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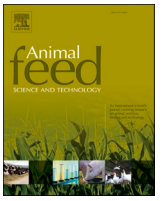

Relationship between predicted in vivo and observed in vivo methane production from dairy cows fed a grass-silage based diet with barley, oats, or dehulled oats as a concentrate supplement

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ABSTRACT

The objectives of this study were to compare predicted *in vivo* methane (CH_4) values based on data from an *in vitro* gas production experiment with observed *in vivo* values measured by the GreenFeed system, and to investigate the effect of the diet fed to donor animals of rumen inoculum (RI) on predicted *in vivo* CH_4 production. For this purpose, we conducted an *in vivo* experiment simultaneously with an *in vitro* gas production experiment. The *in vivo* experiment (previously published) was a 4 × 4 Latin square design including 16 Nordic Red dairy cows. Cows were fed 60% grass silage and 40% concentrate which consisted of either barley, oats with hulls (hulled oats), dehulled oats, or a 50:50 mixture of hulled and dehulled oats on dry matter basis. The *in vitro* experiment was a 2 × 4 factorial design replicated in 4 runs. The *in vitro* diets were incubated for 48 h in two types of RI and formulated according to the diets fed *in vivo*. The RI was obtained from cows fed either barley (two cows) or hulled oats (two cows) as concentrate in the *in vivo* experiment. A set of models were applied to the gas and CH_4 data obtained from the *in vitro* system to predict *in vivo* total gas and CH_4 production. For the comparison between predicted *in vivo* and observed *in vivo* CH_4, two different mean retention times (MRT of 35 and 50 h) in the rumen were used for the predictions. In the *in vitro* experiment, incubation residues were determined for organic matter digestibility and volatile fatty acids at 48 h of incubation. Assuming a MRT of 35 h in the rumen resulted in a significant relationship ($P = 0.04$) between predicted *in vivo* and observed *in vivo* CH_4 yield (g/kg dry matter) with an R-square of 0.91 and a root mean square error of 0.20. Ranking of the diets in terms of their CH_4 production was consistent between the *in vitro* and *in vivo* experiment. There were no significant interactions between diet and RI for any of the investigated parameters ($P = 0.40$). Rumen inoculum did not affect organic matter digestibility or total volatile fatty acid production. In conclusion, there was a good agreement between predicted *in vivo* and observed *in vivo* CH_4 values. In addition, the diet of RI donor animal did not influence the comparison of diets in terms of CH_4 production.

Abbreviations: aNDFom, amylase neutral detergent fiber organic matter; DM, dry matter; CP, crude protein; GF, GreenFeed; iNDF, indigestible neutral detergent fiber; MRT, mean retention time; OM, organic matter; RI, rumen inoculum; TDMD, true dry matter digestibility; TOMD, true organic matter digestibility; VFA, volatile fatty acids.

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1. Introduction

Enteric fermentation of feed in the forestomach of ruminants produce methane (CH_4) which contributes to anthropogenic emissions of greenhouse gases, and thus, climate change. The respiration chamber is considered the standard method to measure enteric CH_4 production *in vivo*. However, the method is both laborious and expensive, and prevents natural behavior as the animals need to be confined in the chamber during measurements (Hellwing et al., 2012). A method with lower costs and labor is the GreenFeed (GF) system (Hristov et al., 2015). A recent study concluded that the CH_4 values measured with the GF system corresponds well with the values measured by respiration chambers (Huhtanen et al., 2019; Alvarez-Hess et al., 2019). For efficient screening of many different diets for their effects on enteric CH_4 production, several *in vitro* methods have been developed where substrates are incubated in buffered rumen inoculum (RI) in a fermentation chamber.

The *in vitro* method developed by Ramin and Huhtanen (2012) uses a fully automated gas production system recording total gas, whereas CH_4 concentrations in head space gas are measured at different time points. A set of equations and dynamic rumen models are further applied to the data obtained from the *in vitro* system to predict *in vivo* CH_4 production. The accuracy of the system was recently confirmed by Danielsson et al. (2017), where predicted *in vivo* CH_4 production was compared with actual *in vivo* measured CH_4. They formulated 49 diets to closely resemble those fed to animals during 13 *in vivo* studies measuring CH_4 with respiration chambers in all studies except one, where the GF system was used. However, there is a notable scarcity of *in vitro* studies conducted in direct relation to an *in vivo* study, particularly those utilizing RI from donor animals on the exact same diet as subjected to *in vitro* incubation. In a study by Hatew et al. (2015), *in vitro* and *in vivo* CH_4 production from different starch sources were compared using RI from diet adapted donor animals in a simultaneously conducted *in vivo* experiment, but no such study has been conducted comparing *in vivo* predicted CH_4 based on the method by Ramin and Huhtanen (2012).

Enteric CH_4 production is affected by dietary factors such as forage quality and forage type (Eugène et al., 2021), grain type and dietary fat content (Alvarez-Hess et al., 2019), and diet digestibility (Blaxter and Clapperton, 1965). A previous *in vitro* study showed 8.9% lower predicted *in vivo* CH_4 production from hulled oats than from barley when incubated on a grass silage-based diet (Fant et al., 2020). Furthermore, Ramin et al. (2021) reported 4.4% lower CH_4 emissions from dairy cows fed hulled oats than from cows fed barley, also on a grass silage-based diet. In the *in vitro* study by Fant et al. (2020), the RI was obtained from cows fed a grass silage-based diet with barley as concentrate. However, the diet fed to the donor animals of RI may influence the results of *in vitro* studies (Yáñez-Ruiz et al., 2016). The choice of diet, particularly focusing on dietary concentrates, has been demonstrated to influence the microbial community and its diversity, subsequently affecting CH_4 production (Danielsson, 2016). It is yet unclear whether the CH_4 mitigating effect of hulled oats, compared with barley, is mediated by changes in the microbial community and thus could influence the results of *in vitro* studies. In addition, no study has investigated the effect of dehulled oats on CH_4 production, although the chemical composition of dehulled oats differs from that of hulled oats. Dehulled oats has a higher content of fat and starch, a lower content of fiber, and a higher digestibility than hulled oats (Biel et al., 2014), factors that are known to affect enteric CH_4 production.

The first objective of this study was to compare predicted *in vivo* CH_4 production based on the *in vitro* method developed by Ramin and Huhtanen (2012) with observed *in vivo* CH_4 production measured by the GF system in a simultaneously conducted *in vivo* experiment. The second objective was to evaluate the effects of the diet of RI donor animal on predicted *in vivo* CH_4 production with barley and three different types of oats as substrates together with grass silage. We hypothesized that predicted *in vivo* CH_4 production values would agree well with the observed *in vivo* CH_4 values.

2. Materials and methods

This study consists of data from two experiments conducted simultaneously during spring 2018. Experiment 1 was an *in vivo* experiment carried out at the Röbäcksdalen experimental farm of the Department of Agricultural Research for Northern Sweden, Swedish University of Agricultural Sciences in Umeå, Sweden (63°45′N; 20°17′E). The results of the *in vivo* experiment are published (Fant et al., 2021), and so only a brief summary of the materials and methods are presented here. Experiment 2 was an *in vitro* gas production experiment carried out in the laboratory at the same department. All experimental procedures were conducted in accordance with Swedish laws and regulations regarding EU Directive 2010/63/EU on animal research and were approved by the Swedish Ethics Committee on Animal Research (Dnr A 17/2016 and A 33/2016, Umeå, Sweden).

2.1. Animals, experimental design, and diets

In Experiment 1, 16 Nordic Red dairy cows were included in a 4 × 4 Latin square design replicated during four periods. Each period lasted 28 d, out of which 18 d (d1 – d18) were used for diet adaptation and 10 d (d19 – d28) were used for data collection and sampling. Cows were divided into four blocks based on parity and milk yield and were randomly allocated to one of four dietary treatments within each block. The dietary treatments consisted of different concentrates: barley, hulled oats (oats with hulls), a mixture of hulled and dehulled oats 50:50 on DM basis, and dehulled oats. The concentrates were a pelleted mixture of the experimental grain component (78.8%), canola meal (18.0%), CaCO3 (1.6%), NaCl (1.0%), MgO (0.4%), and a premix (0.2%), and were obtained from Raisioagro Oy (Ylivieska, Finland). Cows were fed grass silage as the sole forage with a forage to concentrate ratio of 60:40 on DM basis. The grass silage was made from primary growth perennial leys of timothy (*Phleum pratense*). Diets were offered as a total mixed ration and delivered to the feed bunks four times per day by an automatic feeding wagon to ensure *ad libitum* feed access. More details of Experiment 1 are described in Fant et al. (2021).

Experiment 2 was an *in vitro* gas production experiment designed as a 2 × 4 factorial design including the same four dietary

treatments as in the *in vivo* experiment and with two types of RI (barley fed cows vs. hulled oat fed cows). Experiment 2 consisted of four runs; each run was conducted from d21 to d23 (during 48 h) in each corresponding period of the *in vivo* experiment. The concentrates were sampled during the first period and the grass silage was sampled during each period and used to formulate the exact same rations to be incubated *in vitro* as fed to the cows. Rumen inoculum was collected on d21 in each period from two cows receiving the barley diet and from two cows receiving the hulled oat diet. Each run included 24 bottles containing feed samples and buffered RI, and 6 blank bottles containing only buffered RI (3 bottles/inoculum). Days 21–23 were chosen for conducting the *in vitro* experiment because they fell within the data collection period of the *in vivo* experiment. Rumen fluid from experimental cows was collected on d21 to explore the impacts of four different diets on the fermentation pattern *in vivo*.

2.2. *In vivo* methane measurements

During the *in vivo* experiment, CH_4 emissions were recorded by the GF system (C-Lock Inc., Rapid City, SD) as described by Hristov et al. (2015). Emissions were recorded from all 16 cows during the entire experiment, but only data recorded during the last 10 days of each period (d19 – d28) was used for statistical analysis.

The cows had free access to the GF system, except for a requirement of minimum 5 h in between each visit. The cows were motivated to visit the GF system by receiving 8 drops of 50 g concentrate every 40 s during the visit. A mass pellet drop test was performed on the GF unit (50.5 ± 1.81; 10), to ensure correct amounts of concentrate in each drop. The concentrate used as bait was a

Fig. 1. Fermentation unit. The T-tube was used for liquid sampling (volatile fatty acid determination); the red rubber suba seal was used for gas sampling with a gas tight syringe. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.).

commercial concentrate (Komplett Norm 180, Lantmännen Lantbruk AB). Intake of the GF concentrate was taken into account in the calculations of total dry matter intake (DMI).

Calibrations with span gas, which is a mixture of CO_2, CH_4, and O_2, and zero gas (N_2) were performed once a week and CO_2 recovery tests (102.1 ± 3.35; 5) were carried out once before the start of the experiment and then once a month throughout the experiment. Air flow was monitored every day and the air filter was changed when the air flow dropped below 30 L/s.

2.3. *In vitro* incubations

Grass silage, experimental concentrates, and GF concentrate were milled to pass through a 1.0 mm sieve (Retsch SM2000; Rheinische, Haan, Germany) and a total of 1 g feed sample (here refers to diet) was weighed into serum bottles (250 mL; Schott, Mainz, Germany). The serum bottles are hereafter referred to as fermentation units. Since the maximum allowed intake of GF concentrate was 5% of diet DM in the *in vivo* experiment, the *in vitro* diets were formulated as 0.570 g of grass silage, 0.380 g of experimental concentrate, and 0.05 g of GF concentrate.

The RI was collected before morning feeding at 08:00 by stomach tubing (RUMINATOR, Profs Products, Wittibreut, Germany) as described by Geishauser (1993), and filtered through two layers of cheesecloth into two pre-warmed steel thermoses that had previously been flushed with CO_2. The RI was transported to the laboratory within 15 minutes. Each RI was filtered through another four layers of cheesecloth into a measuring cylinder from which 483 mL of rumen fluid was transferred through a funnel into a cylinder containing 483 mL of buffer solution. The buffer solution containing peptone (pancreatic digested casein; Merck, Darmstadt, Germany), and micro- and macro minerals was prepared according to Menke (1988), bubbled with CO_2, and kept submerged in a water bath at 39°C. All cylinders were continuously flushed with CO_2 before and during the handling of RI to ensure an anaerobic environment. To start the incubation, a total of 60 mL of buffered RI was pipetted into each fermentation unit (30 in total) previously flushed with CO_2. The fermentation units were submerged into a water bath at 39°C with continuous agitation.

Each fermentation unit was equipped with two pressure tubes connected to gas recording boxes. For sampling and measurements of CH_4, a metal three-way valve was connected to one of the pressure tubes and a rubber suba seal septa (Z124567–100EA, 13, Sigma–Aldrich) was attached to the third port (Fig. 1). To sample the liquid phase, a plastic tube was inserted into the second pressure tube, forming a T-tube with a valve designed for liquid phase sampling (Fig. 1).

2.4. *In vitro* methane measurements

To measure CH_4 concentrations, gas samples were collected at 3, 6, 18, 30, 42, and 48 h of incubation. Gas samples were drawn from the fermentation units through the rubber suba seal septa (Z124567–100EA, 13, Sigma–Aldrich) with a gastight syringe (Hamilton, Bonaduz, Switzerland). Following each sampling event, the rubber suba seal septa was sealed with Blu Tack (Bostik, Leicester, UK) to ensure an airtight system. A sample size of 0.2 mL gas was injected into a gas chromatograph (Varian Star 3400 CX FID Gas Chromatograph; Varian Inc., Palo Alto, CA), equipped with a thermal conductivity detector. Separations were performed using a stainless steel column with a length of 1.8 m and packed with Haysept T (80–100 mesh). Argon was used as the carrier gas with a flow rate of 32 mL/min and the isothermal oven temperature was set to 32°C. The injector and detector temperatures were set to 110°C and 135°C, respectively. Gas sample peaks were recognized by comparison to a calibration gas (AGA Gas AB, Sundbyberg, Sweden) which was a standard mixture of CO_2 (900 mmol/mol) and CH_4 (100 mmol/mol).

2.5. *In vitro* digestibility and VFA production

At 48 h of incubation, fermentation units were sampled for determination of volatile fatty acid (VFA) concentrations. A sample size of 0.5 mL fluid was drawn from each fermentation unit by a liquid syringe and samples were pooled within treatment and run. Fluid residue samples were transferred to Eppendorf tubes kept on ice and stored at −18°C until VFA analysis. Concentrations of VFA were determined by liquid chromatographic analysis using a Waters Acquity ultra-performance liquid chromatography apparatus (Waters, Milford, MA) with a detection wavelength of 269 nm. In short, a gradient program was used for the analysis with starting conditions of 75% eluent A (0.1% formic acid in water) and 25% eluent B (0.1% formic acid in acetonitrile) from initiation to 1 min. Separations were performed using a Waters UPLC BEH C18 reverse phase column (2.1 × 100 mm, 1.7 μm) at 45°C, at a flow rate of 0.4 mL/min from initiation to 1 min. More details of the applied method are described in Puhakka et al. (2016). The pH value at 48 h of incubation was measured in each fermentation unit after VFA sampling (744 pH Meter, Metrohm Ltd., Herisau, Switzerland).

To determine *in vitro* true dry matter digestibility (TDMD) and *in vitro* true organic matter digestibility (TOMD), feed sample residues from each bottle were transferred to nylon bags with a pore size of 11 μm, as described by Rodrigues et al. (2018). Residues were transferred through a funnel in two stages to avoid loss of particles. Firstly, feed sample residues together with liquid residue were transferred and excess liquid squeezed out through the pores of the bag. Secondly, bottles were rinsed with a small amount of distilled water and emptied through the funnel into the nylon bags. The nylon bags were tightly sealed with straps and boiled in neutral detergent solution for 1 h with addition of heat-stable α-amylase and sodium sulfite to wash away any microbial material attached to feed residues. The bags were rinsed and boiled in water for 10 minutes, dried in an oven at 60°C for 48 h, and weighed to determine TDMD. Residues were further transferred to crucibles (excluding bags) and incinerated at 500°C for 4 h to determine TOMD.

2.6. Chemical analyses

Silage, experimental concentrates, and GF concentrate were analyzed for concentrations of DM, ash, crude protein (CP), amylase neutral detergent fiber organic matter (aNDFom), and indigestible neutral detergent fiber (iNDF). Silage samples were also analyzed for crude fat, and concentrate samples for crude fat and starch concentration. Dry matter concentration was analyzed by drying the feed samples at 105°C for 16 h. Ash concentration was analyzed by incinerating the samples at 500°C for 4 h and OM concentration was calculated as 1000 - ash concentration. Crude protein concentration was analyzed by multiplying N concentration by 6.25, and the N concentration was analyzed according to the Kjeldahl method with a 2020 Digestor and Kjeltec 2460 Analyzer Unit (Foss Analytical A/S, Hillerød, Denmark). Concentration of aNDFom was analyzed by addition of heat stable α-amylase and sodium sulfite (Mertens, 2002) in an Ankom200 Fiber Analyzer (Ankom Technology Corp., Macedon, NY) and expressed free of residual ash. Indigestible NDF concentration was analyzed according to Huhtanen et al. (1994). A sample size of 2 g was weighed into nylon bags with a pore size of 11 μm and incubated for 288 h in triplicates in the rumen of 3 cannulated cows fed a grass silage-based diet (60:40 forage-to-concentrate ratio). The iNDF was expressed free of residual ash. Concentrations of crude fat and starch were analyzed at the Dairy One Forage Laboratory (Ithaca, NY). Crude fat concentration was analyzed by ether extraction and HCl-hydrolysis according to AOAC method 954.02 (AOAC International, 2000), and starch concentration was analyzed with an YSI Analyzer (YSI 2950D-1 Biochemistry Analysers).

2.7. Calculations

Predicted *in vivo* CH_4 production was estimated based on the data obtained from the *in vitro* gas production experiment as described by Ramin and Huhtanen (2012). Cumulative CH_4 production (mL) at each time point (0.2 h) was calculated as follows:

V_{CH4} (mL) = V_{HS} (mL) × CH_4 (mL/mL) + V_{GP} (mL) × A × CH_4 (mL/mL),

where V_{CH4} is the total CH_4 production at each time point; V_{HS} is the headspace volume; CH_4 is the CH_4 concentration in the headspace; V_{GP} is the gas production volume; and coefficient A is the ratio of outflow gas CH_4 concentration to headspace. Coefficient A (0.55) was predicted by applying a mechanistic model outlined by Ramin and Huhtanen (2012). Methane concentration at 0.2-h time intervals was estimated by fitting a logarithmic regression of the measured CH_4 at 6 time points. The data for total gas and CH_4 at 0.2-h time intervals were fitted to the 2-pool Gompertz model, outlined by Schofield et al. (1994), in order to predict kinetic parameters of total gas and CH_4 production at each time point (0.2). The modeling was performed using the NLIN procedure in SAS version 9.4 (SAS Institute Inc., Cary, NC) according to the following equation:

$$V_t = V_1 \times \text{Exp}\{-\text{Exp}[1 - k_1 \times (t - L_1)]\} + V_2 \times \text{Exp}\{-\text{Exp}[1 - k_2 \times (t - L_2)]\},$$

where Vt is measured total gas or CH_4 volume at time t; V_1, k_1, and L_1 are asymptotic cumulative gas production (mL/g of DM), rate (1/h), and lag (h) parameters, respectively, for the first pool (rapid); V_2, k_2, and L_2 are the corresponding parameters for the second pool (slow); and t is incubation time.

To estimate the proportion of CH_4 production reaching asymptotic levels during infinitive residence time of feed in the rumen, the kinetic parameters were subjected to a dynamic, mechanistic 2-compartment rumen model outlined by Huhtanen et al. (2008), with adjustments as specified by Ramin and Huhtanen (2012). Two mean retention times (MRT), 35 and 50 h in the rumen, were assumed for the simulations. A 50 h MRT corresponds to dairy cows at the maintenance level of feed intake and 35 h MRT corresponds to dairy cows fed approximately 20 kg DM per day (Krizsan et al., 2010). Predicted *in vivo* CH_4 production (mL/g of DM) was calculated as CH_4 = proportion of asymptotic CH_4 production × asymptotic CH_4 production (mL/g of DM).

Total VFA concentration (mmol/L) was calculated as a difference between VFA concentration in the sample and mean VFA concentration in blank samples. Total VFA production (mmol/g DM) was calculated as:

Total VFA concentration × 0.06 / incubated DM,

where 0.06 is the volume of the buffered rumen fluid in the serum bottles in L and incubated DM is the amount of DM incubated in the serum bottles (g).

2.8. Statistical analysis

Data were analyzed as a 2 ×4 factorial design with RI, diet, run, and interaction between RI and diet as factors using the MIXED procedure of SAS version 9.4 (SAS Inc., 1985). Data were analyzed according to the following statistical model:

$$Y_{ijk} = \mu + I_j + D_i + R_k + I_j \times D_i + \varepsilon_{ijk}$$

where Y_{ijk} = observation, μ = population mean, I_j = RI effect (j = 2), D_i = diet effect (i = 4), R_k = run effect (k = 4), $I_j \times D_i$ = interaction effect between RI and diet, and ε_{ijk} = residual error. To compare treatment effects, 3 orthogonal contrasts were specified. To compare the effects of barley and oats, the barley diet was compared with the overall mean of the hulled oat, oat mixture, and dehulled oat diets. To investigate the effects of gradual replacement of hulled oats with dehulled oats, linear and quadratic contrasts were specified. Differences were declared significant if $P \leq 0.05$, highly significant if $P \leq 0.01$, and a tendency toward significant was declared if $0.05 < P \leq 0.10$.

The relationship between predicted *in vivo* CH_4 production and observed *in vivo* CH_4 production was examined by using the GLM procedure of SAS according to the following statistical model:

$$Y_i = B_0 + B_1X_{1i} + \varepsilon_i$$

where Y_i is observed *in vivo* CH_4 production (g/kg DMI), B_0 is the intercept, B_1 is the slope for X_1, X_{1i} is the predicted *in vivo* CH_4 production (g/kg DM incubated), and ε_i is the residual error. Residual analysis for CH_4 production was conducted following the methodology outlined by St-Pierre (2003). The procedure involved regressing the centered predicted values against the residuals (observed − predicted) for both 35 and 50 h MRT. To center the predicted values, each predicted value was subtracted from the mean of all predicted values. This approach ensures that the slope and intercept estimates are orthogonal, allowing for independent assessment. In addition, predicted *in vivo* CH_4 production and observed *in vivo* CH_4 production were compared by computing Lin's Concordance Correlation Coefficient (Lin's CCC) for MRT of both 35 and 50 h.

3. Results

3.1. Chemical composition of ingredients and experimental diets incubated in vitro

The dietary concentration of aNDFom varied between 333 and 375 g/kg DM, and was numerically highest in the hulled oat diet and lowest in the dehulled oat diet (Table 1). Starch concentration varied between 146 g/kg DM in the hulled oats diet and 169 g/kg DM in the barley diet. The dietary concentrations of crude fat and CP varied between 36 and 49 g/kg DM, and 165 and 179 g/kg DM, respectively.

3.2. Predicted in vivo gas and CH_4 production

There were no interaction effects of RI and diet on any of the investigated parameters ($P = 0.62$). Total predicted *in vivo* gas production tended to be higher ($P = 0.09$) from the oat diets than from the barley diet, but was not affected ($P = 0.21$) by replacing hulled oats with dehulled oats (Table 2). Asymptotic CH_4 was lower ($P = 0.03$) from the oat diets than from the barley diet, and predicted *in vivo* CH_4 production tended to be lower ($P = 0.06$) from the oat diets than from the barley diet. Both asymptotic CH_4 ($P = 0.08$) and predicted *in vivo* CH_4 production ($P = 0.07$) tended to be higher when diets were incubated in RI from cows fed hulled oats than from cows fed barley.

3.3. Relationship between predicted in vivo CH_4 and observed CH_4 production

Replacing hulled oats with dehulled oats led to a linear increase in observed *in vivo* CH_4 production (g/d, $P = 0.02$) by 3.8% and CH_4 yield (g/kg DMI, $P < 0.01$) by 6.6% (Table 4). Assuming a 35 h MRT in the rumen, predicted *in vivo* CH_4 production increased only numerically ($P = 0.26$) by 3.5%, whereas CH_4 yield increased ($P = 0.05$) by 6.4% when hulled oats was replaced with dehulled oats *in vitro*. Assuming a MRT of 35 h for the predictions of *in vivo* CH_4 resulted in a higher R-square (0.91 vs. 0.56) and a lower root mean square error (0.20 vs. 0.45) (Fig. 2) than assuming a MRT of 50 h (Fig. 3). Using 50 h MRT showed greater mean bias as compared to 35 h MRT ($P < 0.01$ and $P = 0.03$, respectively; Fig. 4). In addition, Lin's CCC was 0.68 and 0.03 for MRT of 35 h and MRT of 50 h, respectively.

3.4. Digestibility and fermentation pattern in vitro

There were no interaction effects of RI and diet on any of the investigated parameters. *In vitro* TDMD and TOMD were not affected

Table 1
Chemical composition of dietary ingredients and experimental diets incubated *in vitro*.

Item	DM (g/kg)	Ash	CP	aNDFom	iNDF[a]	Starch[b]	Crude fat[c]
		g/kg of DM					
Ingredient							
Grass silage	259	65	160	489	90	NA	38
Barley	870	66	168	182	64	410	29
Hulled oats	873	67	174	227	123	350	50
Dehulled oats	886	66	204	116	35	376	64
GF concentrate	878	71	206	205	74	262	64
Diet[d]							
B	522	66	165	358	79	169	36
O	523	66	168	375	102	146	44
ODO	526	66	173	354	85	151	47
DO	528	66	179	333	68	156	49

[a] iNDF = indigestible aNDFom.
[b] Starch concentration in grass silage assumed to be 0 g/kg DM (LUKE, 2023), starch in GF concentrate according to manufacturer (Lantmännen, Sweden).
[c] Crude fat concentration in GF concentrate according to manufacturer (Lantmännen, Sweden).
[d] B = barley, O = hulled oats, ODO = mix of hulled and dehulled oats 50:50 on DM basis, DO = dehulled oats.

Table 2
Effects of rumen inoculum and diet on predicted *in vivo* total gas and methane emissions.

	B Inoculum[a]				O Inoculum				SEM	P-value[b]			
Item	B	O	ODO	DO	B	O	ODO	DO		I	B vs. Oats	Lin Oats	Quad Oats
Total gas, mL/g DM													
Asymptotic gas	321	299	316	319	333	317	311	319	5.4	0.38	0.12	0.30	0.99
Predicted gas[c]	314	296	312	315	333	315	313	318	4.3	0.11	0.09	0.21	0.88
CH_4													
Asymptotic CH_4, mL/g DM	40.4	38.1	38.9	40.2	42.7	39.8	40.0	40.5	1.56	0.08	0.03	0.20	0.85
Rate, 1/h	0.053	0.055	0.056	0.055	0.054	0.056	0.056	0.056	0.0016	0.47	0.10	0.94	0.87
Predicted *in vivo* CH_4[c], mL/g DM	34.9	33.2	34.1	35.0	37.2	34.9	34.8	35.4	1.41	0.07	0.06	0.23	0.86
CH_4/Total gas[d]	0.12	0.11	0.11	0.11	0.12	0.11	0.11	0.11	0.0062	0.64	0.22	0.97	0.54

[a] B = barley, O = hulled oats, ODO = mix of hulled and dehulled oats 50:50 on DM basis, DO = dehulled oats.
[b] I = probability of significant effect of rumen inoculum. Effects tested with orthogonal contrasts for diet were: B vs. Oats = B vs. O, ODO, and DO; Lin Oats = linear effect of replacement O with DO; Quad Oats = quadratic effect of replacement of O with DO.
[c] Predicted *in vivo* total gas and CH_4 production values assuming a mean retention time of 50 h in the rumen.
[d] Ratio of predicted *in vivo* CH_4 (mL/g DM) to predicted total gas production (mL/g DM).

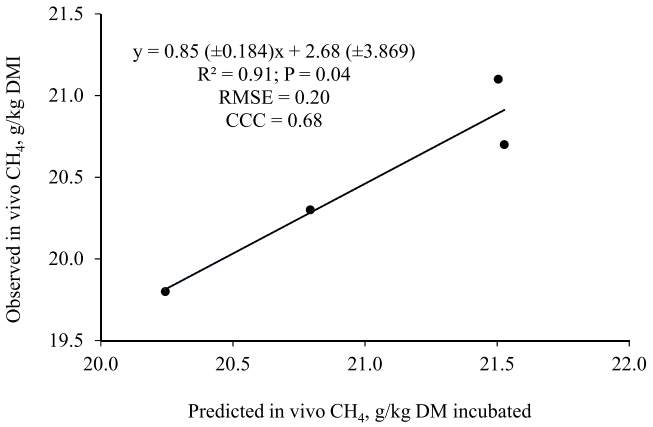

Fig. 2. Relationship between predicted in vivo CH_4 production (g/kg dry matter incubated) and observed in vivo CH_4 production (g/kg dry matter intake) with barley, hulled oats, a mix of hulled and dehulled oats, and dehulled oats as the grain supplement on a grass-silage based diet (each filled dot represents one diet). Predicted in vivo CH_4 was estimated according to Ramin and Huhtanen (2012) assuming a 35 h mean retention time in the rumen. CCC = Lin's Concordance Correlation Coefficient.

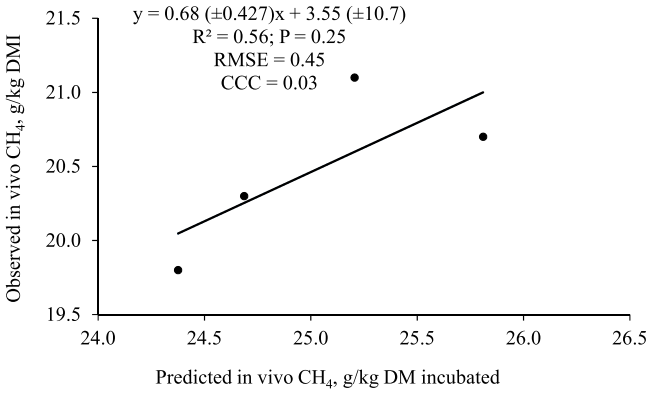

Fig. 3. Relationship between predicted in vivo CH_4 production (g/kg dry matter incubated) and observed in vivo CH_4 production (g/kg dry matter intake) with barley, hulled oats, a mix of hulled and dehulled oats, and dehulled oats as the grain supplement on a grass-silage based diet (each filled dot represents one diet). Predicted in vivo CH_4 was estimated according to Ramin and Huhtanen (2012) assuming a 50 h mean retention time in the rumen. CCC = Lin's Concordance Correlation Coefficient.

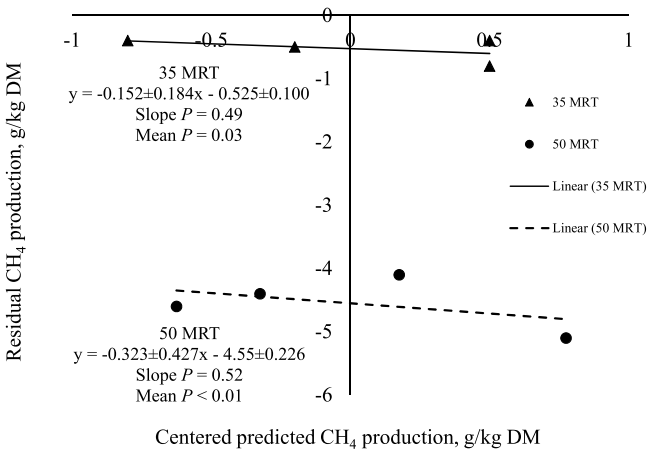

Fig. 4. Relationship between centered predicted *in vivo* CH_4 production and residuals of CH_4 production (observed − predicted) assuming mean retention times (MRT) of both 35 and 50 h in the rumen.

Table 3
Effects of rumen inoculum and diet on true *in vitro* digestibility, pH at 48 h of incubation and fermentation pattern.

	B Inoculum[1]				O Inoculum				SEM	P-value[2]			
Item	B	O	ODO	DO	B	O	ODO	DO		I	B vs Oats	Lin Oats	Quad Oats
TDMD[3]	0.879	0.856	0.876	0.894	0.877	0.853	0.878	0.893	0.0032	0.53	0.11	<0.01	0.07
TOMD[4]	0.908	0.884	0.903	0.921	0.903	0.884	0.905	0.916	0.0021	0.28	0.08	<0.01	0.25
pH 48 h of incubation	6.27	6.33	6.29	6.27	6.23	6.32	6.28	6.25	0.019	0.03	<0.01	<0.01	0.41
Total VFA production, mmol/g DM	7.61	7.40	7.15	7.03	6.97	6.88	7.41	7.36	0.116	0.40	0.65	0.81	0.58
VFA molar porportions, mmol/mol													
Acetate	556	569	563	571	566	574	565	577	6.4	0.23	0.32	0.94	0.62
Propionate	285	282	288	287	277	275	277	272	10.4	0.24	0.91	0.95	0.71
Butyrate	111	101	104	103	109	102	105	103	2.7	0.96	<0.01	0.56	0.35
Isobutyrate	6.41	6.88	6.25	6.52	6.39	6.86	6.71	6.95	0.661	0.59	0.53	0.81	0.52
Valerate	17.6	16.5	16.1	16.2	17.5	17.5	17.5	17.6	0.72	0.01	0.13	0.81	0.80
Isovalerate	2.71	3.37	2.33	2.44	2.45	3.14	4.24	4.79	0.957	0.24	0.38	0.74	0.88

[1] B = barley, O = hulled oats, ODO = mix of hulled and dehulled oats 50:50 on DM basis, DO = dehulled oats.
[2] I = probability of significant effect of rumen inoculum. Effects tested with orthogonal contrasts for diet were: B vs. Oats = B vs. O, ODO, and DO; Lin Oats = linear effect of replacement O with DO; Quad Oats = quadratic effect of replacement of O with DO.
[3] TDMD = true dry matter digestibility.
[4] TOMD = true organic matter digestibility.

by type of RI ($P = 0.28$), but increased linearly ($P < 0.01$) when hulled oats was replaced with dehulled oats (Table 3). The pH was higher ($P < 0.01$) in the oat diets than in the barley diet and decreased linearly ($P < 0.01$) when hulled oats was replaced by dehulled oats. In addition, the pH at 48 h of incubation was slightly lower ($P < 0.01$) when diets were incubated in RI from cows fed hulled oats than from cows fed barley. Total VFA production (mmol/g DM) was not affected by diet or RI ($P = 0.40$). Molar proportions of VFA were not affected by diet, except for butyrate that was lower ($P < 0.01$) in the oat diets than in the barley diet. Rumen inoculum did not affect molar proportions of VFA (mmol/mol), except for valerate that was higher ($P = 0.01$) in the RI from cows fed hulled oats than from cows fed barley.

4. Discussion

The first objective of this study was to compare predicted *in vivo* CH_4 values derived from an *in vitro* gas production experiment with observed values measured by the GF system. This comparison was conducted concurrently with an *in vivo* experiment that ran simultaneously with the *in vitro* experiment. The comparison was made assuming ruminal MRTs of both 50 h (standardized) and 35 h for the predictions of *in vivo* CH_4 production. We obtained low RMSE values (0.20 and 0.45) for both 35 and 50 h MRTs, respectively, indicating a good agreement between predicted and observed values. However, the study compared predicted *in vivo* CH_4 production values using only four diets, because of a limited amount of dietary treatments that could be included in the *in vivo* experiment. Due to the low number of observations (four), the relationship between predicted *in vivo* CH_4 when assuming an MRT of 50 h and observed CH_4 values was not significant.

The second objective was to evaluate the effects of the diet of RI donor animal on predicted *in vivo* CH_4 production with barley and three different types of oats as substrates together with grass silage. Since the different substrates tested in this *in vitro* experiment have already been tested in the *in vivo* experiment, the discussion will focus firstly; on the comparison between the results obtained by the *in vitro* and the *in vivo* experiment, and secondly; on the effects of diet of RI donor animal on *in vitro* results.

4.1. Relationship between predicted in vivo CH_4 and observed CH_4

To our knowledge, this is the first study to compare predicted *in vivo* CH_4 production obtained from an *in vitro* gas production experiment and estimated according to the method of Ramin and Huhtanen (2012) with observed *in vivo* CH_4 production from a feeding trial conducted simultaneously with the *in vitro* gas production experiment. The *in vitro* system used in this study has demonstrated reasonably accurate predictions of *in vivo* CH_4 production, especially when the proportion of concentrate falls within the typical range of Nordic diets (~40% on DM basis) (Danielsson et al., 2017). A rather high R-square value and a low root mean square error were obtained when we compared predicted and observed CH_4 production. This outcome is consistent with what Danielsson et al. (2017) found when they formulated 49 different diets and compared predicted *in vivo* CH_4 production values with actual *in vivo* measurements using the respiration chamber technique. In this study, the prediction errors for an MRT of 35 h and an MRT of 50 h were only 1.0 and 2.2%, respectively, of the observed mean.

In this study, assuming an MRT of 35 h led to lower predicted *in vivo* CH_4 production than assuming an MRT of 50 h in the rumen. These results are expected since both Pinares-Patiño et al. (2003) and Goopy et al. (2013) showed that shorter MRT of feed in the rumen of sheep are associated with lower CH_4 production. Similar results were reported when the mechanistic Nordic dairy cow model was used to simulate the effects of different MRTs on CH_4 production from both sheep and dairy cows (Huhtanen et al., 2016). For a

Table 4
Predicted *in vivo* methane and observed *in vivo* methane production (using GreenFeed) from cows fed barley and different types of oats on a grass silage-based diet.

	Diet[1]					P-value[2]		
Item	B	O	ODO	DO	SEM	B vs Oats	Lin Oats	Quad Oats
Predicted *in vivo* methane production[3]								
MRT35								
CH_4, g/day	502	482	486	499	21.3	0.29	0.26	0.76
CH_4, g/kg DM incubated	21.5	20.2	20.8	21.5	0.91	0.18	0.05	0.88
CH_4, g/kg OM digested	23.6	22.8	22.5	23.5	1.17	0.69	0.69	0.44
MRT50								
CH_4, g/day	601	580	578	585	25.0	0.12	0.78	0.73
CH_4, g/kg DM incubated	25.8	24.4	24.7	25.2	1.07	0.06	0.23	0.86
CH_4, g/kg OM digested	27.7	27.5	27.1	27.8	1.16	0.69	0.69	0.44
Observed *in vivo* methane production[4]								
CH_4, g/day	479	470	474	488	14.1	0.81	0.02	0.49
CH_4, g/kg DMI	20.7	19.8	20.3	21.1	0.67	0.27	<0.01	0.60
CH_4, g/kg of OM digested	30.4	29.9	29.9	29.2	1.50	0.26	0.37	0.63

[1] B = barley, O = hulled oats, ODO = mix of hulled and dehulled oats 50:50 on DM basis, DO = dehulled oats.
[2] Effects tested with orthogonal contrasts for diet were: B vs. Oats = B vs O, ODO, and DO; Lin Oats = linear effect of replacement of O with DO; Quad Oats = quadratic effect of replacement of O with DO.
[3] MRT35, MRT50 = predicted *in vivo* CH_4 with mean retention time in rumen set to 35 h or 50 h, respectively.
[4] Data from Fant et al. (2021), here referred to as Experiment 1.

dairy cow, CH_4 production increased by 0.37 g/kg DM intake per 1 h increase in ruminal MRT. Extending the MRT from 35 h to 50 h corresponds to an increase of 5.55 g CH_4/kg DM, which is close to the observed average increase of 4.03 g CH_4/kg DM in our study.

Assuming an MRT of 35 h for the dynamic modeling of predicted *in vivo* CH_4 production resulted in a lower RMSE value than assuming a MRT of 50 h in the rumen (0.20 vs. 0.45). Furthermore, Lin's CCC of 0.68 when assuming an MRT of 35 h indicates a good agreement between predicted and observed CH_4, whereas the corresponding value (0.03) for assuming an MRT of 50 h indicates a poor agreement (Beck et al., 2023).The method for predicting *in vivo* CH_4 production from *in vitro* data is standardized to feed intake levels at maintenance, and thus a fixed MRT of 50 h is used in the modeling process (Ramin and Huhtanen, 2012). However, this study aimed to utilize both a 50 h and a 35 h MRT, since the latter better corresponds to the intake levels (20 kg DM/d) of lactating dairy cows (Krizsan et al., 2010) and since the average DMI in the simultaneously conducted *in vivo* experiment was 23 kg/d (Fant et al., 2021). With an assumed MRT of 35 h, predicted *in vivo* CH_4 production increased by 6.2% when hulled oats was replaced by dehulled oats (6.6% observed *in vivo*), whereas the corresponding increase with an assumed MRT of 50 h was only 3.4%. The smaller increase of 3.4% when a MRT of 50 h was used in the modeling process resulted in a steeper slope in the regression between observed and predicted CH_4 values. It is well established that a greater retention time in the rumen decreases feed intake and provides more time for feed digestion, especially for digestion of fiber fractions. Since CH_4 production is positively correlated to diet digestibility (Blaxter and Clapperton, 1965), simulating an extended time for digestion of the fibrous oat hulls in the hulled oat diet and the oat mixture diet decreases the difference in CH_4 production observed when simulating shorter time for digestion. Regardless of the assumed ruminal MRT used for modeling predicted *in vivo* CH_4 production, the ranking of diets (barley versus oats, and replacement of hulled oats with dehulled oats) based on their CH_4 production potential remained consistent with the ranking observed in the *in vivo* experiment. This suggests that the method is effective for evaluating a large number of diets to identify the most promising candidates before proceeding to *in vivo* experiments.

4.2. Effects of different substrates on CH_4 production in vitro and in vivo

Predicted *in vivo* CH_4 yield from the barley diet was numerically higher (3.2 and 4.1% for MRT of 35 and MRT of 50 h, respectively) than from the oat diets, whereas the numerical difference between observed *in vivo* CH_4 yields from cows fed the barley and from cows fed the oat diets was only 1.4%. Greater differences in CH_4 production between diets incubated *in vitro* than differences observed *in vivo* have also been reported by other studies, although these studies did not compare predicted *in vivo* CH_4 production from an *in vitro* system with *in vivo* CH_4 production. Martínez-Fernández et al. (2013) compared *in vitro* CH_4 production with *in vivo* CH_4 production in goats when supplementing the diet with various plant compounds. Dietary supplementation of propyl propane thiosulfinate and bromochloromethane decreased *in vitro* CH_4 production by 87 and 96%, respectively, whereas observed *in vivo* CH_4 production decreased by only 33 and 64%, respectively. Hatew et al. (2015) reported similar CH_4 reductions *in vitro* as *in vivo* between diets with a low content of slowly degradable starch and diets with a high content of slowly degradable starch. However, they reported a 27% lower *in vitro* CH_4 production from a diet with high content of rapidly degradable starch than from a diet with low content of slowly degradable starch, whereas the corresponding reduction *in vivo* was only 14%.

Although around 95% of the starch in oats is rapidly degradable and the corresponding part for barley is only 43% (Pan et al., 2021), this difference does not provide a likely explanation for the greater difference in predicted *in vivo* CH_4 production between barley and oats *in vitro* than *in vivo* in this study. In the previous *in vitro* study by Fant et al. (2020), predicted *in vivo* CH_4 production (g/kg DM incubated) was on average 8.9% lower from 8 different oat varieties than from 8 different barley varieties incubated in RI from 2 dairy cows. However, in the *in vivo* study by Ramin et al. (2021), CH_4 production expressed as g/kg DMI decreased by only 4.4% when barley was gradually replaced by oats in the diet of dairy cows using the GF system. Since the CH_4 mitigating effect of oats compared with barley is mostly due to lower digestibility and higher crude fat content of oats (Fant et al., 2020; Ramin et al., 2021), it is likely that the addition of dietary crude fat has a greater effect *in vitro* than *in vivo* as there is no outflow of fatty acids from the fermentation chamber *in vitro*.

4.3. Effects of rumen inoculum on in vitro results

The lack of an interaction effect between diet and RI on predicted *in vivo* CH_4 production suggests that the observed CH_4 reducing effect of hulled oats compared to barley, as reported in prior research (Fant et al., 2021; Ramin et al., 2021), is not influenced by alterations in the rumen microbiome. While this study did not directly contrast barley with hulled oats, the predicted *in vivo* CH_4 production from the hulled oat diet was numerically higher (2.0 mL/g DM) compared with the barley diet.

In our study, we merely observed a tendency for slightly higher (3.7%) predicted *in vivo* CH_4 production when diets were incubated in RI from cows fed hulled oats compared with barley. A study by Martínez et al. (2010) demonstrated greater *in vitro* CH_4 production when substrates were incubated in RI from sheep fed a high forage-to-concentrate ratio (70:30) compared with RI from sheep fed a low forage-to-concentrate ratio (30:70). In addition, the same study reported greater CH_4 production with RI from sheep fed alfalfa hay compared to those fed grass hay. In a separate study by Hatew et al. (2015), a rise in dietary starch levels for RI donor cows led to decreased CH_4 production for the incubated substrates. Notably, the difference in dietary starch concentration in the study by Hatew et al. (2015) was 97 g/kg DM, while in our study, it was merely 23 g/kg DM lower in the hulled oat diet than in the barley diet. The slightly higher numerical CH_4 production observed in the hulled oat diet could potentially be explained by a slightly greater amount of fibrolytic bacteria in the RI from cows fed hulled oats compared to those fed barley (Demeyer and Fievez, 2000). This could be attributed to the higher proportion (25% of grain weight) of fibrous hull in oat grain compared to barley (13% of grain weight), as reflected in the higher NDF concentration of hulled oats compared with barley (Evers and Millar, 2002). In comparison to altering the

forage-to-concentrate ratio and feeding different forage sources, altering the concentrate between barley and different types of oats in the diet of RI donor animals has only minor effects on CH_4 production *in vitro*.

In this study, RI was obtained via stomach tubing rather than from rumen-cannulated cows, which is typically regarded as the standard method (Komarek, 1981). The utilization of rumen-cannulated cows in our research was restricted due to the potential for gas leakage through the rumen cannula during CH_4 measurements via the GreenFeed (GF) system, thus resulting in inaccurate gas data (Hristov et al., 2015). The method of rumen fluid sampling has been shown to influence the pH and volatile fatty acid (VFA) concentrations in ruminal inoculum (RI) (Geishauser and Gitzel, 1996). However, such variations can be mitigated by ensuring the stomach tube is inserted to an optimal depth (Shen et al., 2012). Stomach tubing may introduce salivary contamination of RI, which can have an impact on the accuracy and reliability of gas measurements (Groot et al., 1996). Nonetheless, its influence on the relative ranking of diets concerning CH_4 production is presumed to be minimal.

4.4. Digestibility and fermentation pattern in vitro

The linear increase in digestibility when hulled oats was replaced by dehulled oats is in agreement with the results of the simultaneously conducted *in vivo* study (Fant et al., 2021), although the increase in this *in vitro* study was smaller (3.9% for TOMD) than in the *in vivo* study (8.6% for apparent OM digestibility). The ranking of the four different diets in terms of digestibility was also similar to the ranking *in vivo*, with the dehulled oat diet showing the highest numerical digestibility followed by the barley diet, the oat mixture diet, and the hulled oat diet. Similarly, Mustafa et al. (1998) reported higher effective degradability of naked oats than of barley, and lower effective degradability of hulled oats than of barley.

In contrast to the results observed *in vivo* in the study by Fant et al. (2021), the molar proportion of butyrate was slightly lower in the fluid residues from the incubated oat diets than from the barley diet. However, an *in vivo* study by Vanhatalo et al. (2006), where barley and hulled oats were compared in a grass silage-based diet with similar forage to concentrate ratio as in this study, found a slightly lower proportion of butyrate in rumen fluid from cows receiving oats. Moreover, Vanhatalo et al. (2006) found a lower proportion of valerate in rumen fluid from cows receiving oats. A previous *in vitro* study by Fant et al. (2020) comparing 8 different barley varieties with 8 different oat varieties in a grass silage-based diet found a lower proportion of valerate in the fluid residues from the incubated oat diets than from the barley diets. In this study, however, valerate proportions in fluid residues were only numerically lower for the oat diets than for the barley diet. Studies where the effects of different types of oats on ruminal fermentation pattern are lacking. However, based on the results of this study and previous studies, the effects of barley and oats, and different types of oats, on the ruminal fermentation pattern are small.

5. Conclusions

In conclusion, when assuming an MRT of 35 h in the rumen, predicted *in vivo* CH_4 production values agreed well with the observed CH_4 production values measured by the GF system, despite a low number of observations. Due to the low number of observations ($n = 4$), assuming an MRT of 50 h in the modeling process resulted in a non-significant relationship between predicted and observed values. For future comparisons it is advisable to include a greater number of dietary treatments to be tested. However, ranking of the diets in terms of their CH_4 production was consistent between the *in vitro* and *in vivo* experiment, indicating that the *in vitro* method is well suited for effectively screening the CH_4 mitigating potential of many different diets. In addition, the diet comparison in terms of predicted *in vivo* CH_4 production was not affected by the diet of the RI donor animals when barley and different types of oats were incubated with grass silage *in vitro*. These results indicate that the CH_4 mitigating effect of hulled oats compared with barley is not associated with changes in the microbial community. Finally, the diet of the RI donor animal had only minor effects on the results of the *in vitro* experiment.

Declaration of Competing Interest

We declare that there is no conflict of interest.

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