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# Relationship between *in vitro* and *in vivo* methane production measured simultaneously with different dietary starch sources and starch levels in dairy cattle

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ABSTRACT

To investigate the relationship between *in vitro* and *in vivo* methane ($CH_4$) production measured simultaneously using the same rumen-fistulated cows in both experiments, four dietary treatments based on concentrate that accounted for 400 g/kg of the mixed diet DM, were formulated to contain starch varying in rate of fermentation (slowly (S) vs. rapidly (R): native vs. gelatinized maize grain) and level of inclusion (low (L) vs. high (H): 270 vs. 530 g/kg of concentrate DM). Sixteen rumen-fistulated lactating dairy cows were used in a complete randomized block design with these treatments replicated in four periods of 17 d each. In experiment 1, after 12 d of adaptation, the cows were housed in respiration chambers for 5 d to measure $CH_4$ production. In experiment 2, in each period *in vitro* gas and $CH_4$ production were measured (in duplicate per period) for mixed diet samples from the same diet as fed to the donor cows using rumen inocula adapted to the respective diets for an average of 16 d. In addition, samples of two concentrate ingredients, *viz.* grass silage and beet pulp, were incubated with four different inocula obtained from individual donor cows. Gas production (GP) was measured using automated GP system with $CH_4$ measured at distinct time points. *In vitro* (24-h) $CH_4$ production of mixed diet was lower with R than S (42.9 vs. 49.5 ml/g of incubated organic matter (OM); P=0.004), and higher with L than H (49.8 vs. 42.6 ml/g of incubated OM; P=0.002). A significant interaction effect between source and level of starch (P=0.015) was also found, indicating the $CH_4$ production of the RH diet decreased in particular. *In vivo*, an increased rate of starch fermentation resulted in a lower $CH_4$ per unit of estimated rumen-fermentable OM (eRFOM; 55.6 vs. 61.2 ml/g of eRFOM; P=0.007), and higher level of starch tended (P=0.089) to reduce $CH_4$ per unit of eRFOM, but dietary starch level and source did not affect $CH_4$ per unit of OM consumed. Across the diets tested, 24-h *in vitro* $CH_4$ (ml/g of incubated OM) correlated well with *in vivo* $CH_4$ expressed per unit of eRFOM ($R^2$ = 0.54; P=0.040), but not when expressed per unit of OM ingested ($R^2$ = 0.04; P=0.878). For grass silage (the same trend for beet pulp), inocula adapted to R- and H-based diets compared with S- and L-based diets resulted in a lower

*Abbreviations:* ADFom, acid detergent fibre excluding residual ash; aNDFom, neutral detergent fibre assayed with a heat stable amylase and excluding residual ash; $CH_4$, methane; CP, crude protein; DM, dry matter; GP, gas production; H, high level of starch; L, low level of starch; OM, organic matter; OMI, organic matter intake; eRFOM, estimated rumen-fermentable organic matter; R, rapidly fermentable starch; S, slowly fermentable starch.

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$CH_4$ production (36.1 vs. 44.8 ml/g of incubated OM, R vs. S; and 37.4 vs. 43.4 ml/g of incubated OM, H vs. L; P<0.001). These results indicate that adaptation of rumen inoculum to different diets affects $CH_4$ production of a substrate differently. In conclusion, *in vitro* $CH_4$ measurement can be indicative of the trend of *in vivo* $CH_4$ production from different combinations of sources and levels of starch when *in vivo* $CH_4$ is expressed per unit eRFOM, but not when expressed per unit OM ingested. This study suggests that complexity associated with rumen fermentation conditions needs to be considered to fully predict *in vivo* $CH_4$ production from *in vitro* measurements.

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## 1. Introduction

The *in vitro* gas production (GP) technique is a relatively cheap, well standardized and widely used method to evaluate the nutritive value of ruminant feeds by incubating substrate in buffered rumen fluid (Cone et al., 1996; Dijkstra et al., 2005; Getachew et al., 1998). This technique can also be used to estimate the methane ($CH_4$) production potential of different feeds and the $CH_4$ mitigation potential of feed additives and plant extracts (Bhatta et al., 2012; Getachew et al., 2005,b; Pellikaan et al., 2011a,b). A number of factors with considerable influence on the results obtained with this technique have been reported (as reviewed by Rymer et al., 2005). Amongst the largest sources of variation are the source and activity of the microbial inoculum (Cone et al., 2002). In most *in vitro* studies, the microbial inoculum is obtained from donor animals fed a diet which differs from the substrate used for the *in vitro* incubation. Under such conditions, the fermentation characteristics of the substrate and the amount of $CH_4$ produced were shown to be dependent on the type of available substrate in the diet of the donor animal and its fermentation characteristics (Martinez et al., 2010), due to diet-dependent changes in the microbial type and activity of the rumen inoculum (Boguhn et al., 2013; Fernando et al., 2010).

Feeding high levels of rumen fermentable starch or high concentrate diets were observed to change the type of ruminal micro-flora, including increased amylolytic bacteria and decreased methanogen and fibrolytic bacteria numbers (Martin et al., 2010; Morgavi et al., 2010). Similarly, in contrast to fiber, starch fermentation in the rumen was shown to favor the production of propionate (Bannink et al., 2008), creating an alternative hydrogen sink to methanogenesis. However, there is substantial variation in the *in vitro* fermentation kinetics of different starch sources (Cone and Becker, 2012). Inclusion of these starch sources in the diets of animals also influences rumen fermentation differently, and hence leads to variability in the reduction of $CH_4$ produced (Beauchemin and McGinn, 2005; Popova et al., 2013). Above all, *in vitro* and *in vivo* studies are usually performed separately under different conditions and there is a lack of direct *in vitro-in vivo* comparison, which is essential to demonstrate the robustness or effectiveness of the *in vitro* GP technique in simulating rumen fermentation.

Hindle et al. (2005) compared the *in vitro* and *in vivo* starch degradation from different sources and found a discrepancy between the *in vivo* starch degradation and that estimated from an *in vitro* GP experiment in which the donor animals were not adapted to the diets with the same starch source. The authors suggested *in vitro* GP could provide a more accurate simulation of the *in vivo* fermentation of potato starch if the donor animals are adapted to a diet including this starch source. Cone and Van Gelder (2006) observed that the fermentation rate of native potato starch was considerably enhanced by using rumen fluid of cows adapted to the fermentation of native potato starch, instead of using other rumen fluid of cows fed no potato starch. Although different laboratories around the world are using *in vitro* GP as a well standardized techniques for the evaluation of the nutritional quality of ruminant feeds, studies that have investigated the relationship between the *in vitro* and *in vivo* $CH_4$ production using the same adapted animals for the *in vivo* experiment as for collecting rumen inoculum for the *in vitro* incubations are largely lacking. In an earlier study, Bhatta et al. (2008) found a weak relationship between *in vitro* $CH_4$ production (ml/g DM) estimated by gas production technique with rumen inocula obtained from non-adapted dairy cows and *in vivo* $CH_4$ production (ml/g DM intake) by goats fed different diets and measured in respiration chambers.

The objective of this study was to investigate the relationship between *in vitro* and *in vivo* $CH_4$ production measured simultaneously using the same rumen-fistulated dairy cows in both experiments and with the same mixed diet incubated as substrate *in vitro* and fed to the donor animal of which microbial inoculum is obtained, with the concentrate component of the mixed diet varies in starch rate of fermentation and level of starch. We hypothesized that *in vitro* $CH_4$ measurements from different sources and levels of starch in the diet are related to the *in vivo* $CH_4$ production if both *in vitro* and *in vivo* $CH_4$ measurements are performed simultaneously, using the same animals as donor for microbial inoculum when they are fed and adapted to the same dietary material used as a substrate for *in vitro* incubation.

## 2. Materials and methods

### 2.1. Experimental design, animals and diets

This study consisted of two experiments conducted simultaneously using the same cows. The experiments were conducted at the animal research facility of Wageningen University (Wageningen, The Netherlands). All experimental procedures

were approved by the Institutional Animal Care and Use Committee of Wageningen University and carried out under the Dutch Law on Animal Experimentation.

A total of 16 multiparous lactating Holstein-Friesian dairy cows, all fitted with a permanent rumen cannula were used. Cows were grouped in four blocks using a complete randomized block design based on parity (2.8 ± 1.0; mean ± SD), days in milking (302.4 ± 74.0 d) and fat- and protein-corrected milk (27.1 ± 8.3 kg/d) at the start of the experiment. Cows within a block were randomly assigned to one of the four different dietary treatments that were based on concentrates formulated to contain starch varying in rate of fermentation (slowly (S) vs. rapidly (R): native vs. gelatinized maize grain, respectively) and level of inclusion (low (L) vs. high (H): 270 vs. 530 g per kg of concentrate DM, respectively). Cows received diets consisting of 600 g/kg grass silage and 400 g/kg concentrate (DM basis). The diets were offered individually and in equal meals twice daily during milking at 0600 and 1600 h. The concentrates were in a meal form and mixed with the forage portion manually when fed. Cows had unrestricted access to drinking water. The 16 rumen fistulated cows were part of a larger experiment to investigate effect of starch level and starch source on $CH_4$ production including 24 non-fistulated animals (Hatew et al., 2015).

The two starch sources were selected to create a considerable contrast in starch rate of fermentation. Different levels of starch in the concentrates were achieved by exchanging either native or gelatinized maize grain with fibrous material (beet pulp and palm kernel expeller) on a DM basis. The ingredient composition of the concentrates was reported by Hatew et al. (2015). The main starch source (i.e. maize grain) was supplied by Meneba Meel BV (Rotterdam, The Netherlands). Gelatinization of starch was performed at 80 °C for 2 min using an Insta-Pro extruder (Urbandale, IA, USA). The degree of gelatinization was tested by enzymatic degradation, using an amyloglucosidase test. Gelatinized maize was prepared from the same batch as native maize to avoid differences in chemical composition as would have been the case when using other rapidly fermentable starch sources, such as oats or wheat, instead of gelatinized maize.

Experiment 1 involved the measurement of *in vivo* $CH_4$ emission from cows fed those four dietary treatments replicated in four periods of 17 d each. The first 12 d of each period was used for adaptation to the diet. During the first 8 d of the adaptation period, the cows were fed *ad libitum*. From day 9 onwards, the cows were fed restricted to 95% of the average daily voluntary DM intake based on day 3–8 of the cow consuming the lowest amount of feed in that particular block to avoid the confounding effects of DM intake on $CH_4$ production. After the end of the adaptation period, cows were housed for 5 d in one of two identical climate-controlled open circuit indirect calorimetry respiration chambers for the measurement of $CH_4$ production. Details of the respiration chambers and gas analysis have been described by Verstegen et al. (1987). For welfare reasons, two cows (one rumen fistulated and the other without cannula) receiving the same treatment were housed in one chamber.

Experiment 2 consisted of *in vitro* $CH_4$ measurements using the same four substrates from a sample of the mixed diet as fed to the individual donor cows. This experiment was conducted simultaneously with experiment 1 and, therefore, was replicated over four periods ($n = 4$ per treatment). In each period, six additional substrates (*viz.* the four concentrates, grass silage and beet pulp) were incubated with diet-adapted rumen inocula.

### 2.2. In vitro gas production system

Gas production (GP) profiles of the substrates were measured using a fully automated time related GP system (Cone et al., 1996). Samples of each substrate were freeze-dried and ground over a 1-mm sieve using a Wiley mill (Peppink 100AN, Olst, The Netherlands). Approximately 0.5 g (DM basis) of each substrate was weighed into 250 ml fermentation bottles (Schott, Mainz, Germany). Each substrate was weighed in duplicate bottles within each period and replicated on four separate periods on different days.

In total, 10 different substrates were evaluated with four samples of the mixed diet as fed to the individual donor cows, another four samples of the concentrate included in those diets, and samples of the grass silage and the beet pulp (Table 1). The concentrates were: (1) concentrate composed of the low level of S starch (270 g of S starch per kg of concentrate DM = SL); (2) concentrate composed of the high level of S starch (530 g of S starch per kg of concentrate DM = SH); (3) concentrate composed of the low level of R starch (270 g of R starch per kg of concentrate DM = RL); and (4) concentrate composed of the high level of R starch (530 g of R starch per kg of concentrate DM = RH). Beet pulp and grass silage were included to investigate the effects of diet-adapted rumen inocula on *in vitro* $CH_4$ production of those non-starch substrates. Substrates from the sample of mixed diets and from the concentrates included in those diets were incubated with rumen inoculum obtained from individual donor cows that were adapted to that particular mixed diet and diet consisting of that specific concentrate, respectively. However, beet pulp and grass silage substrates were each incubated with four different rumen inocula obtained from individual cows adapted to each of the four mixed diets.

In each period, rumen fluid was obtained from each of the four individual donor cows that were adapted to one of the four mixed diets on average for 16 d. Equal volumes of rumen fluid (approximately 200–250 ml) were collected from the front ventral, middle ventral and cranial dorsal sac of the rumen of individual donor cows. Rumen fluid from each cow was collected before the morning feeding in a separate insulated flask previously prewarmed and flushed with $CO_2$. All handlings of the rumen fluid were as described by Cone et al. (1996). Prior to inoculation, the fermentation bottles were placed in a shaking water bath (Haake SWB25, Clausthal-Zellerfeld, Germany) maintained at 39 °C and preflushed with $CO_2$. The bottles

**Table 1**
Chemical composition of substrate and total mixed diet.

| Item | DM (g/kg as fed) | Ash | CP | aNDFom | ADFom | Crude fat | Starch | Sugar | Gross energy (MJ/kg of DM) |
|---|---|---|---|---|---|---|---|---|---|
| | | g/kg of DM | | | | | | | |
| Substrate | | | | | | | | | |
| Beet pulp | 913 | 74 | 89 | 416 | 242 | 10 | 7 | 77 | 16.8 |
| Grass silage | 512 | 88 | 148 | 528 | 296 | 38 | NA[c] | 79 | 19.1 |
| Concentrate[a] | | | | | | | | | |
| SL | 889 | 54 | 169 | 311 | 187 | 43 | 275 | 36 | 18.5 |
| SH | 896 | 50 | 170 | 170 | 90 | 34 | 518 | 22 | 18.4 |
| RL | 890 | 47 | 167 | 308 | 181 | 47 | 303 | 33 | 18.7 |
| RH | 883 | 48 | 186 | 153 | 80 | 41 | 542 | 22 | 18.3 |
| Total mixed diet[b] | | | | | | | | | |
| SL | 663 | 74 | 156 | 441 | 252 | 40 | 110 | 62 | 18.8 |
| SH | 666 | 72 | 157 | 385 | 214 | 36 | 207 | 56 | 18.8 |
| RL | 663 | 71 | 156 | 440 | 250 | 42 | 121 | 61 | 18.9 |
| RH | 660 | 72 | 163 | 378 | 210 | 39 | 217 | 56 | 18.7 |

[a] SL and SH are concentrates containing 270 and 530 g of slowly fermentable starch per kg of concentrate DM, respectively; RL and RH are concentrates containing 270 and 530 g of rapidly fermentable starch per kg of concentrate DM, respectively.
[b] Composed of 600 g per kg of grass silage and 400 g per kg of respective concentrate on a DM basis.
[c] NA, not analyzed.

were then inoculated with 60 ml of buffered rumen fluid with a rumen fluid to buffer ratio of 1:2 (v/v) and connected to the fully automated GP apparatus (Cone et al., 1996).

### 2.3. In vitro methane measurement

Methane concentration in the headspace of the fermentation bottle was measured by a gas chromatography (GC; GC8000Top CE Instruments, Milan, Italy). To allow gas sampling from the headspace, the fermentation bottles were fitted with a side port sealed with a screw cap that is fitted with an air-tight septum (GRACE, XLB-11 Septa 7/16, Breda, The Netherlands) as illustrated by Pellikaan et al. (2011a). At distinct time points (0, 2, 4, 8, 12, 24, 30, 36, 48, 60 and 72 h of incubation), 10 μl aliquots of the bottles headspace gas were sampled through this opening using a gas tight syringe (Gastight® # 1701Hamilton 1701 N, 10 μl Syringe, Point Style 5, Bonaduz, Switzerland) and analyzed for $CH_4$ concentration using GC. The GC was fitted with a flame ionization detector (FID) and stainless steel column (6 m long, 0.53 mm i.d., 25 μm film thicknesses) packed with PoraPack Q 50–80 mesh (GRACE, Breda, The Netherlands). The temperatures of the injector, column and detector were maintained at 150, 60 and 150 °C, respectively. The carrier gas was nitrogen, and the pressure for nitrogen, hydrogen and air was set at 100, 50 and 100 kPa, respectively. Peak areas were determined by automatic integration system software for GC (Chrom-Card data system Version 2.4, 2006, Rodano Milan, Italy). The $CH_4$ concentration in the headspace was determined by external calibration using a certified standard containing a known content of $CH_4$ (Linde Gas Benelux, Schiedam, The Netherlands).

### 2.4. Curve fitting and calculations

Cumulative gas production data were fitted iteratively to a monophasic Michaelis–Menten equation (Groot et al., 1996; Eq. (1)) using the non-linear least squares regression procedure in SAS (SAS Institute, Inc., 2010).

$$GP = \frac{A}{1 + (B/t)^C} \quad (1)$$

where GP, cumulative amount of gas produced (ml/g of incubated organic matter, OM); $A$, asymptote gas (ml/g of incubated OM); $B$, time of incubation at which half of the asymptote gas has been formed ($T_{1/2}$, h); $C$, the sharpness of the switching characteristic of the profile; $t$, time (h).

Curve fit parameter estimates of blanks (containing buffered rumen fluid without substrate) were used to correct for gas produced from residual fermentable OM in the inoculum. The cumulative $CH_4$ production was obtained as follows. Measured $CH_4$ concentrations were plotted against time and the monophasic sigmoidal model (Eq. (1)) was fitted to the data points. Next, the model parameter estimates were used to compute $CH_4$ concentrations at each individual valve opening. Cumulative $CH_4$ production was calculated as the sum of the increased amount of $CH_4$ in the bottle headspace between two successive valve openings and the amount of $CH_4$ vented from the bottle as described by Pellikaan et al. (2011b).

$$M = \sum_{i=1}^{n} \{V_{HS}(C_{i+1} - C_i) + G_{i+1}C_{i+1}\} \quad (2)$$

where $M$, cumulative $CH_4$ production (ml/g of incubated OM); $V_{HS}$, bottle headspace volume (ml); $C_i$ and $C_{i+1}$, $CH_4$ concentration in the bottle headspace gas at $i$ and $i+1$ valve openings, respectively; $G_{i+1}$, the amount of gas (ml) vented at $i+1$ valve opening; $n$ = total number of valve openings.

The maximum rate of gas or $CH_4$ production ($R_{max}$, ml/h) and time at which this maximum is reached ($TR_{max}$, h) was calculated based on Eqs. (3) and (4), modified from Yang et al. (2005).

$$R_{max} = \frac{A \times B^C \times C \times TR_{max}^{(-C-1)}}{(1 + B^C \times TR_{max}^{-C})^2} \quad (3)$$

$$TR_{max} = B \times \left[\frac{C-1}{C+1}\right]^{(1/C)} \quad (4)$$

where $A$, asymptote gas or $CH_4$ (ml/g of incubated OM); $B$, time of incubation at which half of the asymptote gas or $CH_4$ has been formed ($T_{1/2}$, h); $C$, the sharpness of the switching characteristic of the profile.

The daily $CH_4$ production based on *in vitro* results was calculated using the observed *in vivo* OM intake of the donor cow multiplied with the 24-h *in vitro* $CH_4$ production per unit of incubated OM.

### 2.5. Comparison of in vitro and in vivo methane production

To investigate the relationship between *in vitro* and *in vivo* $CH_4$ production, only data obtained from the four dietary treatments of both experiments were used. Gas produced during the 24 h of incubation period was assumed as a good estimate of ruminal starch disappearance or the extent of fermentation of starch (Bal et al., 2000), and for the correlations between *in vitro* and *in vivo* $CH_4$ production, the 24-h incubation values were used. Correlations were analyzed with *in vivo* $CH_4$ production expressed in ml per gram of OM consumed, ml per gram of OM digested, and ml per gram of estimated rumen-fermentable organic matter (eRFOM). Details for determination of total-tract apparent digestibility and eRFOM have been reported by Hatew et al. (2015).

### 2.6. Analytical procedures

Diet and substrate samples were freeze-dried, ground using a Wiley mill fitted with a 1-mm sieve and analyzed for DM, ash, N, crude fat, starch, sugar, gross energy, neutral detergent fiber (aNDFom; after a pre-treatment with a heat stable amylase and expressed excluding residual ash), and acid detergent fiber (ADFom; expressed excluding residual ash) following the standard procedures previously reported (Hatew et al., 2015).

### 2.7. Statistical analysis

Data from duplicate bottles for each substrate per period were averaged before statistical analysis. The experimental unit for the *in vitro* measurements was the value of the averaged bottles and for the *in vivo* measurement it was a chamber. The data for one cow fed on RL diet in one respiration chamber had to be excluded from all *in vivo* and *in vitro* analysis due to malfunctioning of the chamber and yielding unreliable data. Data were analyzed by ANOVA using the PROC MIXED procedure in SAS (SAS Institute, Inc., 2010) using the model:

$$Y_{ijk} = \mu + S_i + L_j + P_k + (S \times L)_{ij} + e_{ijk}$$

where $Y_{ijk}$, the response variable (such as $CH_4$, gas, fermentation kinetic parameters); $\mu$, the overall mean; $S_i$, the fixed effect of source of starch ($i = 2$, slowly and rapidly fermentable starch sources); $L_j$, the fixed effect of level of starch ($j = 2$, low and high level of starch in the diet or concentrate); $P_k$, experimental period as a random factor ($k = 4$); $(S \times L)_{ij}$, the interaction of source of starch and level of starch in dietary treatments; $e_{ijk}$, experimental error.

Because of unequal variances, the Kenward-Roger option was used to estimate the denominator degrees of freedom. For each analysis, the first autoregressive, compound symmetry and unstructured covariance structures were tested. Depending on the characteristics of analysis, the covariance structure with the lowest Akaike's information criterion was selected (Littell et al., 1998), which in most cases was the compound symmetry covariance structure. The choice of covariance structure was based on parameters estimated from the restricted maximum likelihood method. To examine the correlation between *in vitro* and *in vivo* $CH_4$ production, Pearson correlation coefficients were estimated using the CORR procedure of SAS.

Results are reported as least square means. Effect of treatments and their interactions were declared significant at $P \leq 0.05$ and a tendency at $0.05 < P < 0.10$.

**Table 2**
*In vitro* gas and methane production of concentrate substrates incubated with diet-adapted rumen inocula, with the diets of the donor cows varying in starch rate of fermentation and level of inclusion.

| Item[c] | Concentrate[a] | | | | SED[b] | P-value | | |
|---|---|---|---|---|---|---|---|---|
| | SL | SH | RL | RH | | Source (S) | Level (L) | S × L |
| Gas production (GP) | | | | | | | | |
| 24-h GP (ml/g of incubated OM) | 329.6 | 327.6 | 311.2 | 278.2 | 14.92 | 0.010 | 0.130 | 0.174 |
| *A* (ml/g of incubated OM) | 390.9 | 389.2 | 362.5 | 323.7 | 24.12 | 0.022 | 0.266 | 0.305 |
| *B* (h) | 7.2 | 7.6 | 6.1 | 6.8 | 0.61 | 0.054 | 0.198 | 0.794 |
| *C* | 1.6 | 1.7 | 1.6 | 1.7 | 0.23 | 0.710 | 0.512 | 0.921 |
| $R_{max}$ (ml/h) | 34.8 | 33.4 | 37.2 | 30.6 | 2.42 | 0.905 | 0.044 | 0.163 |
| $TR_{max}$ (h) | 2.9 | 3.5 | 2.2 | 3.0 | 1.01 | 0.451 | 0.359 | 0.891 |
| $CH_4$ production | | | | | | | | |
| 24-h $CH_4$ (ml/g of incubated OM) | 60.4 | 56.9 | 54.0 | 46.8 | 3.13 | 0.006 | 0.041 | 0.425 |
| *A* (ml/g of incubated OM) | 82.0 | 78.8 | 73.7 | 67.7 | 4.36 | 0.014 | 0.167 | 0.658 |
| *B* (h) | 11.1 | 12.0 | 11.1 | 12.5 | 1.18 | 0.742 | 0.183 | 0.737 |
| *C* | 1.5 | 1.5 | 1.4 | 1.4 | 0.19 | 0.559 | 0.986 | 0.929 |
| $R_{max}$ (ml/h) | 4.4 | 4.1 | 3.9 | 3.0 | 0.49 | 0.045 | 0.095 | 0.420 |
| $TR_{max}$ (h) | 3.5 | 3.8 | 3.2 | 3.3 | 1.36 | 0.712 | 0.815 | 0.956 |
| $CH_4$ (% of total gas) | 18.1 | 17.1 | 17.0 | 16.3 | 0.57 | 0.106 | 0.028 | 0.420 |

[a] Sample from the same concentrate as fed to the donor cows was incubated with inoculum adapted to the respective concentrate included in the mixed diet of the individual donor cow. SL and SH are concentrates containing 270 and 530 g of slowly fermentable starch per kg of concentrate DM, respectively; RL and RH are concentrates containing 270 and 530 g of rapidly fermentable starch per kg of concentrate DM, respectively.
[b] SED, standard error of the difference of means.
[c] *A*, asymptote gas or $CH_4$ production; *B*, incubation at which time half of asymptote gas or $CH_4$ production has been formed; *C*, the sharpness of the switching characteristic for the profile; $R_{max}$, maximum gas or $CH_4$ production rate; $TR_{max}$, time occurrence of $R_{max}$.

## 3. Results

### 3.1. Chemical composition of substrates and donor cow diets

The aNDFom content of the diets varied between 378 and 441 g/kg of DM and starch content between 110 and 217 g/kg of DM (Table 1). Similarly, the concentrates were highly variable in aNDFom and starch contents. The crude protein content was similar for all diets, except a slightly higher level for the RH diet. Among the substrates investigated the aNDFom content of grass silage was higher compared to the other substrates.

### 3.2. In vitro gas and methane production of dietary treatments

Gas production (GP), $CH_4$ production and fermentation kinetics of concentrates incubated with rumen inoculum obtained from donor cows adapted to the diet including that specific concentrate are summarized in Table 2, and those of the mixed diet are presented in Table 3. With incubation of the concentrates, an increasing rate of starch fermentation gave a decreased 24-h GP (294.7 vs. 328.6 ml/g of incubated OM, R vs. S; P=0.010). Similarly, lower asymptote GP (343.1 vs. 390.1 ml/g of incubated OM; P=0.022) and $CH_4$ production (50.4 vs. 58.7 ml/g of incubated OM; P=0.006) were observed with R- compared to S-based concentrates. The half-time of GP showed only a tendency (P=0.054) to be lower for R than for S. The level of starch did not affect both GP and asymptote GP, but H compared with L in the concentrate decreased $CH_4$ production (51.9 vs. 57.2 ml/g of incubated OM; P=0.041).

The asymptote $CH_4$ production was lower (70.7 vs. 80.4 ml/g of incubated OM; P=0.014) for R- compared with S-based concentrates. The $TR_{max}$ for both GP and $CH_4$ production and B for $CH_4$ production were unaffected by source and level of starch in the concentrate. However, the $R_{max}$ of GP was lower (P=0.044) for H than L starch in the concentrate. The $R_{max}$ of $CH_4$ production was reduced (P=0.045) for R compared with S and showed a tendency (P=0.095) to be lower for H than for L. The proportion of $CH_4$ in the total gas varied between 16.3% for RH and 18.1% for SL concentrate, and was less (P=0.028) for H than for L but unaffected by the source of starch in the concentrate.

The *in vitro* gas and $CH_4$ production of diets as fed to the donor cows is presented in Table 3. Diets composed of R compared with S had lower 24-h gas (264.9 vs. 290.7 ml/g of incubated OM; P=0.001) and asymptote GP (296.5 vs. 331.9 ml/g of incubated OM; P<0.001). The higher level of starch in the diet tended (P=0.094) to reduce GP, but did not affect asymptote GP. A significant interaction between source and level of starch indicated that the effects on 24-h GP and asymptote GP were not additive. Lower $CH_4$ production (42.9 vs. 49.5 ml/g of incubated OM; P=0.004) and asymptote $CH_4$ production (57.7 vs. 70.4 ml/g of incubated OM; P=0.002) were observed for the diets based on R compared with S. Higher starch inclusion compared with low starch in the diet reduced $CH_4$ production (42.6 vs. 49.8 ml/g of incubated OM; P=0.002), but asymptote $CH_4$ production was unaffected by the higher level of starch in the diet. A significant interaction effect of source and level of starch on $CH_4$ production was also found.

Dietary treatments had no effect on $R_{max}$ of GP and $TR_{max}$ of both GP and $CH_4$ production (Table 3). However, a significant interaction effect of source and level of starch in the diet on $R_{max}$ of $CH_4$ production was observed, indicating that the effect

**Table 3**
*In vitro* gas and methane production of diet substrates incubated with diet-adapted rumen inocula, with the diets of the donor cows varying in starch rate of fermentation and level of inclusion.

| Item[c] | Diet[a] | | | | SED[b] | P-value | | |
|---|---|---|---|---|---|---|---|---|
| | SL | SH | RL | RH | | Source (S) | Level (L) | S × L |
| Gas production (GP) | | | | | | | | |
| 24-h GP (ml/g of incubated OM) | 284.4 | 297.0 | 280.8 | 249.0 | 7.19 | 0.001 | 0.094 | 0.002 |
| *A* (ml/g of incubated OM) | 325.1 | 338.7 | 313.8 | 279.2 | 9.17 | <0.001 | 0.143 | 0.006 |
| *B* (h) | 7.0 | 7.4 | 6.7 | 7.1 | 0.33 | 0.192 | 0.102 | 0.945 |
| *C* | 1.7 | 1.7 | 1.7 | 1.9 | 0.12 | 0.292 | 0.241 | 0.625 |
| $R_{max}$ (ml/h) | 29.1 | 29.1 | 29.7 | 27.0 | 1.08 | 0.357 | 0.126 | 0.114 |
| $TR_{max}$ (h) | 2.9 | 3.4 | 3.0 | 3.5 | 0.45 | 0.852 | 0.187 | 0.986 |
| $CH_4$ production | | | | | | | | |
| 24-h $CH_4$ (ml/g of incubated OM) | 50.6 | 48.4 | 48.9 | 36.8 | 2.33 | 0.004 | 0.002 | 0.015 |
| *A* (ml/g of incubated OM) | 71.5 | 69.3 | 58.9 | 56.4 | 3.98 | 0.002 | 0.425 | 0.957 |
| *B* (h) | 13.3 | 13.1 | 9.4 | 15.4 | 2.10 | 0.608 | 0.086 | 0.063 |
| *C* | 1.4 | 1.4 | 1.6 | 1.3 | 0.12 | 0.284 | 0.039 | 0.126 |
| $R_{max}$ (ml/h) | 3.6 | 3.7 | 3.9 | 2.6 | 0.40 | 0.206 | 0.069 | 0.038 |
| $TR_{max}$ (h) | 3.5 | 3.0 | 4.0 | 3.3 | 0.68 | 0.447 | 0.252 | 0.819 |
| $CH_4$ (% of total gas) | 18.0 | 16.3 | 17.2 | 14.8 | 0.76 | 0.051 | 0.003 | 0.500 |

[a] Sample from the same diet as fed to the donor cows was incubated with rumen inoculum obtained from individual donor cow adapted to the respective diet. SL and SH are diets containing 270 and 530 g of slowly fermentable starch per kg of concentrate DM, respectively; RL and RH are diets containing 270 and 530 g of rapidly fermentable starch per kg of concentrate DM, respectively.
[b] SED, standard error of the difference of means.
[c] *A*, asymptote gas or $CH_4$ production; *B*, incubation at which time half of asymptote gas or $CH_4$ production has been formed; *C*, the sharpness of the switching characteristic for the profile; $R_{max}$, maximum gas or $CH_4$ production rate; $TR_{max}$, time occurrence of $R_{max}$.

of source of starch depend on level of starch and *vice versa*. Half-time GP was unaffected by treatments, with only a tendency (P=0.086) of higher half-time for $CH_4$ production with H (14.3 h) compared with L (11.4 h). When the amount of $CH_4$ was related to total gas (% of total gas), the difference between treatments still remained significant (P=0.003) for H versus L (15.6 vs. 17.6%), and showed a tendency (P=0.051) to be lower for R- compared with S-based diets.

### 3.3. *In vitro and in vivo methane production of dietary treatments*

The *in vitro* and *in vivo* $CH_4$ production of diets varying in the rate of starch fermentation and level of inclusion are summarized in Table 4. The 24-h *in vitro* $CH_4$ production was less with R than S (42.9 vs. 49.5 ml/g of incubated OM; P=0.004) and with H than L (42.6 vs. 49.8 ml/g of incubated OM; P=0.002). A significant interaction between source and level of starch on the *in vitro* $CH_4$ production indicated that the effect of source of starch was more noticeable with H than L starch in the diet. Treatment differences in 24-h $CH_4$ production were more pronounced than treatment differences in 12-h $CH_4$ production. The daily *in vitro* $CH_4$ production was lower with a higher level compared with a lower level of starch in the diet (661 vs. 813 L/d, respectively; P=0.005). Similarly, R compared with S in the diet resulted in a lower daily *in vitro* $CH_4$ production (684 vs. 790 L/d; P=0.001). In contrast to daily *in vitro* $CH_4$ production, simultaneously measured daily *in vivo*

**Table 4**
*In vitro* and *in vivo* methane production of diets varying in starch rate of fermentation and level of inclusion.

| Item | Diet[a] | | | | SED[b] | P-value | | |
|---|---|---|---|---|---|---|---|---|
| | SL | SH | RL | RH | | Source (S) | Level (L) | S × L |
| *In vitro* methane production | | | | | | | | |
| 24-h $CH_4$ (L/d)[c] | 828 | 751 | 797 | 570 | 39.8 | 0.005 | 0.001 | 0.028 |
| 12-h $CH_4$ (ml/g of incubated OM) | 36.4 | 30.9 | 34.9 | 25.8 | 3.91 | 0.270 | 0.029 | 0.536 |
| 24-h $CH_4$ (ml/g of incubated OM) | 50.6 | 48.4 | 48.9 | 36.8 | 2.33 | 0.004 | 0.002 | 0.015 |
| *In vivo* methane production[d] | | | | | | | | |
| $CH_4$ (L/d) | 597 | 545 | 581 | 557 | 27.8 | 0.916 | 0.081 | 0.488 |
| $CH_4$ (ml/g of OM intake) | 36.5 | 35.6 | 35.7 | 36.0 | 1.31 | 0.796 | 0.750 | 0.518 |
| $CH_4$ (ml/g of digested OM) | 46.7 | 50.7 | 51.6 | 47.5 | 2.79 | 0.685 | 0.968 | 0.075 |
| $CH_4$ (ml/g of eRFOM[e]) | 62.7 | 59.6 | 57.0 | 54.2 | 2.19 | 0.007 | 0.089 | 0.901 |

[a] Sample from the same diet as fed to the donor cows was incubated with rumen inoculum obtained from individual donor cow adapted to the respective diet. SL and SH are diets containing 270 and 530 g of slowly fermentable starch per kg of concentrate DM, respectively; RL and RH are diets containing 270 and 530 g of rapidly fermentable starch per kg of concentrate DM, respectively.
[b] SED, standard error of the difference of means.
[c] Daily *in vitro* $CH_4$ production was calculated using *in vivo* OM intake of the donor cow multiplied with the 24-h *in vitro* $CH_4$ production per unit of incubated OM.
[d] *In vivo* $CH_4$ production of donor cows fed on the same diet as substrate incubated was measured simultaneously in climate-controlled respiration chambers.
[e] eRFOM, estimated rumen-fermentable organic matter based on nylon bag degradation characteristics (for details see Hatew et al., 2015).

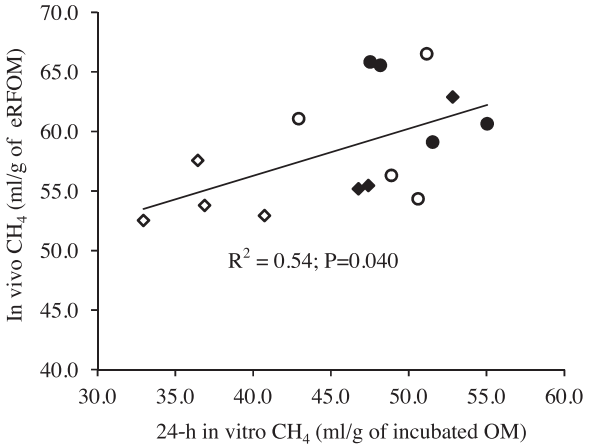


**Fig. 1.** Relationship between *in vivo* (ml/g of eRFOM) and 24-h *in vitro* (ml/g of incubated OM) $CH_4$ production measured simultaneously with dietary starch varying in rate of fermentation and level of inclusion in dairy cows. Sample from the same diet as fed to the donor cows was incubated with rumen inoculum obtained from individual donor cow adapted to the respective diet. ● = diet containing 270 g of slowly fermentable starch per kg of concentrate DM); ○ = diet containing 530 g of slowly fermentable starch per kilogram of concentrate DM); ♦ = diet containing 270 g of rapidly fermentable starch per kg of concentrate DM; ◊ = diet containing 530 g of rapidly fermentable starch per kg of concentrate DM starch). *In vivo* $CH_4$ production of donor cows was measured in climate-controlled respiration chambers.

$CH_4$ production showed only a tendency (P=0.081) to be lower for R- compared with S-based diets. However, *in vivo* $CH_4$ production expressed per unit of eRFOM was affected by starch source (61.2 vs. 55.6 ml/g of eRFOM; S vs. R; P=0.007) and tended (P=0.089) to be affected by starch level (59.9 vs. 56.9 ml/g of eRFOM; L vs. H). But *in vivo* $CH_4$ production expressed per unit of OM intake or per unit of OM digested was unaffected by treatments.

### 3.4. Relationship between in vitro and in vivo methane production

Across the diets tested, 24-h *in vitro* $CH_4$ production per unit of incubated OM correlated well with the *in vivo* $CH_4$ production expressed per unit of eRFOM ($R^2$ = 0.54; P=0.040; Fig. 1), but not when expressed per unit of OM ingested ($R^2$ = 0.04; P=0.878; Fig. 2) or when expressed per unit of OM digested ($R^2$ = 0.05; P=0.868; data not shown).

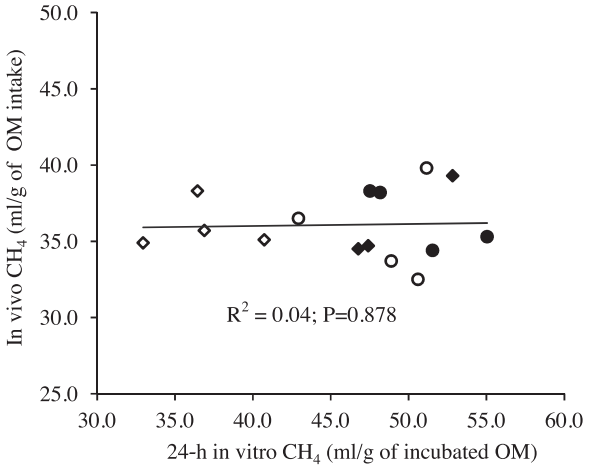


**Fig. 2.** Relationship between *in vivo* (ml/g of OM intake) and 24-h *in vitro* (ml/g of incubated OM) $CH_4$ production measured simultaneously with dietary starch varying in rate of fermentation and level of inclusion in dairy cows. Sample from the same diet as fed to the donor cows was incubated with rumen inoculum obtained from individual donor cow adapted to the respective diet. ● = diet containing 270 g of slowly fermentable starch per kg of concentrate DM); ○ = diet Containing 530 g of slowly fermentable starch per kg of concentrate DM); ♦ = diet containing 270 g of rapidly fermentable starch per kg of concentrate DM; ◊ = diet containing 530 g of rapidly fermentable starch per kg of concentrate DM starch). *In vivo* $CH_4$ production of donor cows was measured in climate-controlled respiration chambers.

**Table 5**
*In vitro* gas and methane production of beet pulp and grass silage incubated with diet-adapted rumen inocula.

| Parameter[c] | Diet[a] | | | | SED[b] | P-value | | |
|---|---|---|---|---|---|---|---|---|
| | SL | SH | RL | RH | | Source (S) | Level (L) | S × L |
| Gas production (GP) | | | | | | | | |
| Beet pulp | | | | | | | | |
| 24-h GP (ml/g of incubated OM) | 347.5 | 337.3 | 336.8 | 306.4 | 8.73 | 0.008 | 0.009 | 0.123 |
| *A* (ml/g of incubated OM) | 381.5 | 372.1 | 367.9 | 341.5 | 11.18 | 0.023 | 0.050 | 0.313 |
| *B* (h) | 5.6 | 5.5 | 5.9 | 7.6 | 0.44 | 0.004 | 0.020 | 0.019 |
| *C* | 1.8 | 1.8 | 2.0 | 2.2 | 0.11 | 0.013 | 0.397 | 0.130 |
| $R_{max}$ (ml/h) | 43.7 | 42.3 | 41.5 | 31.0 | 1.52 | <0.001 | <0.001 | 0.002 |
| $TR_{max}$ (h) | 2.8 | 2.7 | 3.2 | 4.7 | 0.48 | 0.005 | 0.067 | 0.037 |
| Grass silage | | | | | | | | |
| 24-h GP (ml/g of incubated OM) | 243.0 | 240.6 | 233.8 | 205.7 | 4.84 | <0.001 | 0.002 | 0.005 |
| *A* (ml/g of OM) | 302.3 | 295.2 | 291.2 | 243.8 | 7.72 | <0.001 | 0.001 | 0.005 |
| *B* (h) | 8.4 | 7.9 | 8.2 | 9.1 | 0.33 | 0.060 | 0.452 | 0.010 |
| *C* | 1.3 | 1.3 | 1.3 | 1.7 | 0.10 | 0.017 | 0.012 | 0.007 |
| $R_{max}$ (ml/h) | 22.7 | 24.2 | 22.9 | 17.2 | 0.69 | <0.001 | 0.002 | <0.001 |
| $TR_{max}$ (h) | 2.0 | 1.7 | 1.7 | 4.3 | 0.53 | 0.015 | 0.013 | 0.004 |
| $CH_4$ production | | | | | | | | |
| Beet pulp | | | | | | | | |
| 24-h $CH_4$ (ml/g of incubated OM) | 55.4 | 49.5 | 50.0 | 35.8 | 1.81 | <0.001 | <0.001 | 0.010 |
| *A* (ml/g incubated OM) | 71.2 | 70.8 | 70.2 | 62.5 | 2.58 | 0.006 | 0.009 | 0.400 |
| *B* (h) | 12.6 | 13.6 | 13.5 | 20.4 | 1.25 | 0.002 | 0.002 | 0.008 |
| *C* | 1.6 | 1.4 | 1.5 | 1.6 | 0.13 | 0.723 | 0.560 | 0.260 |
| $R_{max}$ (ml/h) | 3.8 | 3.3 | 3.3 | 1.9 | 0.13 | <0.001 | <0.001 | 0.001 |
| $TR_{max}$ (h) | 4.8 | 4.0 | 4.5 | 7.6 | 0.94 | 0.032 | 0.105 | 0.016 |
| $CH_4$ (% of total gas) | 15.9 | 14.7 | 14.9 | 11.7 | 0.49 | <0.001 | <0.001 | 0.020 |
| Grass silage | | | | | | | | |
| 24-h $CH_4$ (ml/g of incubated OM) | 45.8 | 43.7 | 41.0 | 31.1 | 1.40 | <0.001 | <0.001 | 0.003 |
| *A* (ml/g incubated OM) | 68.9 | 66.1 | 65.1 | 44.4 | 3.88 | 0.001 | 0.002 | 0.010 |
| *B* (h) | 14.3 | 14.0 | 15.6 | 14.0 | 1.60 | 0.552 | 0.417 | 0.597 |
| *C* | 1.3 | 1.2 | 1.2 | 1.6 | 0.09 | 0.018 | 0.025 | 0.002 |
| $R_{max}$ (ml/h) | 3.3 | 3.4 | 3.0 | 2.1 | 0.29 | 0.005 | 0.102 | 0.049 |
| $TR_{max}$ (h) | 2.7 | 1.7 | 2.0 | 5.0 | 0.81 | 0.055 | 0.110 | 0.006 |
| $CH_4$ (% of total gas) | 18.9 | 18.2 | 17.6 | 15.9 | 0.81 | 0.011 | 0.071 | 0.461 |

[a] Samples from grass silage and beet pulp were each incubated with four different diet-adapted rumen inocula obtained from individual donor cow adapted to different diets. SL and SH are diets containing 270 and 530 g of slowly fermentable starch per kg of concentrate DM, respectively; RL and RH are diets containing 270 and 530 g of rapidly fermentable starch per kg of concentrate DM, respectively.

[b] SED, standard error of the difference of means.

[c] *A*, asymptote gas or $CH_4$ production; *B*, incubation at which time half of asymptote gas or $CH_4$ production has been formed; *C*, the switching characteristics for the profile; $R_{max}$, maximum gas or $CH_4$ production rate; $TR_{max}$, time occurrence of $R_{max}$.

### 3.5. *In vitro* total gas and methane production of non-starch substrates

Table 5 summarizes the *in vitro* total gas, $CH_4$ production and fermentation kinetics of beet pulp and grass silage, each incubated with rumen inocula adapted to four different diets. Inocula adapted to R- and H-based diets compared with S- and L-based diets, resulted in a lower 24-h GP of beet pulp (321.6 vs. 342.4 ml/g of incubated OM, R vs. S; P=0.008, and 321.9 vs. 342.2, H vs. L; P=0.009) and grass silage (219.8 vs. 241.8 ml/g of incubated OM, R vs. S; P<0.001, and 223.2 vs. 238.4 ml/g of incubated OM, H vs. L; P=0.002). Similarly, 24-h $CH_4$ production was lower for beet pulp (42.9 vs. 52.5 ml/g of incubated OM; P<0.001) and grass silage (36.1 vs. 44.8 ml/g of incubated OM; P<0.001) when the substrates were incubated with inoculum adapted to R- compared with S-based diets. Increasing the level of starch in the diet of the donor cow decreased the 24-h $CH_4$ production for both substrates (42.7 vs. 52.7 ml/g of incubated OM, H vs. L for beet pulp; and 37.4 vs. 43.4 ml/g of incubated OM, H vs. L for grass silage; P<0.001). There was also a significant interaction effect of source and level of starch on 24-h GP of grass silage and 24-h $CH_4$ production for both substrates.

The asymptote GP and $CH_4$ production of both grass silage and beet pulp was lower for R and H compared to S and L starch in the diet of the donor cow (Table 5). A significant interaction effect of source and level of starch was observed for asymptote GP and asymptote $CH_4$ production of grass silage, but not for beet pulp. The effects of diet-adapted rumen inoculum on fermentation kinetics, such as $R_{max}$ of GP were similar for beet pulp and grass silage. A lower $R_{max}$ of GP was observed when both substrates were incubated with rumen inoculum adapted to R- and H- compared with S- and L-based diets, with a significant interaction effect of source and level of starch in the diet. Similar results were obtained for $R_{max}$ of $CH_4$ production of beet pulp and grass silage, except for the lack of an effect of level of starch with grass silage. However, there was a significant interaction effect of source and level of starch in the diet of the donor cow on $R_{max}$ of $CH_4$ production for grass silage showing that an increased level of R and S resulted in a lower and slightly higher $R_{max}$, respectively. The half-time of GP and $CH_4$ production was higher for beet pulp only with R- and H- compared with S- and L-based diets, and with an interaction effect of source and level of starch on half-time.

The proportion of $CH_4$ in the total gas when beet pulp was incubated as substrate was reduced by inocula obtained from R- and H- compared with S- and L-based diets, respectively, with an interaction effect of source and level of starch. With grass silage, the proportion of $CH_4$ in total gas was lower (P=0.011) for R compared with S, and a tendency (P=0.071) for lower $CH_4$ proportion in H versus L was observed.

## 4. Discussion

To our knowledge, this is the first study where *in vitro* $CH_4$ measurements were performed simultaneously with *in vivo* $CH_4$ measurements in order to test the potential of an *in vitro* GP to predict actual $CH_4$ production *in vivo*. This was achieved by using adapted animals in an *in vivo* trial as donor animals for rumen inocula used with the *in vitro* incubations. The *in vitro* $CH_4$ measurements (expressed per unit of OM incubated) at 24-h were shown to be significantly correlated to *in vivo* rumen $CH_4$ production determined from different combinations of sources and levels of starch in the diet when *in vivo* $CH_4$ production was expressed per unit of eRFOM, but were not correlated when expressed per unit of OM ingested or per unit of OM digested.

### 4.1. Relationship between in vitro and in vivo methane production

Given the variation in chemical composition of the substrates and diets (Table 1) and *in situ* rumen degradation characteristics of starch (Hatew et al., 2015), the effect of dietary treatments on *in vitro* $CH_4$ production followed the expected patterns of starch rate of fermentation and level of inclusion when evaluated either by incubating concentrate substrates (Table 2) or mixed diet substrates (Table 3). The fractional degradation rate of the two starch sources varied almost by a factor of three (0.054 vs. 0.155 per h; S vs. R starch) and the estimated amount of rumen degraded starch was 59% higher for R compared with S (Hatew et al., 2015), which shows the markedly differing rates of fermentation of the two selected starch sources. Increased fractional rate of starch fermentation and level of inclusion in the diet reduced 24-h *in vitro* $CH_4$ production by 14%. In agreement with this, Getachew et al. (2005) observed a higher *in vitro* $CH_4$ production for a slowly digestible fraction of the feed (such as structural carbohydrate).

We measured $CH_4$ production of dairy cattle in respiration chambers and used rumen fluid of the very same individual animals to measure $CH_4$ production *in vitro*, allowing *in vitro* $CH_4$ productions to be related with the actual rumen $CH_4$ emissions for individual dietary treatments. The *in vivo* $CH_4$ production expressed in ml per gram of eRFOM was 4.9% lower with the SH than with SL the diet (Table 4). This is similar to a 4.3% reduction in 24-h *in vitro* $CH_4$ production expressed in ml per gram of incubated OM. However, differences in $CH_4$ production were found to be much greater *in vitro* than *in vivo* when comparing other combinations of starch sources and levels. The 24-h *in vitro* $CH_4$ production of the RH diet was 27.3% less compared to the SL diet. This reduction was almost double the difference of 13.6% for *in vivo* expressed in ml per gram of eRFOM. In agreement with our results, more pronounced differences in $CH_4$ production between diets observed *in vitro* than *in vivo* have been reported previously. Martinez-Fernandez et al. (2013) showed a proportionally higher reduction in $CH_4$ production *in vitro* by two plant compounds by as much as 87% and 96% compared with a relatively lower *in vivo* $CH_4$ reduction (33% and 64%, respectively) per unit of DM intake in goats. In their study, the *in vitro* and *in vivo* experiments were not performed in parallel as done in our study, where we used the same animals from the *in vivo* experiment as donors for collecting the diet-adapted rumen inocula for the *in vitro* trial.

The *in vitro* (24-h) $CH_4$ production per unit of incubated OM correlated well with the *in vivo* $CH_4$ expressed per unit of eRFOM ($R^2$ = 0.54; P=0.040; Fig. 1), but not when expressed per unit of OM ingested (Fig. 2) or per unit of OM digested ($R^2$ = 0.05; P=0.868; data not shown). In contrast, 12-h *in vitro* $CH_4$ production was unrelated to *in vivo* $CH_4$ production expressed either per unit of eRFOM ($R^2$ = 0.34; P=0.210), per unit of OM ingested ($R^2$ = 0.03; P=0.908) or per unit of OM digested ($R^2$ = −0.14; P=0.631) (data not shown). *In vivo* OM digestion includes the combined effects of rumen fermentation and large intestine fermentation, may not fully reflect rumen OM fermentation only, whereas the *in vitro* method only simulates rumen fermentation but not intestinal digestion. Even though both *in vitro* and *in vivo* experiments were done simultaneously under the same conditions, the *in vitro* study still did not take into account the influences of complex and dynamic fermentation conditions that occurs during the degradation of feeds in the rumen, such as rumen outflow of unfermented material (Bannink et al., 2011; Dijkstra et al., 2012; Pinares-Patino et al., 2007), or changes in rumen pH and buffering capacity (Dijkstra et al., 2012; Mc Geough et al., 2011) that occur under *in vivo* conditions. The ruminal fluid dilution rate and passage rate of substrate, for instance, were reported to explain about 25% and 28%, respectively, of the variation in $CH_4$ production in cattle (Okine et al., 1989). The latter is probably due to a reduced retention times in the rumen, and hence decreased rate of substrate fermentation and $CH_4$ production. The absence of such rumen processes in the closed *in vitro* GP system used in the current study may explain the absence of a relationship between the *in vitro* $CH_4$ expressed per unit of incubated OM and *in vivo* $CH_4$ expressed per unit of OM ingested or per unit of OM digested.

The *in vivo* $CH_4$ production measured in the current study originated from both rumen and post-ruminal fermentation. Yet the daily *in vitro* $CH_4$ production calculated from 24-h *in vitro* $CH_4$ production multiplied by the *in vivo* OM intake is higher than the actually measured daily *in vivo* $CH_4$ production for all dietary treatments. A similar result was obtained by Bhatta et al. (2007) who reported a lower *in vivo* $CH_4$ production measured by sulfur hexafluoride (SF6) compared with 48-h *in vitro* $CH_4$ (measured by a syringe method) with all diets tested (alfalfa hay, maize silage, Italian ryegrass hay, rice straw and Sudan grass hay). In contrast to the present results, the same authors reported a close correlation between *in vitro* $CH_4$

production estimated from the mean of the two measurement intervals (24- and 48-h) and simultaneously measured *in vivo* $CH_4$ production for most diets mentioned earlier. The discrepancy between the studies might be due to the difference in the techniques used or due to the variations in the fermentation characteristics of the diets investigated. In the present study, $CH_4$ produced during the first 24-h accounted for 54–88% of the asymptote $CH_4$ production, and the gas produced at 24 h of incubation was considered to be a good estimate of the extent of starch fermentation (Bal et al., 2000). The *in vitro* $CH_4$ production at 12-h of incubation was only 35–69% of asymptote $CH_4$ produced, and did not improve correlations with $CH_4$ production *in vivo*, compared with the 24-h *in vitro* $CH_4$ production.

The decline in $CH_4$ produced per unit of incubated OM associated with rumen inoculum adapted to the RH diet might have been caused by a shift towards a more propionate oriented type of fermentation, leading to less $CH_4$ production. Propionate is a sink for hydrogen and, therefore, hydrogen is unavailable as a substrate for methanogens. The decrease in 24-h *in vitro* $CH_4$ production corresponds to the expected relative increase in propionate with R-based diets with a relatively higher contribution of a rapidly fermentable starch with the higher inclusion level. In a previous study, *in vitro* fermentation of different sources of starch showed that rapidly rumen fermentable starch sources resulted in a higher molar proportion of propionate (Cone and Becker, 2012). The absence of significant difference in the *in vitro* GP between L and H starch-based concentrates but a significantly lower $CH_4$ production for H starch-based concentrates (Table 2) might indicate the contribution of the higher level of starch towards propionate production. This could be due to the fact that 1 mol of gas is produced per mole of acetate produced, but no net gas is produced in the propionate fermentation pathway (Firkins et al., 1998) and only fermentation to acetate and butyrate produces $CO_2$ and consequently $CH_4$ (Blümmel and Ørskov, 1993). However, no ruminal VFA concentration and pH were measured in the present *in vitro* study and, therefore, the present results are not conclusive.

### 4.2. Effect of diet-adapted rumen inoculum

Adaptation of the rumen inoculum to different sources and amounts of starch affected the $CH_4$ production from a substrate differently (Table 5). Inoculum obtained from cows adapted to R-based diets resulted in lower $CH_4$ production for both substrates (grass silage and beet pulp). Consistent with our results, Cone and Van Gelder (2006) observed that the fermentation rate of native potato starch was enhanced by using rumen fluid adapted to the fermentation of native potato starch instead of using other rumen fluids. The lower $CH_4$ production with inocula adapted to the R-based diets in the present study might be due to a change in a fermentative activity, i.e. an altered microbial composition, a change in microbial enzyme activity, or a combination of both (Boguhn et al., 2013; Fernando et al., 2010). However, no data were collected on microbial dynamics which might have shown a dependency between the pattern of rumen microbial population and type of diet fed. In line with this, information in the literature shows the extent to which the microbial ecosystem adapts to a particular type of diet. A study by Hristov et al. (2001) reported that rumen protozoa numbers are often lower in cattle fed a high-grain diet with less $CH_4$ being produced, presumably due to a decreased transfer of hydrogen from protozoa to methanogens. Similarly, feeding high levels of rumen fermentable starch or high concentrate diets have been observed to result in changes in the type of ruminal micro-flora, including increased amylolytic bacterial and decreased methanogens and fibrolytic bacterial numbers (Morgavi et al., 2010). Increasing the ratio of concentrate to hay in the diet of donor animals was shown to reduce the initial bacterial concentration and to affect the GP kinetic parameters, such as total GP and rate of GP (Nagadi et al., 2000).

Taken together, results from present study suggest that the complexity of rumen fermentation conditions needs to be taken into consideration in predicting the *in vivo* $CH_4$ production from *in vitro* GP measurements with varying starch sources and levels in the diet. It appears important to consider the diet of the donor animal, since incubation of the same substrate (grass silage or beet pulp) with rumen inocula obtained from donor cows fed on different diets produced variable amount of methane (Table 5). Whether the effects observed in the present study are pertinent to other types of ruminant diets and substrates incubated *in vitro* requires further investigation.

## 5. Conclusions

The potential of an *in vitro* gas production to predict actual $CH_4$ production *in vivo* was evaluated using the same adapted dairy cows in the *in vivo* trial as donor animals for rumen inocula used for the *in vitro* incubations simultaneously. *In vitro* $CH_4$ production is correlated with *in vivo* $CH_4$ production from different combinations of sources and levels of starch in the diet when *in vivo* $CH_4$ production was expressed per unit of eRFOM, but not correlated when expressed per unit of OM ingested or per OM digested.

## Conflict of interest

The authors have no conflict of interest to declare.

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