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Methane emission, digestive characteristics and faecal archaeol in heifers fed diets based on silage from brown midrib maize as compared to conventional maize

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The aim of the present experiment was to compare silage prepared from maize having a brown midrib (BMR) mutation with control (CTR) maize to identify their effects on enteric methane emission, digesta mean retention time (MRT), ruminal fermentation and digestibility. In addition, the utility of archaeol present in faecal samples was validated as a proxy for methane production. Seven German Holstein heifers were fed total mixed rations with a maize-silage proportion (either BMR or CTR) of 920 g/kg dry matter (DM) in a change-over design. Heifers were fed boluses with markers to measure MRT; faeces was collected for 7 days and rumen fluid was collected on the penultimate day. Methane emission was measured in respiration chambers on one day. Data were analysed by t-test and regression analysis. DM intake did not differ between the two diets. The apparent digestibility of DM and most nutrients was unaffected by diet type, but apparent digestibility of neutral and acid detergent-fibre was higher in those heifers fed BMR than in those fed CTR. Comparisons between diets revealed no difference in particle or solute MRT in the gastro-intestinal tract and the reticulorumen. Concentrations of short chain fatty acid and ammonia in rumen fluid and its pH were not affected by silage type. Independent of the mode of expression [l/d, l/kg DM intake, l/kg digested organic matter], methane emissions were not affected by maize-silage type, but with BMR, there was a trend toward lower methane production per unit of digested neutral detergent fibre than there was with CTR silage. Results of the present study show that feeding heifers BMR silage does not increase methane emissions despite a higher fibre digestibility as compared to CTR silage. Therefore, it is assumed that improvements in animal productivity achieved by feeding BMR silage, as some studies have reported, can be obtained without extra environmental cost per unit of milk or meat. Neither faecal archaeol content [µg/g] nor daily amount excreted [mg/d] is suitable to predict methane production in absolute terms [l per day]. However, faecal archaeol content has a certain potential for predicting the methane yield [l per kg DM intake and day] of individual animals.

Keywords: hybrid varieties, methane, retention, rumen fermentation, digestibility, Holstein-Friesian

1. Introduction

Maize with the brown midrib (BMR) mutation is of interest to animal nutrition because inclusion of BMR in the diet has been found to increase intake and milk yield compared to conventional maize silage (Oba and Allen 1999, 2000b; Stone et al. 2012; Holt et al. 2013). These effects likely are caused by higher fibre digestibility (observed in vivo: Rook et al. 1977; Oba and Allen 2000b; Gorniak et al. 2014b; in vitro: Oba and Allen 1999, 2000b) and, therefore, may be accompanied by increased enteric methane emission per unit of input or product. On the other hand, increased dry matter intake (DMI) may lead to shorter passage time (increased passage rate), which lowers methane production. However, the effect on methane production and, thus on the environmental footprint, of feeding BMR instead of conventional maize hybrids is far from clear. Only a few partly inconsistent in vivo reports exist on the effect of BMR feeding on ruminal fermentation (Sommerfeldt et al. 1979; Oba and Allen 2000a; Greenfield et al. 2001; Castro et al. 2010), digesta passage time (Oba and Allen 2000b; Castro et al. 2010; Gorniak et al. 2014a) and enteric methane emissions (Tine et al. 2001) as compared to conventional maize silage. The studies cited focussed on selected variables only, making it difficult to relate the effects of BMR on digestive characteristics to those on methanogenesis.

One limitation when performing studies on methane emissions from ruminants is that they require laborious and costly techniques. Accordingly, there is ongoing research to find proxies for ruminal methanogenesis. One candidate is the compound archaeol, a core membrane lipid ubiquitous in methanogenic Archaea that can be recovered from the faeces of ruminants (Gill et al. 2010). Gill et al. (2011) reported a positive relationship between enteric methane production (expressed per unit of DMI) and faecal archaeol content for individual

data of steers; McCartney et al. (2013b) reported it for individual data of lactating cows, and McCartney et al. (2013a) reported it for treatment means of three studies. However, in the studies cited, regression analysis revealed low coefficient of determination (R^2), and McCartney et al. (2014) found no relationship between archaeol in total rumen content and the corresponding faeces.

The present study tested the hypothesis that feeding silage prepared from BMR instead of a conventional maize hybrid will not alter methane emission per unit of digested organic matter (OM) because of the balance between increased fibre digestibility and shorter reticuloruminal passage time (increased DMI) on a BMR-based diet. In this case, any extra milk or meat derived by feeding this maize hybrid will be obtained without extra environmental cost. This approach validated the utility of faecal archaeol as a proxy for enteric methane production.

2. Materials and methods

2.1. Animals, measurement of zootechnical data and feeding

The BMR-maize genotype used was the experimental hybrid 'SUM 2368' (Saaten-Union GmbH, Isernhagen, Germany). The CTR-maize hybrid was 'Ronaldinio' (KWS-Saat AG, Einbeck, Germany). The latter was chosen as typically representative of medium-maturing silage-maize varieties regarding flowering time, plant height, cold sensitivity, susceptibility to lodging, tillering, maturation, starch content and digestibility (Anonymous 2013). Both BMR and CTR maize hybrids were grown in 2010 at the experimental station of the Institute of Animal Nutrition, Friedrich-Loeffler-Institute, Braunschweig, Germany. Maize plants were harvested in the dough stage. Cutting height was approximately 18–20 cm, chopping length was 5.5 mm and maize grains were broken at harvesting. Harvest dates for BMR and CTR were 12 and 11 October, respectively. Corresponding DM contents at harvest were 334 (BMR) and 344 (CTR) g/kg, and the DM yields per hectare were 17.8 t and 20.8 t,

respectively. Silages were prepared in big bales sealed with stretch foil. The present experiment used the same silages as in studies by Gorniak et al. (2014a; 2014b).

The animal experiment was conducted in compliance with German legislation on the use of experimental animals (Registration No. LALLF M-V/TSD/7221.3-1.1-068/11) in 2011 at the Leibniz Institute for Farm Animal Biology. The experiment used seven ovarectomised German Holstein heifers, averaging 20 months old and 589 ± 47 kg in weight. These heifers previously had participated in an experiment related to reproduction. During a 25-day preliminary period, animals were gradually adapted to a high maize-silage proportion of 920 g/kg DM offered in a total mixed ration (TMR) that also contained hay (50 g/kg DM) and soybean meal (30 g/kg DM). The individual diet was supplemented on top with 200 g/d mineral-vitamin mixture (Rinderstolz 9522, Salvana Tierernährung, Kl.-O. Sparrieshoop, Germany), containing 200 g Ca, 50 g P, 80 g Na, 60 g Mg, 8 g Zn, 5 g Mn, 0.5 g Cu, 60 mg J, 50 mg Se, 40 mg Co, 1,000,000 IU vitamin A, 200,000 IU vitamin D3 and 4500 IU vitamin E per kg DM. Each animal was studied on both diets in turn. Because there were 'only' four respiration chambers, animals were divided into two groups. Hence, the two silage types, BMR and CTR, were compared in a four-period change-over design, with 14-day experimental periods during which the animals were fed BMR and CTR. Intake was measured from days 7–13, and faeces was sampled from days 8–14. A 1-day respiration chamber stay took place on day 12 and collection of ruminal fluid on day 13. Throughout the preliminary and experimental periods, the animals were fed ad libitum twice daily. The TMR offered and the refusals were quantified daily by weighing, and representative samples of each were stored frozen at −20 °C.

2.2. Assessment of digesta passage, digestibility and faecal archaeol excretion

Two markers for measuring MRT, cobalt ethylendiamintetraacetate (Co-EDTA), a solute marker, and chromium (Cr)-mordanted fibre, a 2-mm particle marker, were prepared

according to Udén et al. (1980). Before mordanting with Cr, grass hay was dried at 40 °C and ground through 2-mm square-hole screens with a cutting mill (SM 2000, Retsch, Haan, Germany). Dust and particles smaller than desired were removed by dry sieving by hand with a 2-mm square-hole screen. Then, particles were incubated with 33 g sodium dichromate dihydrate per 100 g particles. After mordanting and washing using a 1-mm square-hole screen, particles were dried at 65 °C. The contents realised in solute and particle markers amounted to 149 g Co/kg Co-EDTA and 61 g Cr/kg DM of Cr-mordanted fibre. The marker dosages applied were 60 g Cr-mordanted fibre per animal (equivalent to approx. 0.1 g/kg body mass) and 6 g Co-EDTA per animal (equivalent to 0.01 g/kg body mass). Markers were fed as a pulse dose shortly before the regular morning feeding on day 8 of the experimental period. For dosing, Co-EDTA was dissolved in approx. 30 ml of tap water, mixed with the particle marker and offered at time zero (t_0) with a portion of feed. The markers were consumed within 20 to 60 min; the average time period for intake was set as t_0 in subsequent calculations. After 60 min, any refusals of marker were removed and the regular feed provided. Faecal grab samples (200 to 400 g) were collected regularly from days 8–14 of the experimental period and stored frozen at −20 °C. The target collection times were 4, 8, 16, 24, 28, 32, 40, 48, 52, 56, 72, 80, 96, 104, 120, 128 and 152 h after marker feeding. Faeces voided in the first two nights (i.e., from days 8–9 and from days 9–10) was sampled and treated as one defaecation unit, and an average time between the last sampling in the evening and the first sampling in the morning was calculated.

In the present study, acid insoluble ash (AIA) was used as internal marker to estimate faecal output and digestibility. For archaeol analysis, one pool sample per animal and diet was generated from faecal subsamples obtained on days 12–14 of the experimental period, close to the time of methane measurement, which took place on day 12. The amount of daily excreted archaeol [mg/d] was calculated from the total faecal dry matter excreted per day (data not shown) and the archaeol content in faecal dry matter [µg/g].

2.3. Rumen fluid sampling

On day 13 of the experimental period, rumen fluid (approx. 300 mL) was collected in a thermos bottle via an oesophageal tube at 1015 h, 3 h after morning feeding. Rumen fluid was strained through four layers of gauze (1-mm pore size) and analysed for pH and ammonia concentration. For details see section 2.5. Rumen fluid was stored frozen at -20 °C until analysis of short chain fatty acids (SCFA).

2.4. Measurement of enteric methane emission

During the 25-day preliminary period, all animals were halter-trained and adapted to the respiration chambers via three stays for several hours without measurement. On day 11 of the experimental period at 1500 h, cows were transferred to open-circuit respiration chambers at an ambient temperature of 15 °C, 60% relative humidity and light cycle ranging from 0600–1900 h (for more details, see Derno et al. 2009). The 24-h measurements of individual methane production were started on day 12 of the experimental period at 0700 h (16 h after transfer to the chambers). Methane concentrations of ingoing and outgoing air were analysed by infrared-absorption (UNOR 600, Maihak, Hamburg, Germany) at time intervals of 6 min. Air flow through the chambers was recorded with a differential-pressure type V cone flow meter (McCrometer, Hemet, CA, USA). Cows were fed ad libitum with equal-sized offers of feed at 0700 and 1500 h. Feed intake was determined by feed-weight reduction as measured by a scale connected to an electronic registration device. Cows had free access to water, and water intake was recorded by water meters. Average body weight was calculated from measurements directly before and after animals were transferred to respiration chambers.

2.5. Laboratory analyses

Samples of TMR, refusals and faeces were freeze-dried for nutrient analysis. From the TMR offered, eight samples (4 periods • 2 silage types) were analysed. Samples of ground feed refusals and faeces from days 8–14 of the experimental week were pooled, resulting in two samples per animal, and diet. Samples were ground through 1-mm round-hole screens with a rotary cutting mill (Brabender, Duisburg, Germany). All analyses, except for archaeol, were made in duplicate. DM contents of the composited samples were determined by drying a subsample at 103 °C to constant weight. Samples were ashed in a muffle furnace at 600 °C to obtain total ash. Total ash was analysed for AIA content by treatment with hydrochloric acid (Van Keulen and Young 1977). Nitrogen (N) was determined by the Dumas method in an Elementar rapid N III Analyser (Elementar Analysensysteme, Hanau, Germany). Crude protein (CP) was calculated as 6.25 • N. Neutral detergent fibre (NDF), acid detergent fibre (ADF) and acid detergent lignin were analysed according to Van Soest (1967) and Van Soest et al. (1991) using the Ankom^{200} Fibre Analyser (Ankom, Macedon NY, USA). NDF was determined with heat-stable amylase but without sodium sulphite in the detergent solution. Both NDF and ADF are expressed exclusive of residual ash. Acid detergent lignin was determined by solubilisation of cellulose with sulphuric acid. In faeces samples, undigested plant N was estimated by analysing N content of the neutral detergent residue. Metabolic faecal N of microbial and endogenous origin was calculated by subtracting faecal NDF-bound N from total faecal N. To determine faecal marker contents, ground samples (0.3 g) were wet-digested by microwave with 8 ml of 65% nitric acid and 2 ml of 30% hydrogen peroxide with a MDS-2000 (CEM GmbH, Kamp-Lintfort, Germany; 630 W, 2450 MHz). Digested samples were analysed for Co and Cr contents by atomic absorption spectroscopy (Perkin Elmer AAS 3300, Ueberlingen, Germany).

Rumen fluid pH was measured directly after collection with a glass electrode (N 1042A, pH meter CG 841, Schott, Mainz, Germany). Ammonia concentration in rumen fluid was determined by the micro-diffusion method (Voigt and Steger 1967). To analyse SCFA, a

mixture of 5 ml rumen fluid and 2 ml iso-caproic acid (internal standard) was centrifuged at 3000 • g at 4 °C for 20 min. Then, the supernatant was filtered through filters with 0.22-µm sized pores to measure SCFA concentration by gas chromatography (Shimadzu GC-14A, Shimadzu Corporation, Kyoto, Japan) on a capillary column (Free Fatty Acid Phase, 25 m • 0.25 mm, Machery-Nagel, Düren, Germany).

To analyse archaeol (2,3-diphytanyl-O-sn-glycerol), lipids were extracted from dried, ground faeces using the methodology of McCartney et al. (2013b). Briefly, as an internal standard, 1,2-di-O-rac-hexadecyl glycerol (M_{std}; Santa Cruz Biotechnology Inc., CA) was added to ~500 mg of each sample before lipid extraction. Total lipid extract was obtained using an extraction procedure modified from the one used by Bligh and Dyer (1959). Acid methanolysis was used to cleave polar head groups from archaeol. Silica column chromatography was applied to separate the total lipid extract into an apolar fraction and an alcohol fraction. For the alcohol fraction, analytes were derivatised to their respective trimethylsilyl (TMS) ethers by adding 50 µl of N,O-bis(trimethylsilyl)trifluoroacetamide containing 1% trimethylchlorosilane and 50 µl pyridine to the sample and heating at 70 °C for 1 h. Samples were dissolved in ethyl acetate prior to analysis by gas chromatography/mass spectrometry using a Trace 1300 GC coupled to an ISQ MS (Thermo Scientific, Hemel Hempstead, UK) equipped with a non-polar fused silica capillary column (CPSil-5CB, 50 m • 0.32 mm • 0.12 mm, Agilent J&W, Santa Clara CA, USA). The following temperature program was used: initial temperature 40 °C, rising to 130 °C at 20 °C/min, then rising to 300 °C at 4 °C/min and holding at 300 °C for 25 min. The ion source was maintained at 300 °C and the transfer line at 300 °C. The emission current was set to 50 µA and the electron energy to 70 eV. The analyser was set to scan m/z 50–650 with a scan cycle time of 0.6 s. As described by McCartney et al. (2013b), a calibration curve was produced by analysing 0.1 µg of the internal standard (M_{std}) together with 0.05 µg, 0.25 µg, 0.5 µg, 1.5 µg and 2.5 µg of archaeol standard (M_x; 1,2-di-O-phytanyl-sn-glycerol; Avanti Polar Lipids Inc., Alabaster,

AL, USA) by GC-MS. Ratio of the peak area of M_x to the peak area of M_{std} was plotted against the ratio of M_x to M_{std}. The resulting regression equation for the slope was rearranged to allow calculation of the content of archaeol present in faecal DM.

2.6. Calculations and statistical analyses

To calculate the variables describing digesta passage through the digestive tract, faecal marker content was corrected for the highest level of the baseline content of Co and Cr in the faeces collected prior to marker application. In some animals, faecal marker content did not decline to the baseline level but declined in small steps to a slightly elevated level. Therefore, faecal marker content below 1% of peak content was set to 0 (adapted from Bruining and Bosch 1992) to avoid an artificial apparent increase in absolute terms in passage measures.

MRT of the digesta in the total gastrointestinal tract (GIT) was calculated according to Thielemans et al. (1978). This method calculates the area under the excretion curve and defines MRT as the time that separates the total area under the excretion curve into two equal parts:

$$MRT = \sum(t_i \bullet dt * c_i) / \sum(dt \bullet c_i) \quad (1)$$

with t_i = time after marker application [h], dt = time interval represented by marker content (calculated as $(((t_{i+1} - t_i) + (t_i - t_{i-1})) / 2)$ and c_i = faecal marker content at time i (mg/kg DM). Solute MRT in the reticulorumen ($MRT_{solute}RR$) was calculated according to Grovum and Williams (1973); this calculation is based on decrease of the faecal solute marker content c_i at time t [mg/kg DM] with time after marker application t_i [h] according to the equation:

$$\ln c_i = -k \bullet t_i + b \quad (2)$$

with k = rate constant [h^{-1}] and b = intercept. The reciprocal of k represents the solute MRT in the RR (Hungate 1966).

$MRT_{particle}RR$ was calculated based on the assumption that solute and particles do not differ in passage characteristics distal to the RR, which has been confirmed empirically by

Grovum and Williams (1973), Kaske and Groth (1997) and Mambrini and Peyraud (1997), using the following equation:

$$MRT_{particle}RR = MRT_{particle}GIT - (MRT_{solute}GIT - MRT_{solute}RR) \quad (3)$$

The selectivity factor (SF)—defined as the quotient of particle over solute MRT, or as the quotient of large over small particle MRT (Lechner-Doll et al. 1990)—was calculated for both the total GIT and the RR.

The scant literature data available so far on individual measurements of methane emission and faecal archaeol were compiled from Gill et al. (2011) and McCartney et al. (2013b) and combined with data from the present study in regression analysis.

Statistical analyses were carried out in Systat 11 (Erkrath, Germany). Data were analysed by the Student's (paired) t-test, the Mann-Whitney rank sum test and linear regression. Apart from the regression equations, standard error of estimate (SEE) and R^2 are given. The significance level was set to $\alpha = 0.05$ and p-values between 0.05 and 0.10 were considered as trends. Results are shown as arithmetic mean values ± standard deviation.

3. Results

3.1. Silage composition, intake and digestibility

The two types of maize silage and the corresponding TMR types had comparable contents of OM, CP, NDF and ADF (Table 1). TMR on a BMR basis contained numerically less ether extract and lignin than did TMR on a CTR basis. Across the entire experimental week, DMI did not differ between diets (Table 2). However, during the 24-hour methane measurement, DMI was higher (n = 7, 8.7 ± 1.7 vs. 7.4 ± 2.1 kg, p = 0.029) in BMR-fed than in CTR-fed cows, although the average daily DMI within silage types did not differ between the 24-hour methane measurement and the entire experimental week (data not shown). Daily excretion of faecal DM estimated with AIA as the internal marker did not differ (p = 0.78) between groups (data not shown). The apparent digestibility of DM, OM, N and ether extract did not differ

between diet types. However, the digestibility of NDF and ADF was higher ($p < 0.05$) in BMR-fed versus CTR-fed heifers (Table 2). Silage type had no effect on water intake (BMR: 16.0 ± 5.8 vs. CTR: 14.7 ± 4.9 l, $p = 0.25$).

Although the maize-silage type had no effect on N intake (Table 2), daily amounts of N excreted tended ($p < 0.1$) to be higher in cows fed BMR than in those fed CTR. Groups did not differ in excretion of N incorporated in faecal NDF (undigested plant N). Metabolic faecal N expressed as percent of total N did not differ between groups. Thus, the slightly higher faecal N excretion of BMR-fed heifers compared to CTR-fed heifers can be attributed to both metabolic N and plant-derived N.

3.2. Ruminal fermentation characteristics and digesta passage time

Groups did not differ in ruminal pH, ammonia concentration, total SCFA concentration, proportions of acetate, iso-butyrate, valerate and caproate in total SCFA nor in acetate:propionate ratio (Table 3). However, in the rumen fluid of BMR-fed cows, the proportion of propionate in total SCFA was higher compared to that of CTR-fed cows, and the proportion of iso-valerate was lower. In addition, there was a trend ($p < 0.1$) toward a higher butyrate proportion.

Figure 1 presents a typical excretion pattern of passage markers. Irrespective of the maize-silage type fed, on average, particle MRT in the GIT and RR were 12 hours longer than that of solute MRT, resulting in a SF of 1.5–1.8 (Table 4). However, comparison between diets revealed no differences in particle or solute MRT in the GIT and RR or in the respective SF.

3.3. Enteric methane production and faecal archaeol excretion

Absolute methane emissions [l/d] and emissions relative to kg DMI or kg OM digested were not affected by maize-silage type (Table 5). However, there was a trend ($p < 0.1$) toward

lower methane emissions in BMR-fed cows than in CTR-fed cows when data were expressed as l/kg NDF digested. The maize-silage type had no effect on the amount of archaeol excreted daily with the faeces (Table 5). Accordingly, silage type had no effect on methane production either per unit of archaeol content or per unit of archaeol amount excreted (Table 5). Methane emission did not increase with increasing levels of archaeol content, both in our dataset alone (methane [l/d] = 245.8 + 2.45 x archaeol [µg/g DM], p = 0.19, SEE = 46.7, R^2 = 0.19, n = 14) and when the literature data were included (methane [l/d] = 364.2 + 0.94 x archaeol [µg/g DM], p = 0.61, SEE = 134.0, R^2 = 0.01, n = 42). With increasing levels of archaeol excretion, methane emission tended to increase (Figure 2A, p = 0.08 in regression equation) with R^2 of 0.37; however, the pattern followed an exponential response, reaching a plateau rather than a linear relationship. Methane yield per unit of faecal archaeol content was lower in BMR-fed than in CTR-fed heifers (Table 5). Methane yield [l/(kg DMI • d)] did not increase with increasing archaeol content [µg/g DM] when considering only our dataset (y = 33.1 + 0.25 x, p = 0.26, SEE = 6.7, R^2 = 0.10, n = 14), but the inclusion of literature data revealed a linear relationship (Figure 2B, R^2 = 0.37).

4. Discussion

4.1. Characteristics of the silages and their inclusion in the test diets

Regarding composition of silage and the resulting TMR, data of the present study are consistent with the literature. The characteristically lower lignin but comparable NDF content of BMR as compared to conventional maize silages has been described repeatedly (e.g., Oba and Allen 1999; Greenfield et al. 2001). However, the animal experiment carried out in the present study was unusual with respect to the very high proportion of experimental silages in the TMR (920 g/kg DM) relative to previous studies (e.g., 450 and 600 g/kg DM in the experiments by Oba and Allen 1999, as well as Greenfield et al. 2001, respectively). Thus, we

aimed at minimising masking effects of other dietary ingredients. This dietary approach was possible due to the use of heifers, which tolerate a diet not balanced for high production trait.

4.2. Intake

The effect on DMI of feeding BMR instead of conventional maize was not entirely clear. On the day when the animals were in the chambers, DMI was significantly higher, but during the experimental week, the small difference (9.9 vs. 9.2 kg/d) was numerical only. The majority of studies comparing BMR and CTR maize silage used lactating cows, and maize silage was not fed separately but as part of a mixed ration. These studies (e.g., Rook et al. 1977; Sommerfeldt et al. 1979; Stallings et al. 1982; Oba and Allen 1999, 2000a; Barriere et al. 2004; Castro et al. 2010) are consistent in reporting improved intake when using BMR as compared to CTR maize silage. There are only a few exceptions, and these report no difference in intake between maize hybrids (e.g., Frenchick et al. 1976; Keith et al. 1979; Greenfield et al. 2001, Singh et al. 2014). There is only one other study (Tine et al. 2001) where a comparably high proportion (980 g/kg DM) of BMR and CTR maize silage was used. However, in that study (Tine et al. 2001), the diet fed to dry cows was restricted to maintenance level; hence, no difference in intake could be observed. Thus, the present study is the first in which such a high proportion of BMR maize silage was fed ad libitum to cattle. It is assumed that in the present study, with similar intakes, both maize-silage types covered energy requirements of non-pregnant heifers.

4.3. Ruminal fermentation characteristics

The high ruminal pH and low ammonia and total SCFA concentrations as compared to other studies (e.g., Gorniak et al. 2014a) can be explained by the high dietary proportion of forage, low DMI and, possibly, salivary dilution. Average pH values above 7 appear too high to be related to diet alone. Studies comparing effects of rumen sampling methods have shown that

rumen fluid samples taken by stomach tube tend to have a higher pH than those obtained via rumen fistula (Raun and Burroughs 1962: pH 6.4 vs. 6.2; Terre et al. 2013: pH 5.9 vs. 5.6), possibly as a result of salivary dilution. However, high pH values appeared consistently, indicating that all rumen fluid samples contained some saliva. In two animals in the present study, pH boli (KB 1000, Kahne Animal Health, Auckland, New Zealand) were placed in the rumen. The average pH value of the 24-hour measurement (at 6-minute intervals) in the two animals was 6.39 and 6.49 during CTR feeding and 6.26 and 6.36 during BMR feeding. Thus, pH values obtained from boli were one unit lower than in rumen fluid obtained via stomach tube (n = 14, Table 3), which may be explained by dilution of rumen fluid with saliva in the case of the oesophageal technique.

Ruminal fermentation characteristics mostly were unaffected by silage type and, thus, did not reflect differences in digestibility, which is in accordance with Oba and Allen (2000a,b). In addition, higher propionate concentrations in the rumen fluid of cows fed BMR compared to those fed CTR have been reported by Block et al. (1981) and can be ascribed to the higher starch content of the BMR maize silage. Furthermore, lower iso-valerate proportions with BMR have been reported by Greenfield et al. (2001) and Gorniak et al. (2014a). The trend toward higher ruminal butyrate proportions of total SCFA with BMR are consistent with a report by Gorniak et al. (2014b) of higher plasma concentrations of β-hydroxy butyric acid (BHBA) in lactating cows fed BMR than in those fed CTR.

4.4. Digesta passage and digestibility

In the present experiment, feeding BMR maize silage rather than CTR did not result in the expected shorter passage time. The major driver of passage time is feed intake (Luginbuhl et al. 1994; Clauss et al. 2007), which did not differ between treatments. Thus, an unchanged digesta passage time appears plausible. In the study by Oba and Allen (2000b), ruminal passage time was shorter and intake was higher in lactating cows fed BMR than in those fed

386 CTR. In the present study, at least by arithmetic means, higher intake (0.7 kg/d) and shorter
387 particle passage time (2 hours) was observed in BMR-fed cows than in CTR-fed cows. An
388 increased rate of solute flow from the rumen increases efficiency of microbial growth
389 (Harrison et al. 1975). Comparable solute passage times in BMR-fed and CTR-fed heifers and
390 similar ratios of particle to solute passage times suggest that treatments did not differ in the
391 degree of digesta washing (in sensu Lentle et al. 2006; Müller et al. 2011). Converting the
392 passage-rate results of Castro et al. (2010) into passage time (passage time [h] = (1/passage
393 rate [%/h]) • 100) and calculating the SF thereof (CTR: 35.2 h/7.8 h = 4.51; BMR: 27.2 h/7.8
394 h = 3.49) reveals a SF higher by one unit in CTR-fed cows than in BMR-fed cows. This
395 translates into a higher relative solute throughput with CTR than BMR in the study by Castro
396 et al. (2010), which could have enhanced microbial yield from the rumen in CTR-fed animals.
397 Castro et al. (2010) studied early lactating Holstein cows, thus, animals with a relatively high
398 protein requirement. The non-lactating, non-pregnant and ovarectomised heifers in the present
399 study presumably had a comparatively low overall protein requirement, rendering
400 unnecessary a higher microbial harvest from the rumen in either group.

401 The higher NDF and ADF digestibility found in cows fed BMR compared to those fed
402 CTR was expected and is in accordance with other studies (Rook et al. 1977; Oba and Allen
403 2000b; Gorniak et al. 2014b). It can be explained by the lower lignin content of BMR, which
404 is less restrictive of both microbial access to and degradation of cellulose and hemicellulose.
405 The studies cited used the higher fibre digestibility in BMR compared to CTR as an
406 explanation for the concomitantly observed higher DMI. Differences in DM digestibility often
407 are observed with different hybrid types (e.g., in sacco/in vitro Singh et al. 2012, 2014).
408 However, in the present study, the 7.5% increase in NDF digestibility with BMR did not lead
409 to an increase in DM and OM digestibility and, thus, did not lead to faster clearance of digesta
410 from the rumen (shorter passage time). Accordingly, other nutrients would have to be digested
411 to a lesser degree. Oba and Allen (2000b) reported that the starch in BMR is less ruminally

degradable than that of conventional maize, which further helps explain higher animal productivity via a larger ruminal bypass of starch. This could have happened even though, because of the higher starch content, BMR silage resulted in higher ruminal propionate proportions. In line with the present results, Tine et al. (2001) and Greenfield et al. (2001) reported improved fibre digestibility but similar intakes in cows fed BMR compared to those fed CTR. The study by Tine et al. (2001) is comparable to the present experiment in terms of silage proportion in the diet (980 g/kg DM) fed to dry cows. Greenfield et al. (2001) fed a mixed ration to late-lactating cows and reported a silage proportion of 600 g/kg DM.

In the present study, ADF digestibility of the CTR diet was lower ($p = 0.023$) than NDF digestibility. However, in BMR-fed cows, ADF digestibility was higher ($p < 0.001$) than NDF digestibility by 1.8% on average, which is unexpected due to the higher proportion of lignin in ADF. Numerically higher ADF digestibility than NDF digestibility has been observed in other studies of dairy cows, as reported in a meta-analysis of Gorniak and Hummel (2015). These authors concluded that linkages with lignin render higher proportions of hemicellulose indigestible (= NDF – ADF) than cellulose, thus balancing the effect of the higher lignin proportion (analysed together within ADF) of ADF. With respect to the present study, this would indicate that lignin rendered hemicellulose less digestible in BMR but not in CTR.

In the current experiment, the larger percentage (~57%) of faecal N consisted of metabolic faecal N, which is in accordance with the literature (Schwarm et al. 2009). Metabolic faecal N (% total faecal N) did not differ between cows fed BMR silage and those fed CTR silage, which is in line with the lack of difference between groups in digesta washing results (the ratio of $MRT_{particle}$ and MRT_{solute}, see previous section). The present study's result is consistent with the findings of Greenfield et al. (2001), in which treatment groups (BMR, CTR) had no effect on duodenal N-flow.

In the present study, faecal output was not measured, but estimated with AIA, which is a suitable marker if animals do not have the opportunity to incidentally ingest sand (Van Keulen and Young 1977; Huhtanen et al. 1994; Kavanagh et al. 2001), such as if it is introduced into the silages by unfavourable harvest procedures. This seems not to have been the case in the present study, as nutrient digestibility levels were similar to results reported by Tine et al. (2001), who fed diets that were predominately maize silage.

4.5. Methane emission

Although arithmetic means of absolute methane emissions were 40 l higher with BMR compared to CTR feeding, the groups did not differ significantly. More importantly, feeding BMR instead of a conventional maize hybrid did not increase methane emission per kilogram of digested OM. In fact, methane emissions expressed per kilogram of digested NDF tended to be lower with BMR than CTR silage, even though the degradation products of fibre, especially hydrogen, are the main substrates for methanogens. The similarity in methane emissions of cows on both diets may be attributed to two factors: higher fibre digestibility, leading to increased methane production, and higher dietary starch content and, thus, ruminal propionate concentration, leading to lower methane production. Both factors presumably levelled each other out. Methane production is known to be correlated to particle-passage time (Okine et al. 1989). Since passage time did not differ between treatments, similar methane emissions appear plausible. Our results are in line with the study by Tine et al. (2001), in which enteric methane production expressed as Mcal/d did not differ between dry cows fed at maintenance a BMR-based diet and those fed a CTR-based diet with a similarly high proportion of maize silage (980 g/kg DM).

4.6. Utility of faecal archaeol as a proxy for enteric methanogenesis of ruminants

462 For practical reasons, establishing faecal archaeol in routine testing of individual animals
463 would be restricted to content [µg/g] rather than daily excretion of archaeol [mg/d]. However,
464 from a theoretical point of view, it is not ideal to predict a variable describing an amount per
465 unit of time (methane in l/day) from a variable based on an amount per unit of mass (faecal
466 archaeol content in µg per g faecal DM). Another way involved predicting methane
467 production relative to DMI from faecal archaeol content, an approach followed by Gill et al.
468 (2011) and McCartney et al. (2013b). McCartney et al. (2013b) argued that it makes sense 'to
469 put it [methane production] on a similar basis to faecal archaeol, which was expressed relative
470 to faecal DM'. This approach has the disadvantage that individual DMI data need to be
471 assessed to calculate methane production in absolute terms [l per day]. In the present study,
472 accuracy of predictions was tested by all three methods described for relating faecal archaeol
473 and enteric methane.

474 Faecal archaeol as a proxy for methane production should be sufficiently robust
475 against various influences, such as diet, intake and digesta passage time. Figure 2B includes
476 results from the literature and our own measurements obtained from various diet types,
477 including grass silage and concentrate offered on a DM basis in ratios of 0.72:0.28 and
478 0.11:0.89 (Gill et al. 2011) or 0.50:0.50 and 0.30:0.70 (McCartney et al. 2013b) and maize
479 silage and concentrate provided in a ratio of 0.92:0.08 (the present study). With silage-based
480 (720 and 920 g/kg DM) diets, faecal archaeol content (median: 20.5 µg/g DM) was 2.6-fold
481 higher ($p < 0.001$, Mann-Whitney rank sum test) than with diets containing high proportions
482 (500, 700 and 890 g/kg DM) of concentrate (median: 8.0 µg archaeol/g DM) (see also data
483 distribution in Figure 2B).

484 The regression in which daily amounts of methane and archaeol excretion were
485 opposed (Figure 2A, present study data only, no literature data available) showed a positive
486 relationship that levelled out from about 70 mg archaeol/d onward. It could be speculated that
487 higher methane emissions are no longer well reflected by archaeol amount after a certain

488 threshold is passed and the methanogenic Archaea further increases in number (estimated by
489 the amount of archaeol in faeces), but metabolic activity per cell decreases concomitantly
490 (i.e., absolute methane emission does not increase any further). Difficulty in relating methane
491 emissions to Archaea counts has been described repeatedly for ruminal conditions (e.g.,
492 compiled by Soliva et al. 2003), but varying archaeol content in cells from different Archaea
493 species and orders cannot be excluded. The relationship of archaeol and methane at low faecal
494 archaeol amounts could not be confirmed when using content instead of amount excreted; this
495 was observed when the scant literature data (Gill et al. 2011, McCartney et al. 2013b)
496 available so far was including (data not shown). These results fit well with the reported lack
497 of relationship between archaeol in total rumen content and corresponding faeces (McCartney
498 et al. 2014), and they support the conclusion by McCartney et al. (2013a) to use faecal
499 archaeol as a general marker for methanogen abundance in the digestive tract rather than as a
500 methane proxy.
501 However, when including literature data (total n = 42), the regression between
502 methane per kg DMI and faecal archaeol content [µg/g] showed a positive relationship with
503 the same R^2 as in the study by McCartney et al. (2013b) (n = 16). In the study by Gill et al.
504 (2011), no regression analysis was given. Obviously, correction for intake is necessary to
505 ensure relationship of faecal archaeol content to methane production. This fits well with the
506 fact that intake is the major driver of methane production and, therefore, must be incorporated
507 into the regression. Self-evidently, the current regression needs to be complemented with
508 more data to validate this first data compilation of individual measurements.
509 Nonetheless, methodological differences between studies should be noted. In the
510 present study, archaeol analysis was carried out in a comparatively larger quantity of sample
511 (500 mg) but without replication (similar to Gill et al. 2011), which potentially could have
512 increased variation in faecal archaeol content. In contrast, McCartney et al. (2013b) used only
513 300 mg of faecal sample for extraction but carried out measurements in triplicate. Regarding

514 the archaeol amount expressed in mg/day (Figure 2A), it must be kept in mind that in the
515 present study, faecal output was not directly quantified but estimated with AIA as an internal
516 marker. In addition, in the present study, similar to the study by McCartney et al. (2013b),
517 methane measurement was performed in respiration chambers, measurements from which
518 likely were more accurate than those using the sulphur-hexafluoride tracer technique
519 performed by Gill et al. (2011).
520
521 **5. Conclusions**
522 Feeding heifers diets based on silage of brown midrib (hybrid: SUM 2368) instead of
523 conventional maize (hybrid: Ronaldinio) did not increase methane emissions per kilogram of
524 digested OM despite better fibre digestibility and unchanged OM digestibility. Therefore, we
525 assume that the higher animal productivity reported by others feeding diets based on brown
526 midrib maize silage can be obtained without extra greenhouse gas emissions per unit of milk
527 or meat. According to the present evaluation, faecal archaeol content has a certain potential to
528 predict the relative, but not absolute, methane-emission potential of individual animals.
529
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534
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670

671 **Table 1.** Chemical composition [g/kg dry matter] of
672 experimental maize silages (conventional or brown
673 midrib) and of respective total mixed ration (TMR), with
674 maize silage representing 920 g/kg of the TMR on a dry
675 matter basis.

	Control	Brown midrib
Maize silage[†] (n = 6)		
Organic matter	954 ± 2	951 ± 3
Crude protein	84 ± 3	82 ± 4
Ether extract	33 ± 3	28 ± 3
Neutral detergent fibre	497 ± 16	472 ± 29
Acid detergent fibre	272 ± 9	249 ± 11
Acid detergent lignin	35 ± 3	15 ± 1
Starch	257 ± 6	283 ± 11
TMR (n = 4)		
Organic matter	952 ± 3	952 ± 3
Crude protein	102 ± 10	99 ± 6
Ether extract	30 ± 3	24 ± 2
Neutral detergent fibre	483 ± 31	471 ± 26
Acid detergent fibre	262 ± 24	260 ± 16
Acid detergent lignin	28 ± 4	16 ± 4

676 **Notes**: Data are means ± standard deviations. [†]Silage data
677 taken from Gorniak et al. (2014a), where the same silages
678 were used in a different experiment.
679

680 **Table 2.** Intake, faecal excretion and digestibility of Holstein heifers fed diets
681 characterised by conventional and brown midrib maize silage.

	Control	Brown midrib	p-Value
	n = 7	n = 7	
Intake			
Dry matter [kg/day]	9.19 ± 1.29	9.88 ± 1.23	0.19
Organic matter [kg/day]	8.77 ± 1.24	9.42 ± 1.18	0.20
Nitrogen [g/day]	149 ± 21	152 ± 17	0.56
Neutral detergent fibre (NDF) [kg/day]	4.46 ± 0.89	4.63 ± 0.38	0.61
Faecal excretion†			
Total N [g/day]	61.2 ± 13.0	74.0 ± 11.7	0.08
NDF-bound faecal N [g/day]	26.1 ± 5.1	31.1 ± 5.5	0.20
Metabolic N‡ [% total faecal N]	57.2 ± 2.6	57.9 ± 5.3	0.60
Apparent digestibility† [%]			
Dry matter	67.9 ± 4.0	70.7 ± 3.4	0.17
Organic matter	70.1 ± 4.0	73.1 ± 3.4	0.14
Nitrogen	58.8 ± 6.9	51.4 ± 5.7	0.11
NDF	59.1 ± 4.6	66.6 ± 6.4	0.03
Acid detergent fibre	56.4 ± 3.9	68.4 ± 6.8	<0.01
Ether extract	86.6 ± 3.4	84.0 ± 3.5	0.29

682 **Notes**: Data are means ± standard deviations. p-values are the result of Student's
683 paired t-test between diet types. †Calculated with acid insoluble ash as a marker.
684 ‡Calculated as the difference of total N and NDF-bound N.
685

686 **Table 3.** Ruminal fermentation characteristics of Holstein heifers fed diets
687 characterised by conventional and brown midrib maize silage.

	Control n = 7	Brown midrib n = 7	p-Value
pH value	7.45 ± 0.30	7.22 ± 0.35	0.16
Ammonia [mmol/l]	4.68 ± 2.21	3.48 ± 1.37	0.21
SCFA total [mmol/l]	34.5 ± 13.0	43.3 ± 10.1	0.24
% of SCFA total			
Acetate	64.3 ± 2.7	64.8 ± 2.4	0.70
Propionate	16.7 ± 2.0	18.2 ± 2.4	0.04
Butyrate	11.8 ± 2.1	13.2 ± 2.4	0.09
iso-Butyrate	0.39 ± 0.51	0.24 ± 0.37	0.60
Valerate	1.27 ± 0.90	1.22 ± 0.94	0.92
iso-Valerate	3.16 ± 1.65	1.32 ± 1.26	0.03
Caproate	1.41 ± 1.36	1.02 ± 0.99	0.58
Acetate:propionate ratio	3.89 ± 0.33	3.61 ± 0.57	0.20

688 **Notes**: Data are means ± standard deviations. p-values are the result of Student's
689 paired t-test between diet types. SCFA, short chain fatty acids.
690

691 **Table 4.** Mean retention time (MRT) of solutes and particles in the gastro-
692 intestinal tract (GIT) and the reticulorumen (RR) as well as the selectivity
693 factor of Holstein heifers fed diets characterised by conventional and brown
694 midrib maize silage.

		Control n = 7	Brown midrib n = 7	p-Value diet type
MRT_{solute} [h]	GIT	26.6 ± 2.2	24.6 ± 5.0	0.33
	RR	17.7 ± 2.6	15.7 ± 3.2	0.29
$MRT_{particle}$ [h]	GIT	38.3 ± 3.0	36.1 ± 3.2	0.14
	RR	29.3 ± 4.9	27.4 ± 4.4	0.18
p-Value marker	GIT	<0.001	<0.001	
	RR	<0.001	<0.001	
Selectivity factor	GIT	1.45 ± 0.21	1.51 ± 0.25	0.57
	RR	1.67 ± 0.29	1.77 ± 0.30	0.47

695 **Notes**: Data are means ± standard deviations. p-values are the result of
696 Student's paired t-test between diet types (control, brown midrib) and
697 between passage markers (MRT_{solute} and $MRT_{particle}$). The selectivity factor
698 is defined as the ratio of $MRT_{particle}$ and MRT_{solute}.
699

700 **Table 5.** Enteric methane production, faecal archaeol and the ratio of methane
701 and archaeol in Holstein heifers (n = 7) fed conventional (control) or brown
702 midrib maize silage.

	Control n = 7	Brown midrib n = 7	p-Value
Methane production			
[l/d]	279 ± 48.9	319 ± 45.1	0.11
[l/(kg DMI • d)]	39 ± 4.8	38 ± 8.7	0.66
[l/(kg dOM • d)]	57 ± 5.1	53 ± 13	0.30
[l/(kg dNDF • d)]	130 ± 17.0	114 ± 28.8	0.098
Faecal archaeol			
Amount excreted [mg/d]	51.9 ± 13.4	71.8 ± 28.3	0.13
Content [µg/g DM]	18.6 ± 8.3	24.9 ± 8.9	0.13
Methane/archaeol			
[l/d]/[mg/d]	5.49 ± 0.84	5.07 ± 2.02	0.55
[l/d]/[µg/g DM]	16.7 ± 6.02	14.1 ± 4.14	0.21
[l/(kg DMI • d)]/[µg/g DM]	2.33 ± 0.65	1.63 ± 0.49	0.028

703 **Notes**: Data are means ± standard deviations. p-values are the result of Student's
704 paired t-test between diet types. DMI, dry matter intake; dOM, digested organic
705 matter; dNDF, digested neutral detergent fibre.

706
707

FIGURE CAPTIONS

Figure 1. Representative excretion pattern of solute (cobalt [Co]-EDTA) and small solid particles (2 mm hay particles; mordanted with chromium [Cr]) in a Holstein Friesian heifer fed a diet characterised by a brown midrib maize silage.

Figure 2. Relationship between daily enteric methane production and faecal archaeol in Holstein heifers (n = 7) fed conventional (control) or brown midrib maize silage. (A) Daily archaeol excretion with faeces; (B) Archaeol content in faecal dry matter (DM) (own measurements (circles) and literature data; symbols represent measurements of individuals). SEE, standard error of estimate.

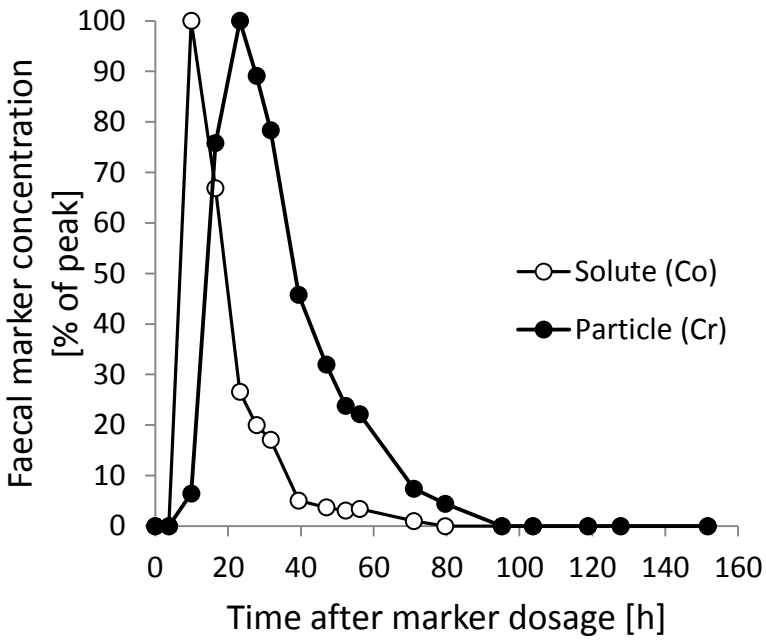
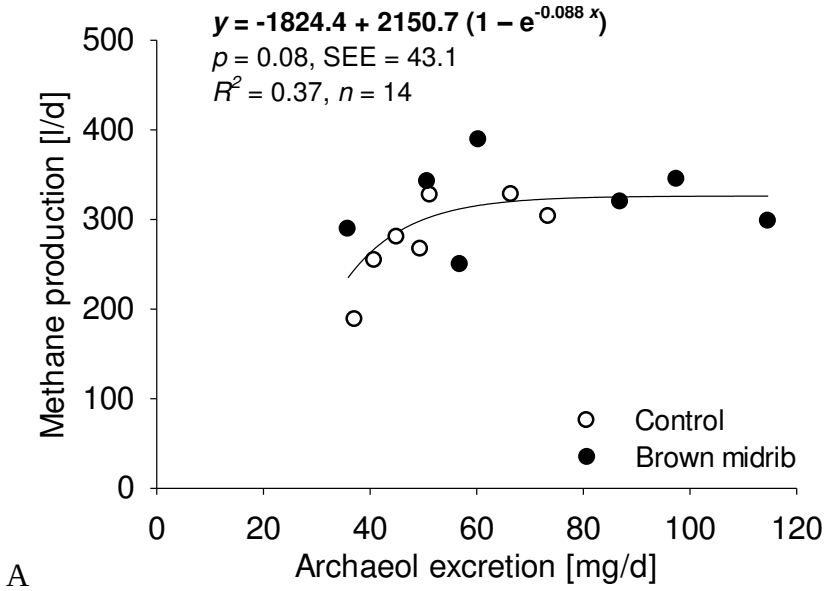
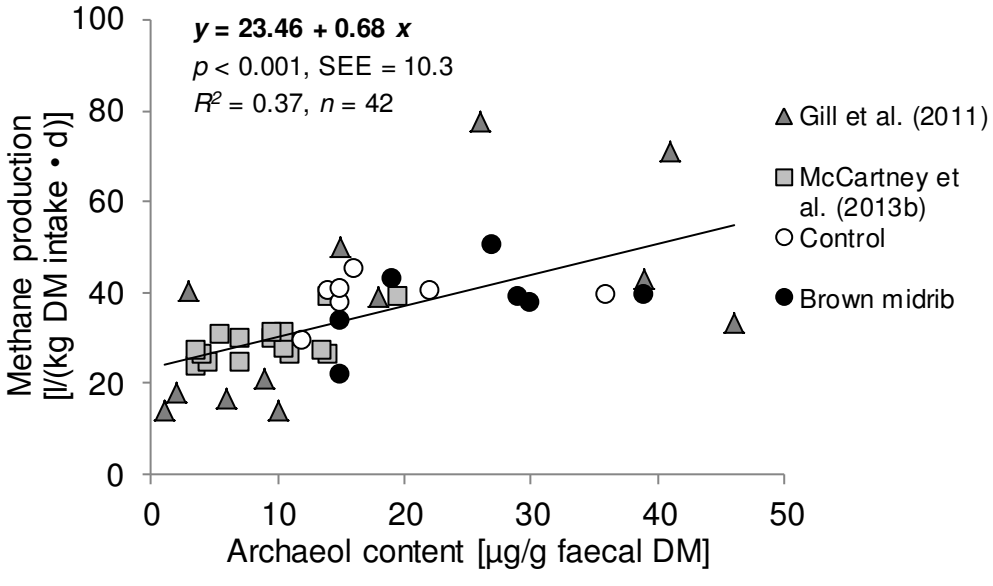

Faecal marker concentration [% of peak]
100
90
80
70
60
50
40
30
20
10
0
0
20
40
60
80
100
120
140
160
Time after marker dosage [h]
Solute (Co)
Particle (Cr)

Figure 1


y = -1824.4 + 2150.7 (1 – e^-0.088 x)
p = 0.08, SEE = 43.1
R2 = 0.37, n = 14
Methane production [l/d]
Archaeol excretion [mg/d]
Control
Brown midrib

A


y = 23.46 + 0.68 x
p < 0.001, SEE = 10.3
R2 = 0.37, n = 42
Methane production [l/(kg DM intake • d)]
Archaeol content [µg/g faecal DM]
Gill et al. (2011)
McCartney et al. (2013b)
Control
Brown midrib

B

Figure 2