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In dairy cattle, the stomach tubing method is not a feasible alternative to the rumen cannulation method to examine *in vitro* gas and methane production

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Declarations of interest: none

Abbreviations: A, the asymptotic methane production; A1, A2, A3, the asymptotic gas production of the soluble fraction, insoluble fraction, and the microbial turnover, respectively; B, the switching characteristic of the methane production profile curve; B1, B2, B3, the switching characteristic of the profile curve of the soluble fraction, insoluble fraction, and the microbial turnover, respectively; C, the halftime to reach the maximum methane production; C1, C2, C2, the half time to reach the maximum asymptotic gas production of the soluble fraction, insoluble fraction, and the microbial turnover, respectively; CAN, rumen cannulation method; CH_4, methane; CON, control diet; DM, dry matter; GC, gas chromatography; GP, gas production; GP% CH_4, the methane production relative to total gas production; LSO, linseed oil diet; NGR, non-glucogenic volatile fatty acid to glucogenic volatile fatty acid ratio; OM, organic matter; OST, oral stomach tube method; R_{max}, the maximum methane production rate; R_{max2}, maximum gas production rate of the insoluble fraction; TMR, total mixed ration; VFA, volatile fatty acid.

ABSTRACT

The objectives were to compare *in vitro* gas and methane (**CH_4**) production kinetics using rumen fluid as inoculum collected via the rumen cannulation (**CAN**) method and with the oral stomach tube (**OST**) method, and to test for interactions between the type of inoculum and the diet fed to the donor cows as well as with the substrate incubated. Four rumen-cannulated lactating Holstein-Friesian cows (364 ± 20 days in milk, 690 ± 36.5 kg of bodyweight, 22.0 ± 6.0 kg of milk/day; mean ± SD) were blocked in pairs balanced over treatment sequence in a 2 × 2 crossover design. Cows were fed a total mixed ration consisting of 40% maize silage, 30% grass silage, and 30% concentrate on dry matter (**DM**) basis, with or without dietary linseed oil (a difference of 23 g/kg DM in fat content between the two diets). Rumen fluid was collected via the CAN and the OST method from each cow at d 17 of each experimental period. Subsequently, a 48 h gas production (**GP**) experiment was performed in which rumen fluid from each donor cow × rumen fluid sampling method was incubated with three substrates, viz. amygold, lupine, and wheat. The pH of the rumen fluid obtained using the OST method was higher than that of the rumen fluid obtained using the CAN method, whereas the total VFA concentration was lower and the VFA molar proportions unaffected. Multiple diet × inoculum and substrate × inoculum interactions in *in vitro* GP and CH_4 kinetic parameters were observed. Furthermore, several GP and CH_4 kinetic parameters, including total GP, absolute, asymptotic, and relative CH_4 production, and maximum CH_4 production rate, were higher for the CAN method compared with the OST method. The opposite was observed for, amongst others, the switching characteristic of the CH_4 production profile and the half time to reach maximum asymptotic GP of the insoluble fraction. In conclusion, rumen fluid obtained using the OST sampling technique differs from that obtained using the CAN method, resulting in differences in *in vitro* gas and CH_4 production profiles between both methods. The multiple interactions indicate that the rumen fluid sampling method can affect the *in vitro* GP and CH_4 kinetics found

for diet and for substrate. The results of this study therefore do not support the OST method as a feasible alternative to the CAN method in dairy cattle to examine *in vitro* gas and CH_4 production.

Keywords: dairy cow, *in vitro* fermentation, oral stomach tube, rumen cannula.

INTRODUCTION

In vitro fermentation techniques have been widely used to evaluate the nutritive value of feeds for ruminants and, in the last decade, to assess the effect of different nutritional strategies on methane (**CH_4**) production (Yáñez-Ruiz et al. 2016). However, many technical factors may influence the results obtained, including the method of collection of rumen fluid as inoculum. Several collection methods are available, with the rumen cannulation method (**CAN**) and the oral stomach tube method (**OST**) being most frequently used (Duffield et al. 2004). The CAN method (i.e., collecting rumen fluid through the fistula from rumen cannulated cows) is considered the reference method for collection of representative samples of rumen digesta and is therefore widely used in ruminant nutrition research (Kristensen et al. 2010). However, access to surgically-modified animals is not universal. Hence, a less invasive alternative has been developed, viz. the OST method. The OST method allows rumen fluid to be obtained through the esophagus.

In the relatively few studies that have compared the CAN and OST method, differences in fermentation profile and microbiota have been reported by some (e.g., Geishauser and Gitzel, 1996; Duffield et al. 2004), but not by others (e.g., Lodge-Ivey et al. 2009; Terré et al. 2013; Henderson et al. 2014; Paz et al. 2016). Part of the discrepancy between studies may reflect differences in the procedures used to avoid salivary dilution and contamination, the type of samples collected, and rumen sampling site (Yáñez-Ruiz et al. 2016). Shen et al. (2012) attributed the differences between CAN and OST samples to rumen sampling site, as a

consequence of the probe in the OST method not being inserted to a depth sufficient to reach the ventral sac of the rumen.

Despite the potential differences in the rumen fluid obtained by the CAN or OST method as inoculum (i.e., pH, volatile fatty acids, microbial composition), to date no research has assessed the effect of the two different rumen fluid sampling methods on *in vitro* gas and CH_4 production. Hence, the objectives of the present study were (i) to compare the extent and the kinetics of *in vitro* gas and CH_4 production upon inoculation using rumen fluid collected via the CAN method with that of rumen fluid collected via the OST method, and (ii) to test for interaction between the rumen fluid sampling method and the diet fed to the donor cows as well as with the substrate incubated.

MATERIALS AND METHODS

Experimental design and rumen fluid collection

The present *in vitro* experiment followed a 2 × 2 × 3 factorial design. Two methods of rumen fluid sampling methods were used, viz. the CAN and the OST method. Additionally, two different diets were fed to the donor cows from which rumen fluid was collected, and three different feed substrates were incubated with the rumen fluid for *in vitro* measurements.

The present study was conducted simultaneously with an *in vivo* experiment previously reported by Van Lingen et al. (2017) where four rumen cannulated multiparous lactating Holstein-Friesian cows were involved for CH_4 measurements in climate-controlled respiration chambers. The experiment was conducted in accordance with Dutch law, and approved by the Animal Care and Use Committee of Wageningen University & Research. In short, the experiment followed a crossover design with two dietary treatments. The dairy cows (364 ± 20 days in milk, 690 ± 36.5 kg of bodyweight, 22.0 ± 6.0 kg of milk/day; mean ± SD) were blocked in pairs according to lactation stage, parity, and milk production. Within each block, cows were

randomly allocated to a dietary treatment sequence in a crossover design with 2 periods and 2 diets: a control diet (**CON**) and linseed oil diet (**LSO**). Cows were fed a total mixed ration (**TMR**) consisting of 40% maize silage, 30% grass silage, and 30% concentrate on dry matter (**DM**) basis, with or without dietary linseed oil (a difference of 23 g/kg DM in fat content between the CON and LSO diet). There were two experimental periods of 17 d each which were composed of a 12-d adaptation period in a tie stall, followed by five d of CH_4 measurements in a climate-controlled respiration chamber. Additionally, there was a 28 d washout period between the two experimental periods to prevent potential carryover effects.

The four cows were previously fitted with a permanent rumen cannula (10 cm i.d., Type 1C; Bar Diamond Inc., Parma, ID, USA) and served as a donor of rumen fluid for the *in vitro* experiment. In two separate runs, with 28 d in between (similar to the length of the washout period) rumen fluid was collected directly after completion of the five d *in vivo* CH_4 measurements (i.e., approximately four h after morning feeding), ensuring that the cows were fully adapted to their respective experimental diets. For the first run, rumen fluid was collected with the OST method immediately followed by collection with the CAN method. The OST consisted of a manual pump, a spiral probe of 190 cm, and a perforated suction head (all H. Hauptner und Richard Herberholz GmbH & Co. KG, Solingen, Germany), which was inserted through the esophagus into the rumen (i.e., the dorsal cranial part of the rumen). Immediately thereafter rumen fluid of the same cow was collected with the CAN method in three equal volumes from the front and middle of the ventral sac and from the caudodorsal region of the rumen using the method described by Van Zijderveld et al. (2011). For the second run, the same methods for rumen fluid sampling were applied, but in the opposite order; first the CAN method directly followed by the OST method. After collecting rumen fluid from individual animals, the rumen fluid was transferred into pre-warmed insulated flasks, previously filled with carbon dioxide and directly transported to the laboratory. The rumen fluid was filtered through a double

layer of cheesecloth to get rid of large rumen fluid particles. During this process the rumen fluid was kept under CO_2 to ensure anaerobic conditions. Prior to inoculation, samples of 600 µL were taken from each insulated flask to determine the volatile fatty acid (**VFA**) concentrations and pH of the rumen fluid as described by Van Gastelen et al. (2017). All other procedures were as described by Cone et al. (1996).

Substrates, *in vitro* gas and methane production

Gas production (**GP**) was determined using a fully automated GP equipment as described in Cone et al. (1996). Three feed ingredients, viz. wheat, lupine, and amygold, were used as substrates. These substrates were selected for their difference in *in situ* fermentation kinetics previously determined by Cone et al. (2008; Table 1) and the subsequently expected differences in *in vitro* GP. The substrates were ground to pass a 1 mm screen using a cross beater mill (Peppink 100 AN, Olst, The Netherlands). The chemical composition of the substrates was determined using the chemical analyses described by Van Gastelen et al. (2017) and are shown in Table 1. The *in situ* fermentation kinetics of organic matter is adapted from Cone et al. (2008). Approximately 0.5 g DM of each substrate was weighed into 250 mL fermentation bottles (Schott, Mainz, Germany). Each experimental treatment (i.e., diet × inocula × substrate) was included in triplicate in fermentation bottles with blanks (i.e., rumen fluid without sample) included in duplicate in each series.

Prior to inoculation, the rumen fluid was filtered through cheese cloth and mixed (1:2 v/v) with an anaerobic buffer/mineral solution containing per litre 8.75 g $NaHCO_3$, 1.00 g NH_4HCO_3, 1.43 g Na_2HPO_4, 1.55 g KH_2PO_4, 0.15 g $MgSO_4.7H_2O$, 0.52 g Na_2S, 0.017 g $CaC1_2.2H_2O$, 0.015 g $MnCl_2.4H_2O$, 0.002 g $CoC1_3.6H_2O$, 0.012 g $FeC1_3.6H_2O$ and 1.25 mg resazurin as described by Cone et al. (1996). Subsequently, the fermentation bottles were pre-flushed with carbon dioxide and placed in a shaking water bath, maintained at 39 °C and 40 movements

per minute. Thereafter, the bottles were inoculated with 60 mL of the filtered and buffered rumen fluid and connected to the fully automated equipment (Cone et al. 1996). Before gas measurements started, the fermentation bottles were fitted with a glass extension and sealed with a screw cap fitted with an air-tight septum (GRACE, XLB-11 Septa 7/16, Breda, The Netherlands), as illustrated by Pellikaan et al. (2011). The screw caps were furnished with a small aperture to allow a fine needle to pass. At distinct incubation times (i.e., 0, 3, 8, 19, 24, 28, 42, and 48 h of incubation), 10 µL aliquots of the headspace gas were collected through this opening with a gas tight syringe (Hamilton 1701N, Point style five needles, 51 mm; Hamilton, Bonaduz, Switzerland). Directly thereafter, the collected headspace gas samples were directly injected into the injector port of the gas chromatography (**GC**; GC8000Top CE instruments, Milan, Italy) to measure the CH_4 concentration in the headspace gas samples as described by Pellikaan et al. (2011) and to quantify cumulative CH_4 production as described by Hatew et al. (2014). After 48 h of incubation, fermentation was terminated and fermentation samples of 600 µL were taken from each bottle to determine the VFA concentrations and pH of the fermentation fluid as described by Van Gastelen et al. (2017).

The cumulative GP and CH_4 production data were fitted using a modified Michaelis-Menten tri-phasic and monophasic equation, respectively, as described by Groot et al. (1996) using the non-linear least squares regression procedure in SAS (PROC NLIN; version 9.3; SAS Institute Inc. Cary, NC, USA). The tri-phasic model followed the procedure of Van Gelder et al. (2005), where phase 1 and 2 are assumed to be related to the fermentation of the soluble and insoluble fraction, respectively, whereas phase 3 represents microbial turnover. Difficulties can arise when estimating the shapes of the separate curves if it is unclear where one phase ends and another starts, resulting in uncertainty in estimated parameters. To resolve this, the asymptote of GP (in mL/g of incubated organic matter; **OM**) for phase 1 (**A1**) was defined as the GP after 3h, for phase 2 (**A2**) as the difference in GP between 3 and 20h, and for phase 3

(**A3**) as the difference in GP between 20 and 48 h (Van Gelder et al., 2005). The parameters **B1**, **B2**, and **B3** represent the switching characteristic of the profile of the soluble fraction, insoluble fraction, and the microbial turnover, respectively (Groot et al. 1996; Cone et al. 1997). The time needed to reach half of A1, A2, and A3 (**C1**, **C2**, and **C3**, respectively) are a measure for the rate of fermentation. The maximum gas or CH_4 production rate (R_{max};,mL/g OM/h) is calculated according to Yang et al. (2005). No correction for blank bottles was made as this may distort, in particular, CH_4 production estimates at early time points which were of interest.

Statistical analysis

Prior to statistical analysis, the triplicate *in vitro* data from the different fermentation bottles were averaged. All *in vitro* data were subjected to ANOVA using the PROC MIXED procedure in SAS (version 9.3; SAS Institute Inc. Cary, NC, USA), based on the model:

$$Y_{ijkl} = \mu + O_g + P_h + D_i + I_j + S_k + (D \times I)_{ij} + (D \times S)_{ik} + (I \times S)_{jk} + (D \times I \times S)_{ijk} + R_l + e_{ijkl}$$

where Y_{ij} = response variable (i.e., VFA, CH_4, GP, or fermentation kinetics parameters); μ = overall mean; O_g = order (g = diet sequence CON-LSO or LSO-CON); P_h = period (h = first or second run); D_i = dietary treatment *in vivo* (i = CON or LSO diet); I_j = inoculum method (j = CAN or OST method); S_k = substrate incubated *in vitro* (k = wheat, lupine, or amygold); two-way and three-way interaction terms between D, I, and S; R = random effect of cow (l = cow 1, 2, 3, or 4); e_{ijk} = residual error. The covariance structure used was variance components (VC) and the Kenward-Roger option was used to estimate the denominator degrees of freedom. The pH, VFA concentration, and VFA molar proportions of the rumen fluid before *in vitro* incubations were analyzed using a similar model that excluded the effect of substrate and its

interactions. Differences between treatment means were compared using the least square means procedure and the Tukey-Kramer method for multiple comparisons when an effect of substrate or interactions were detected at $P \leq 0.05$. Results reported represent least square means and their associated standard errors with statistical significance declared at $P \leq 0.05$ and trends at $0.05 < P \leq 0.10$.

RESULTS

Inoculum used for *in vitro* measurements

No interaction was found between the type of diet fed to the donor cows and the rumen fluid sampling method for any of the rumen fluid parameters (Table 2). The pH of the rumen fluid was lower ($P = 0.004$) for the CAN method (6.11) than for the OST method (6.66), whereas the total VFA concentration was higher ($P = 0.005$) for the CAN method (121 m*M*) than for the OST method (91 m*M*). The type of diet fed to the donor cows did not affect pH, VFA concentration, and VFA molar proportions, except for a tendency ($P = 0.052$) for greater molar proportions of the branched-chain VFA with CON (2.38 % of total VFA) than with LSO (2.09 % of total VFA).

In vitro gas production and kinetic parameters

A substrate × inoculum interaction was observed for A3, C2, and C3 ($P \leq 0.044$); a tendency ($P = 0.074$) for substrate × inoculum interaction occurred for A2 (Table 3). The A3 was greater for the OST method than for the CAN method with lupine, but the sampling method did not affect A3 with amygold and wheat. The C2 was greater for the OST method than for the CAN method with wheat, but did not differ with amygold and lupine. The C3 tended to be greater for the OST method than for the CAN method with lupine, but did not differ with amygold and wheat.

A diet × inoculum interaction was observed for A3 (P = 0.002), and a tendency (P = 0.068) for diet × inoculum interaction was present for B2. The A3 of OST was greater than the A3 of CAN with LSO but not with CON. The B2 of LSO tended to be greater than the B2 of CON with the OST method but not with the CAN method. Finally, diet × substrate interactions occurred ($P \leq 0.038$) for GP, A2, and A3, and tended (P = 0.095) to occur for C1. The A3 of LSO tended to be greater than the A3 of CON, but only with amygold. The total GP and A2 of CON were greater than the total GP and A2 of LSO only with amygold, but not with lupine and wheat.

The rumen fluid sampling method affected ($P \leq 0.001$) A1 and A2, both being higher for the CAN method (91.6 mL/g OM and 190.5 mL/g OM, respectively) relative to the OST method (71.4 mL/g OM and 176.3 mL/g OM, respectively). The B2 was lower (P = 0.012) for the CAN method (2.8) than for the OST method (3.2), whereas C1 was higher (P = 0.011) for the CAN method (0.7 h) than for the OST method (0.5 h). The maximum gas production rate of the insoluble fraction ($\mathbf{R_{max2}}$) tended to be greater (P = 0.060) for the CAN method (22.1 mL/g OM/h) than for the OST method (22.0 mL/g OM/h). Also, the B2 (2.8 vs. 3.2) and the half time characteristic C2 (7.3 h vs. 7.8 h; Table 3; $P \leq 0.034$) were smaller for CON than LSO.

The type of substrate incubated affected the total GP and all kinetic parameters ($P \leq$ 0.034), with the exception of B1 (not significant) and B3 (tendency only; P = 0.090; Table 3). The asymptotic GP of the soluble fraction (A1) was lower ($P \leq 0.004$) for amygold than for both lupine and wheat (71.3 vs. 89.7 and 83.5 mL/g OM, respectively), with no differences observed between lupine and wheat. The B2 was higher ($P < 0.001$) for wheat (3.4) compared to lupine (2.5) and B2 tended to be higher (P = 0.058) for wheat compared to amygold (2.9). The C1 was lower (P = 0.028) for amygold than for lupine (0.5 h and 0.7 h, respectively). The

(R_{max2} was higher for wheat ($P < 0.001$) than for both amygold and lupine (30.2 vs. 16.8 and 16.1 mL/g OM/h, respectively), with no differences observed between amygold and lupine.

In vitro methane production and kinetic parameters

A substrate × inoculum interaction was observed for the switching characteristic of the CH_4 production profile parameter (**B**) ($P = 0.041$; Table 4), which was greater for the OST method than for the CAN method with wheat, but did not differ with amygold and lupine. The total CH_4 production (mL/g OM), the relative CH_4 production (**GP% CH_4**), and the asymptotic CH_4 production (mL/g OM) were higher ($P < 0.001$; Table 4) for the CAN method (53.0, 16.1, and 66.3, respectively) than for the OST method (40.5, 13.5, and 51.5, respectively). Also the maximum CH_4 production rate (**R_{max}**) was higher for the CAN method (3.27 mL/g OM/h) than for the OST method (2.07 mL/g OM/ h), whereas the halftime to reach the maximum CH_4 production (C) tended to be higher ($P = 0.069$) for the OST method than for the CAN method.

The asymptotic CH_4 production (A) tended to be higher ($P = 0.071$) and both the total CH_4 production ($P = 0.008$) and GP% CH_4 were higher ($P = 0.035$) for the CON diet (49.7 mL/g OM and 15.4% of GP, respectively) than for the LSO diet (43.8 mL/g OM and 14.2% of GP, respectively). A similar pattern was observed for R_{max}, being higher ($P = 0.010$) for the CON diet than for the LSO diet (2.94 and 2.40 mL/g OM/h, respectively).

The type of substrate incubated affected all CH_4 production and kinetic parameters. Both total CH_4 production and GP% CH_4 were higher ($P \leq 0.046$) for wheat (54.5 mL/g OM and 16.2 % of GP, respectively) than for both amygold (44.4 mL/g OM and 13.6 % of GP, respectively) and lupine (41.4 mL/g OM and 14.7 % of GP, respectively), with no differences observed between amygold and lupine. The asymptotic CH_4 production (A) increased ($P \leq 0.022$) from lupine (48.9 mL/g OM) to wheat (66.3 mL/g OM) to amygold (70.1 mL/g OM). The R_{max} increased ($P \leq 0.007$) from amygold (1.8 mL/g OM/h) to lupine (2.6 mL/g OM/h) to

wheat (R_{max} of 3.5 mL/g OM/h). Additionally, the halftime to reach the maximum CH_4 production (C) was higher ($P < 0.001$) for amygold (31.8 h) than for both lupine and wheat (14.5 and 11.8 h, respectively), with no differences observed between lupine and wheat.

Fermentation end products and pH

No significant interactions were observed for ruminal pH and the fermentation end products after 48 h of *in vitro* incubation in the rumen fluid (Table 5). The molar proportion of acetate tended to be affected by a D × I interaction ($P = 0.073$), and both propionate molar proportion and pH tended to be affected by a D × S interaction ($P = 0.091$ and $P = 0.069$, respectively). Ruminal pH and the molar proportions of propionate were lower ($P < 0.001$) for the CAN method (6.53 and 21.2 % of total VFA, respectively) than for the OST method (6.64 and 24.2 % of total VFA, respectively). The VFA production, the molar proportion of acetate, and NGR were higher ($P < 0.006$) for the CAN method (67 mmol/L/48 h, 60.0 % of total VFA, and 4.07, respectively) than for the OST method (60 mmol/L/48 h, 57.6 % of total VFA, and 3.45, respectively).

Ruminal pH was lower ($P = 0.010$) and the molar proportions of propionate tended to be lower ($P = 0.094$) for the CON diet (6.56 and 22.1 % of total VFA, respectively) than for the LSO diet (6.62 and 23.2 % of total VFA, respectively), whereas the VFA production and the molar proportion of acetate were higher ($P < 0.015$) for the CON diet (67 mmol/L/48 h and 59.4 % of total VFA, respectively) than for the LSO diet (61 mmol/L/48 h and 58.2 % of total VFA, respectively; Table 5).

Amygold had the lowest ($P < 0.001$) ruminal pH and molar proportions of branched-chain VFA (6.48 m*M* and 3.6 % of total VFA, respectively), lupine the highest ruminal pH and proportions of branched-chain VFA (6.72 m*M* and 4.5 % of total VFA, respectively), with wheat in between (6.56 m*M* and 3.9 % of total VFA, respectively). The molar proportions of

acetate were higher ($P < 0.001$) for amygold (59.9 % of total VFA) than for wheat (57.9 % of total VFA), whereas the molar proportions of butyrate were higher ($P < 0.001$) for wheat (14.3 % of total VFA) than for both amygold and lupine (11.4 and 11.0 % of total VFA, respectively), with no differences observed between amygold and lupine. The non-glucogenic VFA to glucogenic VFA ratio (**NGR**) was lower ($P = 0.013$) for amygold and lupine (3.66 and 3.53, respectively) than for wheat (4.08).

DISCUSSION

Inoculum used for *in vitro* measurements

The ruminal pH was higher for OST than for CAN in the present study, which is in accordance with Duffield et al. (2004), Terré et al. (2013), and Wang et al. (2016), who all reported a higher ruminal pH when sampling rumen fluid using OST compared with using CAN. Using the OST method in the present study resulted in collection of rumen fluid from the dorsal cranial part of the rumen. This is based on multiple checks of the position of the OST suction head in rumen cannulated cows. The pH within this region of the reticulo-rumen is generally higher compared with other sites (e.g. Duffield et al. 2004; Li et al. 2009) as a result of rumination and the entry of saliva. In addition, the flow of saliva induced by the OST method and the saliva contamination generally occurring with the OST method, may also contribute to the higher pH (Shen et al. 2012). The latter, viz. flow and contamination of saliva, may also explain the decreased total VFA concentration in rumen fluid samples collected via OST compared with CAN. In agreement with the results of the present study, previous studies indicated a decreased total VFA concentration when using the OST method (e.g., Terre et al. 2013; Wang et al. 2016). Ramos-Morales et al. (2014) did not statistically test for differences between the rumen fluid samples collected via CAN and OST, but did observe a 10 to 25% lower total VFA concentration for OST compared with CAN. Raun and Burroughs (1962)

already reported a lower total VFA concentration in rumen fluid collected using the OST and attributed this result to dilution by salivary contamination. This suggests that saliva contamination, either due to the flow at the sampling site or due to the collection procedure, cannot totally be avoided when using the OST method. Interestingly though, and in agreement with other studies (e.g., Lodge-Ivey et al. 2009; Terre et al. 2013; Wang et al. 2016), despite the differences in total VFA concentration, the molar proportions of the individual VFA (except for a tendency for branched-chain VFA) were not affected by the rumen fluid sampling method.

The results of the present study indicate that type of diet (i.e., CON and LSO) does not affect ruminal pH, total VFA concentration, and molar proportions of individual VFA of the rumen fluid used as inoculum for *in vitro*, with the exception of branched-chain VFA (tendency only). This is contrary to the findings of other studies (e.g., Martin et al. 2016; Van Gastelen et al. 2017), which reported a shift in VFA pattern toward proportionally more propionate and less acetate when a linseed oil containing diet was fed. The lack of effect in the present study might be related to the number of cows used. The four cows were suitable for the objective to test for effects of rumen fluid sampling method, but perhaps not sufficient for testing the effect of diet on rumen fermentation characteristics.

In vitro gas production and kinetic parameters

The diet × inoculum interactions and the substrate × inoculum interactions found in the present study indicate that the rumen fluid sampling method can influence the *in vitro* GP kinetics for diet (i.e., CON vs. LSO) and for substrates (i.e., amygold, lupine, and wheat). These interactions suggests that the OST method is not a feasible alternative to the CAN method in dairy cattle to examine *in vitro* GP, perhaps caused by differences in the microbial population, such as concentrations, relative abundance, activity, or a combination of these. The OST method results in the collection of samples containing a high proportion of liquid, whereas the

CAN method allows both liquid and small solid digesta fraction to be obtained (Yáñez-Ruiz et al. 2016). The latter, which is related to inconsistent recovery of both liquid and solid phases (Duffield et al., 2004; Shen et al., 2012), has therefore limited the use of OST for rumen microbial ecology investigations (Paz et al. 2016). Henderson et al. (2014) demonstrated that the microbial communities of liquid rumen fraction differ considerably from the microbial communities of the solid and total rumen fractions, suggesting that the liquid rumen fraction is not representative to the total rumen fraction.

In the same study, differences were detected in the relative abundance of specific taxa in rumen fluid samples collected via the CAN and the OST method, but the rumen fluid samples collected via the CAN and OST method could not be readily distinguished by principle coordinate analysis (Henderson et al. 2014). Similarly, Paz et al. (2016) reported that the bacterial community composition was not affected by sampling method, and Lodge-Ivey et al. (2009) demonstrated that the microbial communities were clustered by animal and ruminant species (i.e., sheep vs. cows) rather than by sampling method. The results of these studies suggest that differences in microbial communities between sampling methods are negligible. However, Henderson et al. (2014) already stated that despite the overall resemblance of the microbial community structure, one should not overlook the differences in relative abundances of certain microbial groups.

The latter becomes evident in the study of Ramos-Morales et al. (2014), where the authors concluded that the OST method is a good alternative to assess the structure and the composition of the rumen microbial community. However, the authors statistically tested for effects of species (sheep vs. goat), diet (forage vs. forage plus concentrate), and sampling time (pre-feeding and post-feeding) for three microbial groups. In four out of the nine possible combinations of main factors, using rumen fluid collected via the CAN method resulted in a different statistical outcome than using rumen fluid collected via the OST method. This

illustrates that the different rumen sampling methods result in differences between the rumen fluid collected. The OST method may not allow the collection of small fiber-rich particles and therefore may underestimate the numbers of microorganisms associated to plant material.

The dietary effects found on GP kinetics are in agreement with Klop et al. (2017) and might be associated with the toxic effect of the poly-unsaturated fatty acids present in linseed oil on fibrolytic bacteria (Nagaraja et al. 1997; Yang et al. 2009; Martin et al. 2010). Fibrolytic bacteria ferment mainly fiber and produce both acetate and butyrate, resulting indirectly (via release of CO_2 from the bicarbonate buffer) in GP. Furthermore, the effect of substrate on GP kinetics is in accordance to their chemical composition (i.e., lupine containing high amounts of fibers and almost no starch relative to both amygold and wheat).

In vitro methane production and kinetic parameters

The substrate × inoculum interaction for parameter B as well as the main effect of rumen fluid sampling method on almost all CH_4 production and kinetic parameters, suggests that different rumen fluid sampling methods affect the fermentation activity measured *in vitro*. Hence, the OST method appears not to be a feasible alternative to the CAN method in dairy cattle to examine CH_4 production kinetics *in vitro*, most likely due to differences in the archaea population between both inocula. Henderson et al. (2014) reported a 1.2 fold increase in the relative abundance of *Methanobrevibacter ruminantium* in rumen fluid collected using the CAN method, whereas the relative abundance of the *Methanoplasmatales* was 1.9 fold increased in rumen samples collected using the OST method. Additionally, Ramos-Morales et al. (2014) reported a numerical lower ruminal concentration of methanogenic archaea in rumen fluid obtained using the OST method compared with the CAN method for both sheep and goats. This illustrates that both the ruminal concentration of archaea and the relative abundance of certain archaea is affected by rumen fluid sampling method, explaining the differences observed

401 in the present study in *in vitro* CH_4 production. Furthermore, a higher concentration of protozoa
402 is generally associated with increased CH_4 production due to the attachment of methanogenic
403 archaea to the surface of protozoa (Moss et al. 2000). Yang and Vargas (1989) observed that
404 the ventral site of the rumen contains more protozoa than the dorsal site of the rumen. In the
405 present study, the OST rumen fluid samples were collected at the dorsal cranial part of the
406 rumen, whereas the CAN rumen fluid samples were collected at the ventral part of the rumen.
407 Hence, the number of protozoa could have been lower in the rumen fluid collected using the
408 OST method compared with the rumen fluid collected with the CAN method, explaining the
409 lower levels of *in vitro* CH_4 production.

410 The dietary effects on *in vitro* CH_4 production and kinetics are in agreement with Klop
411 et al. (2017), where donor cows were fed diets with and without lauric acid. The lower *in vitro*
412 CH_4 production observed in the present study when rumen fluid was obtained from a LSO fed
413 cow is most likely due to changed concentrations of ruminal archaea (Veneman et al. 2015;
414 Martin et al. 2016) or their activity, or due to a changed archaea to bacteria ratio. A decrease in
415 archaea to bacteria ratio with LSO compared with CON diets was observed by Van Gastelen et
416 al. (2017), who argued that a reduced archaea to bacteria ratio suggests that per unit substrate
417 fermented by bacteria, a smaller archaeal concentration is present to form CH_4.

418 The composition of the substrate fermented *in vitro* determines the production of
419 hydrogen that serves as a substrate for methanogenic archaea (Yáñez-Ruiz et al. 2016). The
420 higher *in vitro* CH_4 production with wheat than with amygold or lupine, despite having the
421 highest starch content and the lowest fiber content, might be related to lactate formation. Heldt
422 et al. (1999) reported that formation of lactate is commonly associated with rapid fermentation
423 of starch found in the cereal grains, which subsequently can result in increased levels of butyrate
424 (Heldt et al. 1999). The formation of butyrate, which coincides with the production of hydrogen,

was also observed in the present study and might explain the higher CH_4 production observed for wheat.

Fermentation end products and pH

Despite numerical differences, no interactions were observed in any of the fermentation end products and pH after 48 h *in vitro* incubations. This suggests that the sampling method used for rumen fluid collection (i.e., CAN and OST), results in the same conclusions regarding the effect of substrates and diet fed to the donor cows on fermentation. However, numerical as well as significant differences between the two sampling methods should be taken into account. The ruminal pH was for example higher after 48 h of *in vitro* incubation using the OST method compared with the CAN method. This difference in pH was also observed in the rumen fluid, but the difference has become smaller (i.e., on average 9% higher in rumen fluid vs. on average 2% higher after 48 h *in vitro* incubation). There were no differences in molar proportions of VFA in rumen fluid, but after 48 h *in vitro* incubations the molar proportion of acetate was lower and that of propionate higher for rumen fluid obtained using the OST method compared with the CAN method. This is in line with the lower absolute CH_4 production and GP% CH_4 observed for the OST method compared with the CAN method.

In accordance with Zhang et al. (2008), a shift in VFA pattern toward proportionally more propionate and less acetate was observed after 48 h *in vitro* incubation when rumen fluid from LSO fed cows was used, which most likely results from the inhibitory effect of linseed oil on fiber degradation, whereas the degradation of other carbohydrates remains unaffected (Doreau and Chilliard, 1997). This is also in accordance with the lower VFA production observed after 48 h *in vitro* incubation when rumen fluid from LSO fed cows was used. Additionally, the pH after 48 h *in vitro* incubations decreased from lupine to wheat to amygold,

449 which is most likely related to the ash content of the substrates as minerals have a buffering
450 capacity, decreasing pH (Dijkstra et al., 2012).
451
452 ## CONCLUSION
453 Rumen fluid obtained using the oral stomach tube sampling technique differs from that
454 obtained using the rumen cannulation method, resulting in differences in *in vitro* gas and CH_4
455 production profiles between both methods. Multiple interactions between substrate and the
456 rumen fluid sampling method, and between diet fed to donor cows and rumen fluid sampling
457 method, were observed. Although the OST method can be applied to a much larger population
458 of dairy cows than the CAN method, the results of this study do not support the OST method
459 as a feasible alternative to the CAN method in dairy cattle to examine gas and CH_4 production
460 *in vitro*.
461
462 ## ACKNOWLEDGEMENTS
463 The authors gratefully acknowledge the staff of the experimental facilities “Carus”
464 (Wageningen, the Netherlands) for their assistance during the implementation of the *in vivo*
465 experiment, as well as the laboratory staff of the Animal Nutrition Group (Wageningen, the
466 Netherlands) for their skilled assistance during the *in vitro* experiment.
467
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589

Table 1. Chemical composition and fermentation kinetics of the feed ingredients incubated for *in vitro* measurements

Item	Wheat	Lupine	Amygold
Analyzed chemical composition (g/kg DM, unless otherwise stated)			
DM (g/kg product)	890	910	915
Ash	19	29	11
Crude protein	132	315	116
Crude fat	18	57	30
NDF	120	292	496
ADF	34	209	133
ADL	6	4	10
Starch	681	3	272
Sugar	29	64	22
In situ fermentation kinetics of organic matter (%, unless otherwise stated; adapted from Cone et al. 2008)			
Washout fraction	46	27	21
Undegradable fraction	3	2	4
Potential degradable fraction	51	71	75
Fractional degradation rate of the potential degradable fraction (/ h)	0.175	0.073	0.030

590

591

Table 2. The pH, total volatile fatty acid (VFA) concentration in m*M*, and VFA molar proportions as % of total VFA of rumen fluid collected via the CAN[1] or OST[2] method from dairy cows fed diets with (LSO) or without (CON) linseed oil

Diet	Inocula	pH	Total VFA	Acetate	Propionate	Butyrate	Branched-chain VFA[3]
CON	CAN	6.12	123	66.4	16.3	13.6	2.34
CON	OST	6.62	94	66.7	16.6	12.8	2.42
LSO	CAN	6.10	119	66.3	16.4	13.9	2.03
LSO	OST	6.70	87	66.6	16.4	13.5	2.14
	SEM	0.139	8.8	0.73	0.56	1.02	0.215
Inocula (I)		0.004	0.005	0.232	0.414	0.102	0.476
Diet (D)		0.809	0.523	0.801	0.921	0.195	0.052
Period		0.851	0.258	0.008	0.318	0.017	0.537
Order		0.911	0.801	0.514	0.430	0.978	0.828
D×I		0.727	0.806	0.898	0.445	0.616	0.922

[1] Rumen cannulation method.

[2] Oral stomach tube method.

[3] The sum of isovaleric and isobutyric acid.

592

Table 3. Total gas production (GP) and kinetic parameter from fermentation of rumen fluid collected via the CAN[1] or OST[2] method from dairy cows fed diets with (LSO) or without (CON) linseed oil incubated with different substrates

Substrate	Diet	GP[3]		A1[4]		A2[4]		A3[4]		B1[5]		B2[5]		B3[5]		C1[6]		C2[6]		C3[6]		R_{max2}[7]	
		CAN	OST	CAN	OST	CAN	OST	CAN	OST	CAN	OST	CAN	OST	CAN	OST	CAN	OST	CAN	OST	CAN	OST	CAN	OST
Amygold	CON	356.1^{a}	328.4^{a}	78.8	62.8	201.6^{a}	195.1^{a}	75.7^{a,d,g}	68.5^{a,d,g}	0.9	0.9	2.5	2.8	5.0	5.0	0.7	0.6	8.0^{d}	8.2^{d}	23.5^{d}	24.4^{d}	18.8	18.5
Amygold	LSO	327.4^{b}	280.4^{b}	82.8	60.6	162.4^{b}	132.2^{b}	82.2^{a,d,g}	87.5^{a,d,h}	1.0	1.2	2.7	3.7	5.3	6.0	0.4	0.4	8.6^{d}	8.7^{d}	26.4^{d}	29.0^{d}	15.0	14.9
Lupine	CON	304.1^{b,c}	266.5^{b,c}	103.8	79.4	171.3^{b}	157.9^{b}	28.9^{b,e,g}	27.4^{b,f,g}	1.0	0.9	2.3	2.4	23.0	11.6	0.9	0.6	6.9^{ef}	7.1^{ef}	17.0^{e}	19.8^{d,e}	17.2	16.1
Lupine	LSO	290.6^{c}	257.3^{c}	100.9	74.6	165.8^{b}	128.6^{b}	23.8^{b,e,g}	54.1^{b,f,h}	1.0	2.1	2.3	3.2	11.2	6.5	0.8	0.6	7.1^{ef}	7.5^{ef}	16.3^{e}	26.0^{d,e}	16.2	14.8
Wheat	CON	346.6^{a}	326.3^{a}	91.6	80.5	219.7^{ac}	216.0^{ac}	35.2^{b,ef,g}	28.1^{b,ef,g}	0.9	0.7	3.3	3.3	7.7	12.5	0.8	0.3	6.3^{f}	7.5^{de}	23.3^{d,e}	19.4^{d,e}	33.7	26.8
Wheat	LSO	340.1^{a}	325.1^{a}	91.6	70.3	222.0^{c}	228.1^{c}	26.5^{b,ef,g}	26.7^{b,ef,h}	1.0	1.1	3.4	3.6	7.5	13.9	0.7	0.6	6.7^{f}	8.1^{de}	23.2^{d,e}	19.4^{d,e}	31.8	28.6
	SEM	11.14		6.67		8.30		4.39		0.38		0.28		4.82		0.12		0.34		2.76		1.90	
Inocula (I)		< 0.001		< 0.001		0.006		0.200		0.360		0.012		0.801		0.011		0.006		0.390		0.060	

Diet (D)	0.003	0.345	< 0.001	0.022	0.101	0.011	0.396	0.216	0.034	0.193	0.151
Substrate (S)	< 0.001	< 0.001	< 0.001	< 0.001	0.474	< 0.001	0.090	0.034	< 0.001	0.014	< 0.001
Period	0.112	0.027	0.399	< 0.001	0.063	0.563	0.010	0.804	0.001	0.060	0.032
Order	0.912	0.768	0.401	0.050	0.462	0.567	0.494	0.512	0.136	0.982	0.252
D×S	0.038	0.660	< 0.001	0.014	0.644	0.588	0.312	0.095	0.892	0.603	0.403
S×I	0.259	0.378	0.074	0.014	0.591	0.331	0.131	0.229	0.032	0.044	0.184
D×I	0.765	0.285	0.202	0.002	0.182	0.068	0.589	0.397	0.816	0.378	0.573
D×S×I	0.587	0.827	0.264	0.124	0.569	0.550	0.889	0.702	0.977	0.661	0.739

[1] Rumen cannulation method.

[2] Oral stomach tube method.

[3] Cumulative gas production measured after 48 h of incubation in mL/g organic matter.

[4] Asymptote of gas production for the soluble fraction (A1), the insoluble fraction (A2), and the microbial turnover (A3) in mL/g organic matter.

[5] The sharpness of the switching characteristic for the profile of the soluble fraction (B1), insoluble fraction (B2), and the microbial turnover (B3). Dimensionless.

[6] Incubation time at which half of maximum gas production has been formed for the soluble fraction (C1), insoluble fraction (C2), and the microbial turnover (C3) in h.

[7] Maximum gas production rate of the insoluble fraction (R_{max2}) in mL/g organic matter/h.

[a,b,c] Values within a colomn (per variable) with a different superscript indicate a significant difference for the diet × substrate interaction.

[d,e,f] Values within a variable with a different superscript indicate a significant difference for the substrate × inoculum interaction.

[g,h] Values within a variable with a different superscript indicate a significant difference for the diet × inoculum interaction.

593

Table 4. Methane production and kinetic parameters from fermentation of rumen fluid collected via the CAN[1] or OST[2] method from dairy cows fed diets with (LSO) or without (CON) linseed oil incubated with different substrates

Substrate	Diet	Methane[3]		GP% CH_4[4]		A[5]		B[6]		C[7]		R_{max}[8]	
		CAN	OST	CAN	OST	CAN	OST	CAN	OST	CAN	OST	CAN	OST
Amygold	CON	54.2	44.8	15.2	13.6	85.7	66.8	1.17^{a}	1.31^{ab}	30.3	28.1	2.62	1.98
Amygold	LSO	46.9	31.6	14.3	11.2	73.0	54.7	1.29^{a}	1.42^{ab}	30.2	38.5	1.77	0.97
Lupine	CON	50.9	38.7	16.7	14.5	58.7	42.9	1.46^{ab}	1.95^{b}	11.7	13.6	3.52	2.48
Lupine	LSO	44.6	31.2	15.4	12.2	50.2	43.7	1.65^{ab}	1.57^{b}	10.6	22.4	3.22	1.34
Wheat	CON	62.4	47.5	17.9	14.5	67.9	48.8	1.70^{b}	2.45^{c}	10.7	12.6	4.24	2.81
Wheat	LSO	59.1	49.0	17.4	15.1	62.6	51.8	1.82^{b}	2.39^{c}	9.5	14.3	4.27	2.84
	SEM	4.09		1.02		5.86		0.180		4.17		0.331	
P-values													
Inocula (I)		< 0.001		< 0.001		< 0.001		< 0.001		0.069		< 0.001	
Diet (D)		0.008		0.035		0.071		0.850		0.198		0.010	
Substrate (S)		< 0.001		0.001		< 0.001		< 0.001		< 0.001		< 0.001	

Period	0.416	0.696	0.211	0.971	0.640	0.897
Order	0.650	0.728	0.428	0.948	0.398	0.318
D×S	0.196	0.293	0.317	0.648	0.667	0.116
S×I	0.995	0.944	0.625	0.041	0.761	0.254
D×I	0.849	0.651	0.331	0.165	0.108	0.393
D×S×I	0.590	0.554	0.821	0.447	0.754	0.634

[1] Rumen cannulation method.

[2] Oral stomach tube method.

[3] Cumulative methane production measured after 48 h of incubation in mL/g organic matter.

[4] Relative methane production measured after 48 h of incubation expressed as a percentage of cumulative gas production at 48 h.

[5] Asymptotic methane production in mL/g organic matter.

[6] The sharpness of the switching characteristic of the methane production profile. Dimensionless.

[7] Incubation time in h at which half of the maximum methane production has been formed.

[8] Maximum methane production rate in mL/g organic matter/h.

[a,b,c] Values within a variable with a different superscript indicate a significant difference for the substrate × inoculum interaction.

594

Table 5. The fermentation end products produced in *in vitro* incubations of rumen fluid collected via the CAN[1] or OST[2] method from dairy cows fed diets with (LSO) or without (CON) linseed oil incubated with different substrates

Substrate	Diet	pH		VFA production[3]		Acetate[4]		Propionate[4]		Butyrate[4]		Branched-chain VFA[4,5]		NGR[6]	
		CAN	OST	CAN	OST	CAN	OST	CAN	OST	CAN	OST	CAN	OST	CAN	OST
Amygold	CON	6.38	6.46	75	66	61.1	60.3	21.3	23.3	11.8	10.8	3.7	3.6	4.03	3.59
Amygold	LSO	6.49	6.60	65	54	60.2	57.8	21.3	26.7	12.8	10.3	3.7	3.2	4.06	2.96
Lupine	CON	6.68	6.74	64	66	60.5	58.5	20.8	23.4	11.5	10.6	4.6	4.6	4.02	3.43
Lupine	LSO	6.66	6.82	66	56	59.8	55.8	22.4	26.4	11.1	10.6	4.2	4.5	3.69	2.97
Wheat	CON	6.47	6.64	67	62	59.3	56.9	20.7	23.3	13.9	13.3	3.9	4.0	4.28	3.64
Wheat	LSO	6.52	6.61	66	59	59.3	56.1	20.4	22.0	14.1	15.9	3.9	3.9	4.31	4.09
	SEM	0.037		3.8		0.83		1.24		1.03		0.24		0.291	
P-values															
Inocula (I)		< 0.001		0.006		< 0.001		< 0.001		0.252		0.678		< 0.001	
Diet (D)		0.010		0.015		0.004		0.094		0.379		0.138		0.330	
Substrate (S)		< 0.001		0.817		0.002		0.053		< 0.001		< 0.001		0.013	

Period	< 0.001	0.391	0.442	0.657	0.871	0.775	0.751
Order	0.715	0.332	0.937	0.664	0.552	0.927	0.455
D×S	0.069	0.258	0.299	0.091	0.409	0.777	0.185
S×I	0.815	0.572	0.344	0.541	0.216	0.174	0.654
D×I	0.646	0.219	0.073	0.311	0.659	0.882	0.692
D×S×I	0.184	0.655	0.783	0.367	0.355	0.408	0.374

[1] Rumen cannulation method.

[2] Oral stomach tube method.

[3] Volatile fatty acid production in mmol/L/48 h calculated as the difference between the total volatile fatty acids in the inoculum and the total volatile fatty acids after 48 h of *in vitro* incubation.

[4] Molar proportions as % of total VFA.

[5] The sum of isovaleric and isobutyric acid.

[6] Non-glucogenic VFA to glucogenic VFA ratio = [(acetate + 2 × butyrate + 2 × isobutyrate + valerate + isovalerate)/(propionate + valerate + isovalerate); Cone and Becker, 2012]

595