experiment we aim to gain more scientific evidence for the nutritional strategy to decrease the methane production by replacing grass silage with maize silage. Our ultimate goal is to put this nutritional strategy into practice on dairy farms in The Netherlands and develop a simple method (i.e. indicator in the milk) to estimate CH4 production in individual cows and dairy herds, in order to increase our understanding of factors that contribute to variation in methane emission and thereby for interventions leading to its reduction.

A second aim, within the setup of the methane experiment, is to develop a method for rapid assessment of nutritional status of dairy cattle. The method includes measurement of isotopic enrichment in metabolic end-products in breath (CO2) and milk (fat, protein and lactose) after intravenous administration of isotopically labelled nutrients. Ultimately this method could be used to determine which nutrient is limiting for milk production.

1.c. Lekensamenvatting:
Een indicator voor methaanproductie bij melkkoeien: kuilgras versus snijmais.
Een Nederlandse melkkoe produceert dagelijks gemiddeld 350 gram methaan. Het gas wordt voornamelijk in de pens van de koe geproduceerd bij de voedselvertering. Een kwart van methaangas in onze atmosfeer is afkomstig van melkkoeien. Voor de verduurzaming van de melkproductie in Nederland is het van het grootste belang om de uitstoot van broeikasgassen, in het bijzonder methaan, te verminderen.
Tijdens dit project wordt een indicator ontwikkeld in melk om methaan gas uitstoot bij individuele koeien te meten. Met deze te ontwikkelen indicator in melk zal de methaanuitstoot niet alleen bij individuele koeien kunnen worden gemeten maar ook op kudde niveau. Voor melkveehouders is deze indicator een middel om methaangasuitstoot op hun boerderijen te monitoren en te sturen. Op deze manier zal het project een waardevolle bijdrage leveren aan de verdere verduurzaming van de primaire sector.
Om de indicator te ontwikkelen, worden melkkoeien gebruikt. Aangezien het uiteindelijke doel van dit project het verminderen van methaan uitstoot bij melkkoeien is, wordt tijdens dit onderzoek ook gebruik

gemaaktvan melkkoeien. Er worden in totaal 28 melkkoeien gebruikt, verdeeld over 4 groepen. Elke groep zal een ander rantsoen krijgen: (1) 100% kuilgras, (2) 67% kuilgras met 33% snijmais, (3) 33% kuilgras en 67% snijmais, en (4) 100% snijmais. Deze rantsoenen variëren in de hoeveelheid vezels en zetmeel, met de gedachte om verschillen in methaan uitstoot te creëren. Onderzoek met andere voedermiddelen heeft namelijk aangetoond dat een rantsoen rijk aan vezels resulteert in een hogere methaan uitstoot ten opzichte van een rantsoen rijk aan zetmeel. Kuilgras en snijmais zijn de meest gebruikte voedermiddelen in de Nederlandse melkveehouderij, maar de specifieke kennis over methaanproduktie bij vervanging van kuilgras door snijmais (of omgekeerd) is vrijwel afwezig.
De koeien worden gedurende 12 dagen in een grupstal gehouden voor de adaptatieperiode. Tijdens deze periode raken de koeien geadapteerd aan het nieuwe rantsoen en aan het aangebonden staan. Na deze 12 dagen, worden de koeien overgeplaatst naar de klimaatkamers. Ze worden individueel gehuisvest in de klimaatkamers, maar door de aanwezigheid van ramen kunnen ze elkaar zien en horen. In deze klimaatkamers blijven de koeien hetzelfde rantsoen ontvangen en staan ze aangebonden. Ze zullen bijna 5 dagen in deze klimaatkamers staan, zodat we de methaan uitstoot kunnen meten. Elke dag wordt tijdens het ochtend en avond melken, melkmonsters genomen. Na deze 5 dagen, worden de melkkoeien weer teruggebracht naar de stal.
Naderhand zal de melk geanalyseerd worden op samenstelling. Het is de bedoeling dat de samenstelling van de melk in relatie gebracht kan worden met de hoeveelheid methaan uitstoot. Er zal uiteindelijk, aan de hand van de relatie, een indicator in melk ontwikkeld worden om methaan gas uitstoot bij koeien te meten.

Binnen de opzet van het hiervoor beschreven onderzoek kan nog een tweede onderzoeksvraag worden beantwoord. In voedingsonderzoek, ook bij melkvee, wordt in toenemende mate gebruik gemaakt van labeling met stabiele isotopen. Dergelijke studies bieden de mogelijkheid het gebruik van individuele nutriënten te volgen en worden dan ook voornamelijk ingezet bij kwantitatieve studies naar de effecten van voeding op productie. Het voorgestelde experiment is gebaseerd op de hypothese dat de manier waarop een melkkoe haar metabolieten gebruikt afhankelijk is van haar voedingsstatus. Er kan dus een relatie worden verwacht tussen de voedingsstatus van een koe en de recovery van een geïnjecteerde dosis van een met isotopen gelabelde metaboliet in de melk. Een dergelijke relatie kan, zonder kwantitatief te zijn, zeker informatief zijn over de voedingsstatus van de koe. Deze techniek zou in de praktijk op koppelniveau kunnen worden toegepast.

2. Gepland vanaf: 03-11-2012 **tot** 21-12-2012

3. Specificatie diergroepen:

Cows for experiment	32	runderen	All cows (with and without rumen fistula, inclusding the reserve cows) for the methane experiment
Cows for testing infusion technique	2	runderen	Cows used to test the intravenous infusion technique

4.a. Nadere aanduiding gebruikte dieren:
28 lactating dairy cows (4 treatments, 7 cows per treatment), of which 12 cows will be rumen fistulated (i.e., only cows who already have a fistula; no new surgery is required). The 28 lactating dairy cows will be grouped into 7 blocks of 4 cows according to lactation stage, parity, milk production, fat- and protein levels in the milk and presence of a fistula. By doing this, 7 blocks are made, each with relative similarly cows. Therefore, it is expected that the dry matter feed intake will be quite similar for all 4 animals of one block. Within blocks, animals will be randomly assigned to one of the four experimental diets, resulting that all treatments occur within a block. In addition, 1 reserve block of 4 reserve cows will be made. These reserve cows will only be used if other cows would become unsuitable for use in the experiment.

Moreover, 2 cows will be used to test the intravenous infusion technique. The intravenous infusion technique will be tested first on these two animals, in order to refine the technique and to minimize the stress induced for the animals in the running experiment.

4.b. Motivatie waarom is gekozen voor deze diersoort:
The aim is to reduce methane emission from dairy cows, thus these are the target animals. Fistulated cows are needed for measurements of rumen volatile fatty acid (VFA) concentration and pH. Also, the standard in vitro technique will be applied, in which rumen fluid of individual cows is used as inoculum to study fermentation in vitro.

4.c. Toelichting voor het aantal gebruikte dieren:
During this experiment, the experimental unit will be one individual lactating dairy cow. There will be 7 cows assigned to each dietary treatment (i.e. 4 treatments, see following section). Thus, this results in 28 cows in total. This number of cows per treatment is the minimal number that allows sufficient statistical power and is based on the variation in methane emission found in previous studies with a similar experimental set-up. The recent studies of [redacted] in [redacted] have shown that a number of 10 cows per experimental treatment is sufficient to detect ($P<0.05$) treatment effect of approximately 10%. However, [redacted] used a different experimental unit. The 10 lactating cows were paired and the experimental unit was 2 cows per respiration chamber. This results in 5 experimental units. In this type of experimental setting, a lower number of cows will not allow a methane difference to be significant. For the present experiment, the cows will be housed individually in a respiration chamber instead of in pairs. Using 5 experimental units, as done by [redacted], is not sufficient because using pairs of cows per chamber as an experimental unit gives less statistical variation than using only 1 cow per chamber as an experimental unit. Therefore, it is estimated that 6 individual cows per treatment should give a similar statistical power compared with the 5 units (i.e. 2 cows per chamber) per treatment. However, chamber techniques differ worldwide (e.g., accuracy depends on frequency of gas sampling). Therefore, 7 cows per treatment will be required. Based on various rumen models, and depending on the actual nutritional quality of the grass and the maize silage, the predicted difference in methane production between grass silage and maize silage diets is some 10-15%.

Of the 28 cows, 12 cows will be rumen fistulated (i.e. only cows who already have a fistula). Fistulated cows are needed to sample rumen fluid for determination of pH and volatile fatty acid (VFA) concentration, and as inoculum for the gas production technique. The aim is to use 3 fistulated cows per treatment. This number is based upon a study with a block-design with 3 fistulated cows per treatment by Abrahamse et al. (2009), showing that significant pH differences of 0.1 units could be detected. In addition, differences in the ratio between non-glucogenic and glucogenic VFAs (and therefore the ratio between VFAs that release hydrogen and VFA that act as hydrogen sinks; the balance of which is strongly related to methane production) of 0.1 were detected. It is therefore expected that using 3 fistulated cows per treatment will result in finding differences of approximately the same level.

Moreover, 2 cows will be used to practice the intravenous infusion technique. The intravenous infusion technique will be practiced first on these two animals, in order to refine the technique and to minimize the stress induced for the animals in the running experiment.

4.d. Herkomst:
Cows for experiment A. van gereg. fok/toeleveringsbedrijf in Nederland
Cows for testing infusion technique A. van gereg. fok/toeleveringsbedrijf in Nederland
Toelichting:
Lactating dairy cows of experimental accommodations [redacted]) and [redacted].

In total, 34 cows will be selected from [redacted], of which 12 fistulated cows. Only cows that have been fistulated for previous purposes will be used; no new surgery will be done for this experiment. As much as possible cows of [redacted] will be chosen, provided that cows match in parity, lactation stage and milk production per group of 4 animals (since there are 4 treatments).

5.a. Accommodatie: [redacted]

Accommodation is in the dairy cattle tie-stall and climate respiration chambers at [redacted].

The lactating dairy cows for this experiment will be selected from [redacted] and [redacted]. The cows from [redacted] will be transported to [redacted], placed together in a group at [redacted] after arrival and receive a pre-adaptation period of at least 5 days. The lactating cows from [redacted] will be housed separately in the free barn from the already present animals, to avoid unrest due to arrival of new animals. During this period, they will receive the basic ration fed at [redacted].
During the first 12 days during then experimental period, the cows are housed in the tie-stalls. The period in the tie-stalls is considered to be the adaptation period in which the dairy cows can adapt to the experimental diet and get accustomed to being tied down. A previous study of Van Zijderveld et al. (2011) showed that an adaptation period of 12 days is sufficient. Methane production in respiration chambers seems rather constant after entering the respiration chambers on day 13, and there is no clear day-effect which would have been expected if adaptation have been incomplete. The tie-stalls will be illuminated by daylight and TL-lamps which will be switched on between 5:30 a.m. and 21:30 p.m..
After the adaptation period in the tie stalls, the measuring period begins by transporting the 4 cows to the calorimetric respiration chambers. During this measuring period, the cows will be fed the very same restricted ration as before. The cows will be moved to the respiration chambers on day 13 at 15:00h and will be removed from the chambers on day 17 at 9:00h. In these respiration chambers, the TL-lamps will be switched on between 5:30 a.m. and 21:30 p.m. At night, a small lamp will illuminate the chambers. The relative humidity in the chambers will be set at 70% and the temperature at 16 C. The methane production will be determined during 3 days of the measuring period (i.e. 2nd-4th day of the measuring period). Van Zijderveld et al. (2011) has applied this procedure before. Cows will be housed individually in a respiration chamber (i.e. 1 cow per chamber). The individually housed cows are able to see each other through windows and are capable of hearing the sounds another cow makes.

5.b. Huisvesting & Verzorging:
Cows are fed and milked twice daily. Drinking water is supplied at libitum. During the adaptation period and in the respiration chambers, feed intake and milk production will be measured twice daily. This provides the opportunity to recognize health issues. All subjects will be monitored on a daily basis for clear signs of difficult of refusing to get up, eat, or any other remarkably reason, and if any of this occurs and other health problems a veterinarian will be called immediately. In general, cows will be withdrawn from the experiment when a situation occurs that affects the characteristics to be measured. In case of suspicion of health issues, experts (i.e., the caretaker of the animals and [redacted], article 14 expert on laboratory animals) will be consulted, after which it will be decided if veterinary treatment should be obtained. When antibiotics have to be supplied, or in case of health issues or medicine supplies which might interfere with metabolism and feed intake, the cow will be withdrawn from the study.

As mentioned before, the cows from [redacted] will receive a pre-adaptation period of at least 5 days. It is expected that this pre-adaptation period of 5 days is sufficient, for two reasons. Firstly, these 5 days will give the cows from [redacted] a sufficient amount of time to recover from their transport to [redacted]. In addition, >80% of the micro flora in the rumen is adapted to a newly introduced ration after 5 to 7 days (Boeckaert et al., 2008). Complete adaptation to the [redacted] ration is not required, because the pre-adaptation phase is immediately followed by a 12 day adaptation phase, in which all cows will become adapted to the experimental diets. After 12 days, we expect that all cows are adapted to the experimental diets and have developed a stabile rumen micro flora. The five-day pre-adaptation period functions mainly as a synchronization period, during which all cows (i.e. both from [redacted] and [redacted]) get accustomed to the same total mixed ration and it will prevent that the cows from [redacted] will have an extreme change of diets in a short time period. All cows will be group-fed in this period and feed intake will not be monitored.

5.c. Voeding:

This experiment has a completely randomized block design in order to examine the effect of starch level in the diet on methane emission in dairy cattle. The cows will receive 1 of the 4 experimental diets. The diets will consist of grass silage and / or maize silage in order to create different fiber and starch levels in the diet.
The four diets are (% of roughage compared to the total the total amount of roughage in the diet):

1. 100% grass silage (GS100)
2. 67% grass silage and 33% maize silage (GSMS67:33)
3. 33% grass silage and 67% maize silage (GSMS33:67)
4. 100% maize silage (MS100).

These four different diets are included for several reasons. Firstly, we want to find a linear relation between the nutritional replacing strategy and the production of methane. If we would only use the two extreme diets (i.e. 100% grass silage and 100% maize silage), we can only assume a linear relation. Therefore, the two diets in between (i.e. diet 2 and 4, a combination of grass silage and maize silage) are needed to be able to find a linear relation. Secondly, we want to find an indicator for methane in the milk. For this we need diets commonly used in practice. As mentioned before, grass silage and maize silage are the most common feed ingredients in dairy cattle diets. In the Netherlands for example, on average some 30% of the diet of dairy cattle is grass silage, and 25% is maize silage.
The four diets are not isocaloric. We want to measure methane production in relation to the amount of feed intake. Since feed intake is a major determinant of methane production, we decided that restriction of the feed intake is required (see restriction method below). Therefore, all cows within the same block will have a rather similar dry matter intake, resulting in a similar feed intake between the different diets. This has to come with the compromise that the four diets are not isocaloric.

The cows will be milked and fed twice daily (06.00 and 16.00 hours) and have ad libitum access to clean drinking water. All cows will receive their experimental diet as a total mixed ration. The forage:concentrate ratio will be 80:20 based on dry matter content. Each ration will fulfil requirements and will not cause any feed-related health issues such as acidosis. The proportion of concentrate in the diet will be kept low in order to create large differences in nutrient composition between the experimental diets, but concentrates need to be included in the ration to meet the requirements for maintenance and lactation. The composition of the concentrate will be the same for all four treatments. The composition of the concentrates will be chosen carefully, in order to meet the minimal OEB (rumen degradable protein balance) and DVE (intestinal available protein) requirements according to Dutch standards for dairy cattle (Centraal Veevoer Bureau). In addition, the concentrate will contain minerals and nutrients (premix).

The experimental diets will be fed individually and ad libitum during the first 7 days of the adaptation period in the tie stalls. From day 8 till 17 (i.e. last 5 days adaptation period and measuring period) the feed intake will be restricted per block to 95% of the ad libitum feed intake of the cow consuming the lowest amount of feed during day 5 till 8 within a block (Van Zijderveld et al., 2011). The feed restriction is required to reduce variation in feed intake between animals, since feed intake in itself is a major determinant of methane production. As mentioned before, by grouping cows into groups, we assume that the dry matter feed intake will be quite similar for all 4 animals of one block. However, even a small difference in milk production can be accompanied with a large difference in feed intake (i.e. cows that differ of 2.2. kg milk production, differ approximately 1 kg of dry matter feed intake when fed ad libitum). Thus, restricted feeding is needed, because the variation in methane production will be unacceptable due to the variation in feed intake level between the cows when fed ad libitum. This could potentially make it more difficult to find statistical valid conclusions. Feeding restrictedly will lead to a more similar feed intake within blocks and the reduced variation will help to detect smaller differences between treatments.
The feed intake will be restricted to have identical dry matter intake for cows in the same block as much as possible throughout the whole experiment, provided that the cow with the highest feed intake in the block is never restricted to less than 82% of the ad libitum feed intake.

When animals consume lower amounts of feed than they are used to, less nutrients are available for production. We expect milk production to adjust to the feed intake level. Previous studies in [redacted] have demonstrated that this method does not lead to problems. Staff of [redacted] and researchers of [redacted] that will be involved in the trial, are experienced with this method. To prevent problems with negative energy balance as much as possible, we will not use cows that have

only been in lactation for 8 weeks or less.

6.a. Proefschema / proefbehandelingen:
This experiment has a completely randomized block design in order to examine the effect of starch level in the diet on methane emission in dairy cattle. As mentioned previously, 28 cows will be grouped into 7 blocks of 4. Within each block, cows will be randomly assigned to the four different diets. These four diets differ in starch level and consists of grass silage and/or maize silage (see section 5c).

Experimental set-up:
The cows from [redacted] will be transported to [redacted], placed together in a group at [redacted] after arrival and receive a pre-adaptation period of at least 5 days. The lactating cows from [redacted]d will be housed separately in the free barn from the already present animals, to avoid unrest due to arrival of new animals. After at least 5 days, the cows are moved to the tie-stalls. In every block, the 4 animals will be moved to the tie-stalls. The period in the tie-stalls is considered to be the adaptation period in which the dairy cows can adapt to the experimental diet and get accustomed to being tied down. A previous study of Van Zijderveld et al. (2011) showed that an adaptation period of 12 days is sufficient. In addition, during this adaptation period the feed intake can be controlled and measured.
During the first 7 days feed is supplied ad libitum. After 7 days, feed supply is restricted. This procedure has been used in a number of experiments before (Van Zijderveld et al., 2011) and is needed since feed intake is a major determinant of methane production. During the last 2 days of the adaptation period, samples of rumen fluid will be taken from fistulated cows to measure volatile fatty acid concentration and pH levels. Rumen samples will be taken once before morning feeding and at 6 time points after morning feeding; 1, 2, 3, 4, 6, and 8h after feeding.
In addition, we want to define and establish mixed population reflecting the metabolic map in the rumen. For this, we will take 3 rumen samples from fistulated cows. On day -1 (i.e. 1 day before start adaptation period in tie stalls), day 10 and day 17 we will take both rumen fluid and rumen fiber fraction samples at 3 hours after morning feeding.
After the adaptation period in the tie stalls, the measuring period begins by transporting the 4 cows to the respiration chambers. During this measuring period, the cows will be fed the very same restricted ration as before. The cows will be moved to the respiration chambers on day 13 at 15:00h and will be removed from the chambers on day 17 at 9:00h. Cows will be housed individually in a respiration chamber (i.e. 1 cow per chamber). The individually housed cows are able to see each other through windows and are capable of hearing the sounds another cow makes.
The methane production will be determined during 3 full days of the measuring period (i.e. starting at 09.00h on day 14 until 09.00h on day 17). Methane emission is expressed per day. Van Zijderveld et al. (2011) has applied this procedure before. If measurements would only be done for 2 days, this could affect the accuracy of the methane measurements (e.g. when something disturbs the data on one day, like an error in the measurement equipment). Also for measuring the energy balance, which has been done many times in Wageningen, a measurement period of 3 days has been shown to give reliable data.
Methane emission, O2 consumption, CO2 production, urine plus faeces production and milk production will be registered. The ration components and the manure collected over the whole period in the chambers will be analyzed for amongst others organic matter, cellulose, hemicellulose, starch and crude fat. The milk samples will be analysed on composition, fatty acid profile, fat, protein, lactose and urea levels.
In addition, we will take hair samples from all 32 cows. We will pull approximately 20 hairs per cow from the crown of their head. DNA will be isolated from the hair follicle to determine the genotype for each cow. These genotypes will be related to the measured methane emission from these cows in the climate chambers.
After the measurements in the respiration chambers, most cows are transported back to the free barn of [redacted]. Cows from [redacted] are transported back all at once after the last group of cows has been in the respiration chambers. At the end of each experimental period, the respiration chambers have to be cleaned completely using as little cleaning water as possible. All urine, faeces and cleaning water will be collected in a large tank. This will be mixed for homogeneity and sampled for dry matter, ash, nitrogen, crude fat, neutral detergent fiber and gross energy determination.

The isotope experiment will be carried out within the previously explained experimental setup. The

experimental procedure per cow will be as follows:
Infusion grade isotopes will be dissolved in sterile water. Solutions will be prepared in collaboration with the pharmacy of [redacted] and administered to the animals as a sterile nutrient solution.
On day 2 in the respiration chambers, directly after morning milking around 6am, a labeled nutrient solution (13C-glucose and 15N-glycine) will be injected in the jugular vein. 4 g 15N-glycine and 2 g of 13C-glucose will be dissolved in a volume of 500 ml sterile water. Before injection, a catheter (Type: Intraflon i.v. 12G 80mm) will be placed into the jugular vein to make sure that the entire dose is injected in the blood stream. To facilitate catheter insertion, a small incision in the skin will be made. After isotope administration the catheter will be flushed with saline and subsequently removed. The skin will be closed with suturing staples. Based on advice from an experienced veterinarian, it was decided that no local anaesthetics will be administered before making a small incision (5-10mm) in the skin. However, after making a small incision in the skin, an anaesthetic in the form of lidocaine spray will be applied on the skin, to minimize discomfort during catheterization and application of the suturing staples. Most likely, the discomfort to the cows caused by puncturing the skin for the injection (of the anaesthetic), will be similar to the discomfort caused by making the actual incision. Moreover, infiltration with lidocaine results in a depot above the jugular vein, which may complicate insertion of the catheter. It is also possible to accidentally puncture the jugular vein while injecting the anaesthetic. The latter may cause a hematoma which would again complicate insertion of the catheter.
The bottle used for injection of the isotope solution will be weighed before and after injection to determine the infused volume. On day 4 in the respiration chambers, 9 g of 13C-NaAcetate will be infused in a volume of 500 ml sterile water following the same procedure as for infusion of isotopically labeled glucose and glycine. The amounts of isotopically labelled nutrients have been chosen in such a way to detect sufficient amounts of isotope in milk by combustion-IRMS.
Duplicate samples of the morning milking of day 2 and 4 in the respiration chambers will be taken for background measurement of isotopic enrichment. Isotopic enrichment in breath will be measured at 9 minute intervals. After infusion of isotopically labelled material, milk from the 3 subsequent milkings (duplicate sample per milking) will be collected. The third milking after administration of 13C-acetate will be collected outside the chambers, but after a similar time interval as for 13C-glucose. For each milking, a representative subsample of the milk will be analysed for milk composition(i.e. fat, protein and lactose) and a second subsample will be fractionated. After freeze drying, the different milk fractions will be analysed for isotopic enrichment using combustion IRMS. During this procedure enrichment with 15N and 13C can be measured simultaneously.

6.b. Mate van ongerief:

Cows for experiment B. Gering/Matig
Cows for testing infusion technique A. Gering

6.c. Waaruit bestaat het ongerief en hoe bent u tot uw inschatting van de mate van ongerief gekomen?

- Housing the cows tied during the adaptation period and in the respiration chambers
- Opening/closing the fistula for collecting rumen fluid (only applicable for the cows with a rumen fistula)
- Individual housing of animals in chambers.
- During the period that the animals will be housed in the respiration chambers, an intravenous catheter will be inserted twice for injection of a solution isotopically labelled nutrients into the bloodstream.

The discomfort has been estimated based on the above and based on previous experiments within [redacted] chambers with dairy cattle.

7. Welke maatregelen heeft u getroffen om het ongerief tot een minimum te beperken?
Anesthesie:

Cows for experiment D. Is wel toegepast.
Cows for testing infusion technique D. Is wel toegepast.

Pijnbestrijding:

Cows for experiment A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.
Cows for testing infusion technique A. Wordt niet toegepast omdat hiertoe geen aanleiding bestaat.

Re-use of previously fistulated animals (only applicable for the cows with a rumen fistula).
Placing animals from [redacted] in a separate group after arrival at [redacted].
The effect of individual housing on animals is minimised by allowing animals to see each other through the windows in the chambers and when animals make a sound, the other cows will hear that as well.
Practicing the intravenous infusion technique under instruction of an experienced veterinarian. To do this, two cows from the [redacted] herd will be used. Also for the first infusions, adequate supervision will take place.
After making a small incision in the skin, an anaesthetic in the form of lidocaine spray will be applied on the skin, to minimize discomfort during catheterization and application of the suturing staples.

8. Toestand van dieren na einde van de proef:

Cows for experiment Het dier is na de proef in leven gelaten.
Cows for testing infusion technique Het dier is na de proef in leven gelaten.

Toelichting:

Cows will return to the accommodation after the experiment ([redacted])

9. Welke alternatieven (vervanging, verfijning, vermindering) zijn voor de beschreven experimenten overwogen en waarom zijn deze verworpen?

Replacement:
In vitro analyses of some previous studies have been shown not to be representative for in vivo measurements. It is also difficult to represent processes like pH, VFA absorption rate and passage rate in vitro; such processes are expected to have a major impact on methane. Therefore this study should be performed in vivo with the target animal (dairy cow) itself. The measurement of pH and of VFA concentration and type, major elements associated with methane production, also has to be performed in the target animal (fistulated animals) as the buffered in vitro systems do not allow such effects to be evaluated.

Refinement:
Social isolation will be prevented by housing cows in individual respiration chambers with windows in the walls; the sounds of the animals will be heard by other animals as the walls of the chambers are quite thin. In the adaptation area they will be housed in larger numbers.
Transport of cattle from [redacted] will be transported for some 80 km distance and will be housed separately in the free barn from the already present animals, to avoid unrest due to arrival of new animals; from their special, separate part of the free barn, they will move after a minimal of 5 days to the tie-stall.
Of the fistulated animals, only animals that already have a fistula will be used. To make optimal use of the available fistulated cows, cows of [redacted] will be used. Therefore no new surgery is required.

Reduction:
We will use the lowest number of animals possible to obtain the desired statistical accuracy (see part 4c). By doing some measurements repeatedly in the same animals (pH and VFA measurements) we try to obtain as many data as possible from one animal. In addition, we will also use data from previous experiments ([redacted]) in order to find a robust indicator for methane emission with the use of a minimal number of cows.
Moreover, answering multiple research questions within the same experimental setup will further contribute to reduction as it is not needed to setup a separate experiment.

10. Namen van direct betrokkenen bij de dierproef (artikel 9- en 12-functionarissen):

Tabel registratiecode opties voor aanvraag 2012086.d (K14):

	1	2	3	4	5	6	7	8	9	10	11	12	13	
					37	1	1	01						
Cows for experiment	**1**	**45**	**1**	**32**					**01**	**4**	**1**	**2**	**3**	
Cows for testing infusion technique	**1**	**45**	**1**	**2**						**01**	**4**	**1**	**1**	**3**

Uw aanvraag 2012086.a, door u aangemeld vanuit DRS heeft van de DEC de status: 'Wijzigen' gekregen.

De DEC onderschrijft het wetenschappelijke belang van de proef, maar heeft nog enkele vragen ter verheldering waar ze graag een antwoord op wil, alvorens tot een definitieve afweging te komen:
De DEC verzoekt u bij 1.a (te beantwoorden vraag) de te beantwoorden vragen en bijbehorende hypothesen van de voorliggende proef beknopt samen te vatten.
Daarnaast verzoekt de DEC u bij 1.b. (maatschappelijke en wetenschappelijke relevantie) beknopt aan te geven, welke praktische toepassing in de praktijk u uiteindelijk voor ogen hebt (gras vervangen door mais, koeien op stal, ...).
Bovendien verzoekt de DEC u bij 3. (specificatie diergroepen) en bij 6.b. (mate van ongerief) en 6.c. (bronnen van ongerief) de proefgroepen te splitsen, aangezien niet alle dieren hetzelfde ongerief ondergaan.
Tevens verzoekt de DEC u de keuze voor de dieetgroepen 2 en 3 beter te onderbouwen, aangezien het haar niet duidelijk is, wat deze groepen toevoegen als het gaat om het ontwikkelen van een indicator. Hiervoor zou in de ogen van de DEC kunnen worden volstaan met de twee uiterste groepen.
De DEC vraagt zich af, of een preadaptatieperiode van 5 dagen lang genoeg is, om de dieren te laten wennen aan de microflora van [redacted] en verzoekt u bij 5.b. (huisvesting & verzorging) hierop in te gaan (verwacht u hiervan een effect?).
Daarnaast verzoekt de DEC u bij 5.c. (voeding) aan te geven of het voer isocalorisch is en waarom dat al dan niet nodig is.
De DEC verzoekt u de incisie t.b.v. het aanbrengen van de katheter vooraf te laten gaan door een verdoving en verzoekt u dit toe te voegen bij 6.a. (proefschema) en 7. (maatregelen ter beperking van ongerief) of te beargumenteren, waarom dit niet kan.
Tot slot heeft de DEC nog enkele redactionele opmerkingen:
Zij verzoekt u in het vervolg het proefplan beknopter op te schrijven en 1.a. toe te spitsen op de concrete onderzoeksvragen van de voorliggende proef en de bijbehorende hypothesen.
Tevens verzoekt zij u de dubbele teksten te verwijderen.
Daarnaast verzoekt zij u de dieren "koeien" te noemen in plaats van "subjects".
Bovendien verzoekt zij u de criteria, op grond waarvan de dieren worden geselecteerd te verplaatsen van 5.c. naar 4.a.
Tenslotte verzoekt zij u de passage "The effect of individual housing on animals is minimised .....the other cows will hear that as well" bij 6.c. (bronnen van ongerief) te verplaatsen naar 7.

Na beantwoording zal de proef door de kleine commissie worden besproken en zo mogelijk afgehandeld.

Uw aanvraag 2012086.b, door u aangemeld vanuit DRS heeft van de KC de status: 'Wijzigen' gekregen.

S.v.p. indeling proefgroepen wijzigen.

Indien de status op 'wijzigen' is gezet en u wilt deze aanvraag gaan wijzigen, dan selecteert u deze aanvraag en kiest u vanuit het menu 'bewerken aanvraag', en dan de optie 'wijzigen'. Er wordt dan een kopie van de originele aanvraag gemaakt. Deze kopie kunt u vervolgens wijzigen, en opnieuw aanmelden.

Met vriendelijke groet,

[redacted]
Secretaris DEC

Uw aanvraag 2012086.c, door u aangemeld vanuit DRS heeft van de KC de status: 'Positief advies na behandeling DEC' gekregen.
De DEC is van mening dat het doel van de proef opweegt tegen het te verwachten maximaal gering/ matige ongerief dat de dieren ondergaan en dat de vraag m.b.t. alternatieven voldoende is beantwoord.

Met vriendelijke groet,
[redacted]
Secretaris DEC

Uw aanvraag 2012086.c, door u aangemeld vanuit DRS heeft van de PD de status: 'Wijzigen' gekregen.

Indien de status op 'wijzigen' is gezet en u wilt deze aanvraag gaan wijzigen, dan selecteert u deze aanvraag en kiest u vanuit het menu 'bewerken aanvraag', en dan de optie 'wijzigen'. Er wordt dan een kopie van de originele aanvraag gemaakt. Deze kopie kunt u vervolgens wijzigen, en opnieuw aanmelden.

Na aanmelding zal de aanvraag door de proefdierdeskundige beoordeeld worden.

Met vriendelijke groet,

[redacted]
Proefdierdeskundige

Uw aanvraag 2012086.d, door u aangemeld vanuit DRS heeft van de PD de status: 'Positief advies' gekregen.

Met vriendelijke groet,

[redacted]
Proefdierdeskundige