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Using archaeol to investigate the location of methanogens in the ruminant digestive tract

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ABSTRACT

The quantification of archaeol, a methanogen membrane lipid, may provide an alternative method to estimate methanogen abundance. The focus of this study was to determine the location of methanogens in the ruminant digestive tract using this biomarker. Archaeol was quantified in samples obtained from four lactating cows with rumen cannulae that grazed on either white clover (WC) or perennial ryegrass (PRG) in a changeover design study with three 3-week periods. Faeces were collected over the final 5 d of each period and total rumen contents (TRC) were obtained on the final 2 days (day 1: 9am; day 2: 3pm). Solid-associated microbes (SAM) and liquid-associated microbes (LAM) were also isolated from the TRC. Concentrations of archaeol in the TRC showed a significant diet by time interaction, which may be related to diurnal grazing patterns and different rumen conditions associated with PRG or WC diets. There was significantly more archaeol associated with SAM than LAM, which may reflect difficulties of methanogen proliferation in the liquid phase. Faeces had higher concentrations of archaeol than SAM and LAM which was unexpected, although, losses of methanogens may have occurred during isolation (i.e. attachment to protozoa and very small particles), or the methanogens associated with SAM may have been underestimated. There was no significant relationship between faecal and TRC archaeol concentrations. Finally, there was a significant positive relationship between rumen pH and concentrations of archaeol in SAM and LAM, which may be caused by pH and/or WC diet effects. In conclusion, archaeol is potentially a useful alternative marker for determining the abundance of methanogens in the ruminant digestive tract. This work has also highlighted the difficulties associated with methanogen quantification from microbial isolates, and the need for more representative rumen sampling in future studies.

Key words: archaeol, faeces, methanogen, rumen.

1. Introduction

This study investigated an alternative approach to study methanogen abundance involving the use of the lipid biomarker archaeol (2,3-diphytanyl-O-*sn*-glycerol). This is a core membrane lipid that is ubiquitous in methanogens, and is readily quantified by gas chromatography mass spectrometry. Previous studies have already assessed archaeol as a potential faecal biomarker for methanogens and methane production (Gill et al., 2011; McCartney et al., 2013a), where a significant positive relationship was found between total methanogens and methane production when looking at treatment means. Archaeol has also recently been assessed in parallel with quantitative real-time PCR (qPCR) techniques and it was found that archaeol was a useful complementary approach for estimating methanogen abundance (McCartney et al., 2013c).

The purpose of this study was to use archaeol as a total methanogen proxy to provide insights into the location and abundance of methanogens in the digestive tract of cows grazing either perennial ryegrass (PRG) or white clover (WC). Archaeol was quantified in samples from the rumen, including total rumen contents, liquid-associated microbes (LAM) and solid-associated microbes (SAM), and in faeces.

2. Materials and Methods

2.1. Animal study

This study was conducted under a license issued under the UK Animal (Scientific Procedures) Act (1986). Four lactating Holstein-Friesian cows that had previously been prepared with rumen cannulae (Bar Diamond, Parma, ID) were used. At the start of the experiment the animals had an average BW of 694 kg (s.d. = 46.4), a BCS of 2.7 (s.d. = 0.51) and were 104 days in milk (s.d. = 23.6). The BCS was determined according to Mulvany (1977). Animals were strip-grazed on pure stands of either perennial ryegrass (*Lolium perenne* cv. Fennema) or white clover (*Trifolium repens* cv. AberHerald) that had been re-growing for 21 to 27 days in a changeover design with three 3-week periods. In period 1, two animals grazed WC and 2 animals grazed PRG. In period 2, two animals switched diet, and in period 3 all animals switched diet. As a result two animals received the same diet in periods 1 and 2. Measurements were taken from all four animals in each period, resulting in six measurement periods per dietary treatment. All cows were supplemented with 4kg/d of pelleted concentrates (Table 1) and received 25 g/d of poloxalene (Bloat Guard, Agrimin Ltd., Brigg, Lincolnshire, UK). Cows were milked twice-daily (approx. 08:00 and 16:00 h) with milk yields recorded electronically (Tru-Test NZ Ltd, Auckland, NZ) and samples for analysis of milk components taken on four consecutive milkings at the end of each period. Milk samples were sent to NMR laboratories (National Milk Records plc, Chippenham, UK) for infrared milk analysis of milk fat, protein and lactose.

Herbage samples (20 random snips to 6 cm) were collected daily and bulked weekly for feed composition analysis (described below). Cows were dosed *via* the rumen cannula with plastic pellets of different colours to identify their faeces in the field

92 (pellets were removed prior to analysis). Faecal samples were collected daily from the
93 field over 5 days at the end of each experimental period (bulked proportional sample
94 of all faeces deposited over the previous 5 days) and then lyophilized and finely
95 ground for analysis. The dried faecal samples were stored at room temperature.
96 Rumen contents were obtained by total rumen evacuation on the final 2 days of each
97 period (day 1: 9am; day 2: 3pm). A 5% sub-sample was taken throughout the
98 evacuation procedure (every twentieth bail) and composited for subsequent analysis
99 and the remaining rumen contents were returned immediately on completion of the
100 evacuation procedure. The weight, DM and NDF content of the total rumen contents
101 was determined. Rumen fluid samples were taken using automatic samplers every 2
102 hours for the 2 days prior to rumen evacuation for ammonia and VFA analysis. The
103 pH was recorded (Oakton PC510 pH meter; Eutech Instruments BV, Nijkerk, The
104 Netherlands) twice a day at morning and evening milking on the final 2 days of the
105 experimental period. All chemical analysis of feed and rumen contents was performed
106 using methods outlined by Dewhurst et al. (2000). Briefly, the DM was determined by
107 toluene distillation, VFA concentrations were determiend using gas chromatography,
108 crude protein was determined using a Kjeldahl procedure with Cu catalyst on
109 'Kjeltec' equipment (Perstorp Analytical Ltd., Berkshire, UK). Detergent fibre
110 analyses, NDF (with amylase) and ADF were determined using 'Fibretec' equipment
111 (Perstorp Analytical Ltd., Berkshire, UK). Water-soluble carbohydrate were
112 concentration was determined using an automated anthrone method (Method 9a;
113 Technicon Industrial Systems, Tarrytown, NY), and starch by incubating with
114 buffered amyloglucosidase prior to the automated anthrone method. Rumen ammonia
115 concentration was determined using a test kit (No. 66-55; Sigma-Aldrich Co. Ltd.,
116 Poole, UK) on a discrete analyser. *In vitro* DOMD based on incubation with pepsin-

cellulase was performed according to Jones and Hayward (1975). At the end of the experiment the animals had an average bodyweight of 665 kg (SD = 29.7) and an average BCS of 2.8 (SD = 0.34).

2.2. Isolating SAM and LAM

SAM and LAM fractions were obtained from the whole rumen contents collected at the 9am rumen evacuation following the protocol of Merry and McAllan (1983). To obtain the LAM fraction, total rumen contents were hand squeezed through 4 layers of cheesecloth to obtain 1000 mL of liquid before centrifugation for 10 min at low speed (500 × *g*). The supernatant was then centrifuged at high speed (25,000 × *g*) for 25 min. After discarding the supernatant, the remaining pellet washed in saline (9g NaCl/L) before another high-speed centrifugation step for 25 min. The supernatant was discarded and the pellet washed in distilled water. After one final high-speed centrifugation step for 15 min, the supernatant was discarded and the remaining pellet freeze-dried before storage at -20ºC. To obtain the SAM fraction, 500 g of rumen contents that had previously been retained within the cheese cloth was gently washed with saline (9g NaCl/L). This was then hand squeezed twice before processing with 320 mL of saline in a stomacher (Seward Ltd, West Sussex, UK) for 5 min. After the contents were centrifuged at low speed, with the resultant supernatant then centrifuged at high speed. After discarding the supernatant, the pellet was washed with saline then centrifuged at high speed for 25 min. The pellet was then re-washed with distilled water, and centrifuged at high speed for 15 min. After discarding the supernatant, the remaining pellet was freeze-dried prior to storage at -20ºC.

2.3. Archaeol analysis

Quantification of archaeol was achieved according to the protocol of McCartney et al. (2013a). In summary, around 300 mg of dried, ground faeces and total rumen contents were weighed in triplicate, and around 200 mg of the dried LAM or SAM fraction was weighed singly. An internal standard 1,2-di-*O*-hexadecyl-*rac*-glycerol (43.4 µg) was added to the weighed samples prior to extraction. The total lipid extract (TLE) was extracted using a modified monophasic extraction procedure, followed by removal of sugar- and phosphate-head groups by acid methanolysis. The TLE was chromatographically separated over an activated silica column to isolate a fraction containing hydroxylated compounds, which were analysed as the corresponding trimethylsilyl ethers by GC/MS. Archaeol was identified on the basis of its characteristic mass spectrum and retention time, and quantification was achieved by using the internal standard and an external calibration curve of 1,2-di-*O*-phytanyl-*sn*-glycerol normalized against the internal standard.

2.4. Statistical analysis

One sample of rumen contents was lost prior to analysis, so all data from this cow-period were excluded from subsequent analysis, hence there were 6 replicates for PRG and 5 replicates for WC. Analysis of variance for effects on archaeol, rumen contents (kg), DM of rumen contents, rumen DM (kg), NDF concentrations and rumen pH were conducted using residual maximum likelihood (REML) analysis of linear mixed models with 'diet' × 'time' as fixed effects and 'period' + 'cow' as random effects. 'Time' was defined as either morning (approx. 9am) or afternoon

(approx. 3pm). 'Diet', 'time' and their interaction each used one d.f., there were 2 d.f. for period and 3 d.f. for cow, leaving 15 residual d.f. REML was also used to analyse milk yield and composition data with 'diet' as a fixed effect and 'period' + 'cow' as random effects. In this case there were 1 d.f. for diet, 2 d.f. for period, 3 d.f. for cow and 5 residual d.f. Comparisons of archaeol concentration in the different fractions used REML, with sample type as a fixed effect (1 d.f. for sample type and 22 residual d.f.). A repeated measured analysis of variance (procedure AREPMEASURES) was applied to rumen ammonia and VFA data with 12 time points, with 'diet' × 'time' as fixed effects and 'period' + 'cow' as random effects. Simple linear regression analysis was also used to explore relationships between archaeol concentrations with rumen pH. All statistical analyses were carried out using Genstat (14th Edition, VSN International Ltd). A *P*-value of <0.05 was considered significant, and a *P*-value of <0.1 was considered a trend.

3. Results

The chemical composition of feeds is provided in Table 1. The WC had a high CP content, low NDF content and was highly digestible, whereas the PRG diet also had a relatively high CP content, but had moderate levels of NDF and was moderately digestible. The average milk yield was 26.4 kg/d (SD = 4.17), and the average milk fat, protein and lactose content of the milk was 3.38 % (SD = 0.50), 3.06 % (SD = 0.23) and 4.47 % (SD = 0.13), respectively. There were no significant effects of dietary treatments on milk yield and composition

All of the rumen measurements were significantly affected by the diet consumed, though in the case of faecal archaeol concentration, this was most evident as a significant 'diet' × 'time' effect (Table 2). Animals grazing WC had a lower pH, lower total rumen volume and lower NDF content in their rumen contents than animals grazing PRG. Total volume of rumen contents was also affected by 'time', being higher for the 3pm measurements compared to the 9am measurements.

There were significant effects of diet on rumen ammonia-N and VFA concentrations (Table 3). The higher VFA concentrations reflect the higher rate of fermentation of WC in comparison with PRG (Dewhurst et al., 2009), whilst the higher rumen ammonia-N also reflects the higher N content of WC. Sampling time only affected concentrations of ammonia, propionic acid and *iso*-valeric acid, and there were no significant 'diet' × 'time' interactions.

The average coefficient of variation for the archaeol triplicate measurements for total rumen contents and faeces was 7.79% (SD = 0.55). The concentrations of archaeol (mg/kg DM) found in total rumen contents, faeces, and SAM and LAM fractions are presented in Table 4. Faeces contained the highest concentration of archaeol, followed by the SAM fraction, total rumen contents, and then the LAM fraction. Archaeol concentration in the SAM fraction was significantly higher than in the LAM fraction (LAM = 3.64 mg/kg DM; SAM = 6.67 mg/kg DM; SED = 0.587; $P < 0.001$). Archaeol concentration in the faeces was significantly higher than in the total rumen contents (total rumen contents = 4.60 mg/kg DM; faeces = 11.08 mg/kg DM; SED = 0.7224; $P < 0.001$). There was no significant relationship between faecal archaeol and total rumen archaeol concentrations ($P = 0.521$).

Regression analysis showed a significant relationship between average rumen pH and archaeol concentration in the SAM (Figure 1), and LAM fractions (Figure 2). There were no significant relationships between average rumen pH and total archaeol concentration in the whole-rumen contents (WRC) (P = 0.130) or faecal archaeol concentration (P = 0.687).

4. Discussion

4.1. Rumen measurements

Conditions in the rumen were altered when animals grazed predominantly on WC instead of PRG. The lower NDF content of the white clover herbage was paralleled by lower concentrations in the total rumen contents. WC also led to significantly lower average rumen pH at both sampling times. The lower pH with WC is probably due to the effect of high fermentation rates and subsequent higher VFA production (Van Kessell and Russell, 1996; Lana et al., 1998). VFA concentrations were also higher for WC in this study. Much of the removal of VFAs by passive diffusion through the rumen epithelium is a consequence of rumen movements, which is triggered by the presence of particulate matter in the rumen (Lana et al., 1998). Less efficient removal of VFAs on a WC diet could be explained by the reduced amounts of ruminal digesta and therefore VFAs can accumulate in the rumen and reduce pH further. Another explanation for lower pH could be the reduced levels of rumination and saliva production with WC (Rutter et al., 2002).

4.2. Archaeol in rumen digesta

Concentrations of archaeol in rumen digesta showed a significant ‘diet’ by ‘time’ interaction. This effect is probably related to the diurnal grazing patterns and the different rumen conditions associated with PRG or WC diets. Cows tend not to graze at night and have a major grazing bout after morning milking (Rutter et al., 2002), so morning samples include a lot of material that has been in the rumen overnight, whilst the afternoon samples include relatively much more recently-ingested herbage. The low concentrations of archaeol in the afternoon for cows grazing WC suggest that methanogens proliferated more slowly with this diet. This may be the consequence of a number of factors, including the lower pH, lower NDF or higher rumen passage rates. Whilst we may expect higher DMI for legumes (Dewhurst et al., 2009), intake was not recorded in this study and the absence of milk production responses suggests that any increase would have been modest. In this study, the pH of the rumen contents for animals grazing WC was at the lower end of the normal range, which would inhibit methanogens (Janssen, 2010) with a smaller and less active population as a consequence (Van Kessell and Russell, 1996). Furthermore, the high passage rate of legumes from the rumen (Dewhurst et al., 2009) restricts the opportunity for methanogens to colonize the feed substrate. Higher growth rates are required for methanogens to maintain themselves in the rumen at higher passage rates (Janssen, 2010).

4.3. Comparison of archaeol concentration in SAM and LAM

There was a higher concentration of archaeol, and by implication methanogens, in the SAM fraction in comparison with the LAM fraction. This is in agreement with the qPCR studies of Gu et al. (2011), Pei et al. (2010) and Morgavi et al. (2012) that reported a higher abundance of methanogens associated with the solid rather than the liquid fraction.

The lower abundance of methanogens in the LAM fraction in this study probably reflects the difficulties of methanogens establishing and proliferating in the liquid phase. This is because methanogens are most efficient when located closely to symbiotic fermentative microbes in a matrix (Conrad *et al.*, 1985) as it is required for them to carry out 'interspecies hydrogen transfer' which is important for methanogenesis and consequent growth of methanogens (Van Nevel and Demeyer, 1996). However, the liquid-phase lacks particulate (or matrix) material on which these associations could occur. Furthermore, the rate of passage of the liquid fraction from the rumen is higher than for the solid fraction (typical values being 7.5 *vs.* 3.5% h^{-1}, respectively; Evans, 1981a,b). This may affect methanogen abundance in the liquid fraction as methanogens are slow growing (Pei *et al.*, 2010) and therefore they may not have time to proliferate before they are effectively 'washed out' from the rumen.

4.4. Comparison of archaeol concentration in faeces and isolated microbial fractions

Faeces had a higher concentration of archaeol (mg/kg DM) than the SAM and LAM fractions. This was unexpected as pure microbial fractions should contain much higher levels of methanogens (and archaeol) than faeces. It is unlikely that hindgut

fermentation would have contributed to the archaeol content in faeces, as a previous study by Gill et al. (2010) found no detectable levels of archaeol in the faeces of animals that carry out extensive hindgut fermentation (i.e. horses). In addition, the contribution of archaeol by non-methanogenic Archaea is also possibly negligible, as studies have found <0.05% non-methanogenic Archaea in the rumen (Shin et al., 2004; Lee et al., 2012). One explanation for the discrepancy is that there must be other methanogen fractions in the rumen that are not represented by LAM or SAM. For instance, protozoa losses could occur when the rumen contents were strained through the cheesecloth, as the pore size of cheesecloth is typically around 50 μm, whereas protozoa can range 15 to 250 μm (Mould et al., 2005). Loss of protozoal associated methanogens could be considerable, as it is estimated that methane production from protozoa-associated methanogens is as much as 37% (Finlay et al., 1994). The main source of the discrepancy, however, is likely to be the inefficient detachment of solid-adherent micro-organisms from rumen solids. Legay-Carmier and Bauchart (1989) used diaminopimelic acid (DAPA) as a bacterial marker and found that 50-60% of rumen bacteria could not be detached from rumen solids. Furthermore, very fine particles (<0.1 mm) were associated with 70-80% of total rumen bacteria, of which only 12-15% of bacteria could be extracted. Similarly, Martín-Orúe et al. (1998) found that only 17-21% of bacteria could be extracted from the solid fraction of rumen digesta. The colonisation of methanogens on feed is mostly in association with bacteria, so failure to isolate the bacterial population could also mean that methanogens were not isolated. This problem is exacerbated because bacteria and archaea associated with small particles that have been present in the rumen for a longer period of time are most likely to leave the rumen via the reticulo-omasal orifice (Poppi et al., 1981).

Since methanogens are slow growing, it seems possible that there would be higher levels on small particles that have been present in the rumen for longer – further exacerbating the under-sampling of archaea. Sampling of SAM and LAM at a single time of day (9am) is a limitation of this study, which could explain some of the discrepancy in archaeol concentration between microbial fractions and faeces; future work should consider diurnal variation in the composition of SAM and LAM. Furthermore, the archaeol concentrations in the faeces are more likely to be associated with methanogen concentrations in digesta in the reticulum (i.e. that is about to leave the reticulo-rumen) as opposed to concentrations in the WRC. This is because digesta from the reticulum is very similar to that found in the duodenum (Hristov, 2007) and no further selective retention of particles occurs after this site (Ahvenjärvi et al., 2001).

4.5. Comparison of archaeol concentration in whole rumen contents and faeces

There was no significant relationship between archaeol concentration in whole rumen contents and faeces, even at the individual animal level. Furthermore, there were significant differences between observed faecal archaeol concentrations and 'expected' faecal archaeol concentrations (obtained by adjusting the total rumen archaeol for the estimated digestibility of the feed (Table 1) and disappearance of feed post-rumen (Agricultural Research Council, 1980)). The expected and actual faecal archaeol concentrations were 9.12 and 11.4 mg/kg DM respectively for the PRG diet, and 8.60 and 10.7 mg/kg DM respectively for the WC diet. Faecal archaeol was approximately 25% higher than expected. Both of these observations point to the lack of a strong relationship between the population of methanogens present in the rumen

and the numbers of methanogens leaving the rumen. Possible explanations for this effect could include differences in the selective retention of digesta (and thus methanogens) in the rumen. A number of factors could influence this selectivity, including diet, dry matter intake, protozoal population size and physiological effects (e.g. stage of lactation; McCartney et al., 2013b). Another potential reason for higher faecal archaeol concentrations is the heterogeneous nature of the rumen contents. Ahvenjärvi et al. (2001) argued that a heterogeneous distribution of rumen microbial markers in different particle size fractions would lead to incorrect total rumen estimates. In particular, inconsistent distribution of the very fine particles (< 0.1 mm) which tend to settle in the reticulum (Hristov, 2007) could skew microbial abundance estimates as they make up 70-80% of total rumen bacteria (Legay-Carmier and Bauchart, 1989). Variation in the abundance of microbes within different sites in the rumen may explain why there was no clear relationship between TRC and faecal archaeol concentrations. It is likely that archaeol concentrations from reticulum digesta would have a better relationship with faecal archaeol concentrations because the reticulum contains a higher proportion of material that is ready to be expelled from the rumen.

4.6. Relationships between archaeol concentration in microbial fractions and rumen pH

There was a strong relationship between rumen pH and the concentration of archaeol in SAM, with a weaker relationship found for the concentration of archaeol in LAM. These results are in agreement with a previous study by Hook et al. (2011) who showed a positive correlation between rumen pH and methanogens per grams of wet

365 weight (pgww) for rumen solids (P = 0.018; R^2 = 0.97), but no relationship between
366 pH and methanogens pgww in rumen fluids. It was clear there was an inhibition of
367 methanogens by lower pH in these fractions. This may have been caused by the
368 reduced dehydrogenase activity of the fermentative bacteria *via* the increased partial
369 pressure of hydrogen in the rumen. As a consequence, less products of fermentation
370 (i.e. CO_2) would have been available to the methanogens for growth (Janssen, 2010).
371
372 Variation in relationships between archaeol concentration and rumen pH for the
373 different fractions may be related to differences in the composition of the cell
374 membrane. For example, some methanogens may contain a higher proportion of
375 tetraethers in their cell membrane (Schouten et al., 2013). Furthermore, we have
376 shown significant effects of diet forage/concentrate ratio on the archaeol/tetraether
377 ratio in faeces (McCartney et al., 2013d). In theory, the methanogens could adapt their
378 membranes to contain more tetraethers in more 'challenging' rumen environments in
379 order to minimise the loss of protons and thus conserve energy. In this case, the liquid
380 fraction may contain a higher proportion of tetraethers in order for the slow-growing
381 methanogens to survive with a higher flow rate through the rumen.
382
383 The poorer relationship between pH and archaeol concentration in LAM may be due
384 to the considerably lower abundance of methanogens associated with the fluid
385 fraction. Alternatively, it may be that SAM are more sensitive to the effects of the WC
386 diet and low pH. Interestingly, no significant effect of pH was seen for archaeol
387 concentration in the WRC. This may be because methanogens in the loosely-adherent
388 SAM and LAM fractions are more sensitive to changes in the rumen than
389 methanogens found elsewhere such as protozoa and small, highly digested particles.

For example, protozoa have a stabilizing effect on pH (Veira et al., 1983) so effects will not be as large. In addition, the high density of microbes in the small, highly digested particles may exhibit a protective mechanism against pH. For example, Li et al. (2001) found that a higher density of microbes and the production of biofilms improved cell survival when exposed to low pH.

5. Conclusions

The quantification of archaeol from the ruminant digestive tract has provided an insight into the abundance and kinetics of methanogens when animals were consuming a WC based diet in comparison to a PRG diet. The combination of the assumed higher passage rates (Dewhurst et al., 2009) and low rumen pH for the WC diet reduced archaeol concentrations in all samples studied, and particularly affected archaeol concentration in SAM. Archaeol concentrations in the isolated microbial fractions were lower than expected, which highlights the importance of representative rumen sampling and the efficient extraction of methanogens from the feed.

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411 **REFERENCES**

412 Agricultural Research Council. 1980. The nutrient requirements of ruminant
413 livestock. Commonwealth Agricultural Bureau, Farnham Royal, UK.

414 Ahvenjärvi, S., Skiba, B., Huhtanen, P., 2001. Effects of heterogeneous digesta
415 chemical composition on the accuracy of measurements of fiber flow in dairy cows. J.
416 Anim. Sci. 79, 1611-1620.

417 Beauchemin, K.A., Kreuzer, M., F. O'Mara, F., McAllister, T.A., 2008. Nutritional
418 management for enteric methane abatement: a review. Aust. J. Exp. Agric. 48, 21-27.

419 Conrad, R., Phelps, T.J., Zeikus, J.G., 1985. Gas metabolism evidence in support of
420 the juxtasposition of hydrogen-producing and methanogenic bacteria in sewage sludge
421 and lake sediments. Appl. Environ. Microbiol. 50, 595-601.

422 Dewhurst, R.J., Moorby, J.M., Dhanoa, M.S., Evans, R.T., Fisher, W.J., 2000. Effects
423 of altering energy and protein supply to dairy cows during the dry period. 1. Intake,
424 body condition, and milk production. J. Dairy Sci. 83, 1782-1794.

425 Dewhurst, R.J., Delaby, L., Moloney, A., Boland, T., Lewis, E., 2009. Nutritive value
426 of forage legumes used for grazing and silage. Irish J. Agric. Food Res. 48, 167-187.

427 Ding, X., Long, R., Zhang, Q., Huang, X., Guo, X., Mi, J., 2012. Reducing methane
428 emissions and the methanogen population in the rumen of Tibetan sheep by dietary
429 supplementation with coconut oil. Trop. Anim. Health Prod. 44, 1541-1545.

430 Evans, E., 1981a. An evaluation of the relationships between dietary parameters and
431 rumen solid turnover rate. Can. J. Anim. Sci. 61, 97-103.

432 Evans, E., 1981b. An evaluation of the relationships between dietary parameters and
433 rumen liquid turnover rate. Can. J. Anim. Sci. 61, 91-96.

434 Finlay, B.J., Esteban, G., Clarke, K.J., Williams, A.G., Embley, T.M., Hirt, R.P.,
435 1994. Some rumen ciliates have endosymbiotic methanogens. FEMS Microbiol. Lett.
436 117, 157-162.

437 Frey, J.C., Pell, A.N., Berthiaume, R., Lapierre, H., Lee, S., Ha, J.K., Mendell, J.E.,
438 Angert, E.R., 2009. Comparative studies of microbial populations in the rumen,
439 duodenum, ileum and feces of lactating dairy cows. J. Appl. Microbiol. 108, 1982-
440 1993.

441 Gill, F.L., Dewhurst, R.J., Dungait, J.A.J., Evershed, R.P., Ives, L., Li, C.S., Pancost,
442 R.D., Sullican, M., Bera, S., Bull, I.D., 2010. Archaeol – a biomarker for foregut
443 fermentation in modern and ancient herbivorous mammals? Org. Geochem. 41, 467-
444 472.

445 Gill, F.L., Dewhurst, R.J., Evershed, R.P., McGeough, E., O'Kiely, P., Pancost, R.D.,
446 Bull, I.D., 2011. Analysis of archaeal ether lipids in bovine feces. Anim. Feed Sci.
447 Technol. 166-167, 87-92.

448 Gu, M.J., Alam, M.J., Kim, S.H., Jeon, C.O., Chang, M.B., Oh, Y.K., Lee, S.C., Lee,
449 S.S., 2011. Analysis of methanogenic archaeal communities of rumen fluid and rumen
450 particles from Korean black goats. Anim. Sci. J. 82, 663-672.

451 Hammond, K.J., Hoskin, S.O., Burke, J.L., Waghorn, G.C., Koolaard, J.P., Muetzel,
452 S., 2011. Effects of feeding fresh white clover (*Trifolium repens*) or perennial
453 ryegrass (*Lolium perenne*) on enteric methane emissions from sheep. Anim. Feed Sci.
454 Technol. 166-167, 398-404.

455

456 Hook, S.E., Steele, M.A., Northwood, K.S., Wright, A.D.G., McBride, B.W., 2011.
457 Impact of high-concentrate feeding and low ruminal pH on methanogens and protozoa
458 in the rumen of dairy cows. Microbial Ecol. 62, 94-105.

459 Hristov, A.N., 2007. Comparative characterization of reticular and duodenal digesta
460 and possibilities of estimating microbial outflow from the rumen based on reticular
461 sampling in dairy cows. J. Anim. Sci. 85, 2606-2613.

462 Huguet, C., Urakawa, H., Martens-Habbena, W., Truxal, L., Stahl, D.A., 2010.
463 Changes in intact membrane lipid content of archaeal cells as an indication of
464 metabolic status. Org. Geochem. 41, 930-934.

465 Hummel, J., Südekum, K.H., Bayer, D., Ortmann, S., Streich, W.J., Hatt, J.M.,
466 Clauss, M., 2009. Physical characteristics of reticuloruminal contents of oxen in
467 relation to forage type and time after feeding. J. Anim. Phys. Anim. Nutr. 93, 209-
468 220.

469 Janssen, P.H., 2010. Influence of hydrogen on rumen methane formation and
470 fermentation balances through microbial growth kinetics and fermentation. Anim.
471 Feed Sci. Technol. 160, 1-22.

472 Jones, D.I.H., Hayward, M.V. 1975. The effect of pepsin treatment of herbage on the
473 prediction of dry matter digestibility from solubility in fungal cellulose solutions. J.
474 Sci. Food Agric. 26, 711-718.

475 Lana, R.P., Russell, J.B., Van Amburgh, M.E., 1998. The role of pH in regulating
476 methane and ammonia production. J. Anim. Sci. 76, 2190-2196.

477 Lee, H.J., Jung, J.Y., Oh, Y.K., Lee, S.S., Madsen, E.L., Jeon, C.O., 2012.
478 Comparative survey of rumen microbial communities and metabolites across one

caprine and three bovine groups, using bar-coded pyrosequencing and ^{1}H nuclear magnetic resonance sprectroscopy. Appl. Environ. Microbiol. 78, 5983-5993.

Legay-Carmier, F., Bauchart, D., 1989. Distribution of bacteria in the rumen contents of dairy cows given a diet supplemented with soya-bean oil. Br. J. Nutr. 61, 725-740.

Li, Y.H., Hanna, M.N., Svensäter, G., Ellen, R.P., Cvitkovitch, D.G., 2001. Cell density modulates acid adaptation in *Streptococcus mutans*: implications for survival in biofilms. J. Bacteriol. 183, 6875-6884.

Martín-Orúe, S.M., Balcells, J., Zakraoui, F., Castrillo, C., 1998. Quantification and chemical composition of mixed bacteria harvested from solid fractions of rumen digesta: effect of detachment procedure. Anim. Feed Sci. Technol. 71, 269-282.

McCartney, C.A., Bull, I.D., Yan, T., Dewhurst, R.J., 2013a. Assessment of archaeol as a molecular proxy for methane production in cattle. J. Dairy Sci. 96, 1211-1217.

McCartney, C.A., Bull, I.D., Dewhurst, R.J., 2013b. Chemical markers for rumen methanogens and methanogenesis. Animal 7 (s2), 409-417.

McCartney, C.A., Bull, I.D., Waters, S.M., Dewhurst, R.J. 2013c. Technical note: Comparison of biomarker and molecular biological methods for estimating methanogen abundance. J. Anim. Sci. 91, 5724-5728.

McCartney, C.A., Bull, I.D., Dewhurst, R.J. 2013d. Changes in the ratio of tetraether to diether lipids in cattle faeces in response to altered dietary ratio of grass silage and concentrates. Advances in Animal Biosciences, Proceedings of the 5th Greenhouse Gases and Animal Agriculture Conference 4, 563.

Merry, R.J., McAllan, A.B., 1983. A comparison of the chemical composition of mixed bacteria harvested from the liquid and solid fractions of rumen digesta. Br. J. Nutr. 50, 701-709.

503 Morgavi, D.P., Martin, C., Jouany, J.P., Ranilla, M.J., 2012. Rumen protozoa and
504 methanogenesis: not a simple cause-effect relationship. Br. J. Nutr. 107, 388-397.

505 Mould, F.L., Kliem, K.E., Morgan, R., Maurico, R.M., 2005. In vitro microbial
506 inoculum: A review of its function and properties. Anim. Feed Sci. Technol. 123-124,
507 31-50.

508 Pei, C.X., Mao, S.Y., Cheng, Y.F., Zhu, W.Y., 2010. Diversity, abundance and novel
509 16S rRNA gene sequences of methanogens in rumen liquid, solid and epithelium
510 fractions of Jinnan cattle. Animal. 4, 20-29.

511 Poppi, D.P., Minson, D.J., Ternouth, J.H. 1981. Studies of cattle and sheep eating leaf
512 and stem fractions of grasses. III. The retention time in the rumen of large feed
513 particles. Aust. J. Agri. Res. 32, 123-127.

514 Rutter, S.M., Orr, R.J., Penning, P.D., Yarrow, N.H., Champion, R.A., 2002.
515 Ingestive behaviour of heifers grazing monocultures of ryegrass or white clover. Appl.
516 Anim. Behav. Sci. 76, 1-9.

517 Schouten, S., Hopmans, E.C., Sinninghe Damsté, J.S., 2013. The organic
518 geochemistry of glycerol dialkyl glycerol tetraether lipids: A review. Org. Geochem.
519 54, 19-61.

520 Shin, E.C., Choi, B.R., Lim, W.J., Hong, S.Y., An, C.L., Cho, K.M., Kim, Y.K., An,
521 J.M., Kang, J.M., Lee, S.S., Kim, H., Yun, H.D., 2004. Phylogenetic analysis of
522 archaea in three fractions of cow rumen based on the 16S rDNA sequence. Anaerobe
523 10, 313-319.

524 Van Kessel, J.A.S., Russell, J.B., 1996. The effect of pH on ruminal methanogenesis.
525 FEMS Microbiol. Ecol. 20, 205-210.

526 Veira, D.M., Ivan, M., Jui, P.Y., 1983. Rumen ciliate protozoa: effects of digestion in
527 the stomach of sheep. J. Dairy Sci. 66, 1015-1022.

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533 Figure captions

534 **Figure 1.** Relationship between rumen pH and the concentration of archaeol (mg/kg
535 DM) in the liquid- and solid-associated microbial fraction

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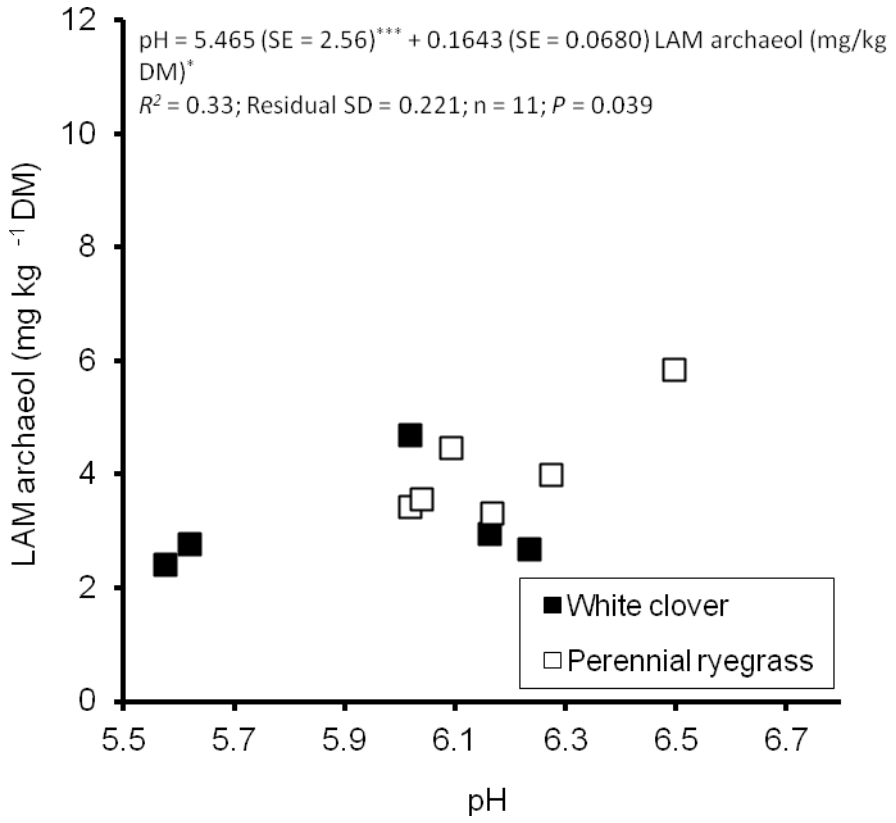

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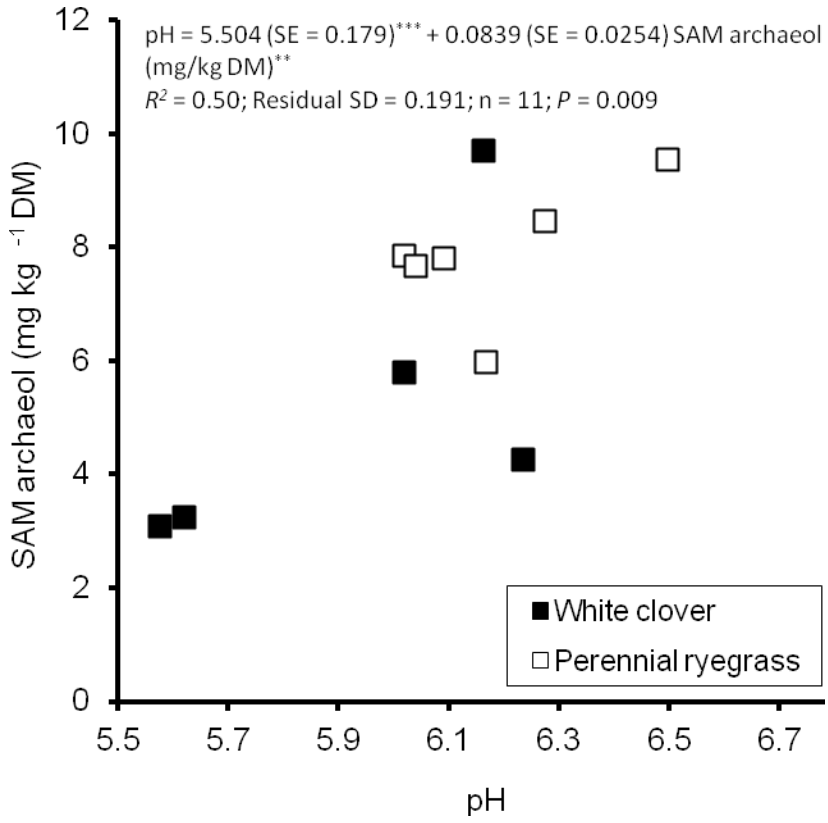

Figure 1.

Relationship between rumen pH and the concentration of archaeol (mg/kg DM) in the liquid- and solid-associated microbial fraction

Table 1. Chemical composition of the feeds used in this study

Component (g/kg DM, unless stated otherwise)	Perennial ryegrass	White clover	Concentrates
DM (g/kg)	113	94.0	870
OM	899	886	914
NDF	486	231	279
ADF	269	211	142
Ether extract	30.6	19.9	54.1
Crude protein (N×6.25)	216	309	200
Starch	-	-	283
Neutral cellulase-gammanase digestibility	-	-	823
Digestible organic matter	663	749	-
Water-soluble carbohydrates	96.1	51.0	89.2