Methyl-coenzyme M reductase A as an indicator to estimate methane production from dairy cows

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ABSTRACT

The evaluation of greenhouse gas mitigation strategies requires the quantitative assessment of individual methane production. Because methane measurement in respiration chambers is highly accurate, but also comprises various disadvantages such as limited capacity and high costs, the establishment of an indicator for estimating methane production of individual ruminants would provide an alternative to direct methane measurement. Methyl-coenzyme M reductase is involved in methanogenesis and the subunit α of methyl-coenzyme M reductase is encoded by the *mcrA* gene of rumen archaea. We therefore examined the relationship between methane emissions of Holstein dairy cows measured in respiration chambers with 2 different diets (high- and medium-concentrate diet) and the *mcrA* DNA and *mcrA* cDNA abundance determined from corresponding rumen fluid samples. Whole-body methane production per kilogram of dry matter intake and *mcrA* DNA normalized to the abundance of the *rrs* gene coding for 16S rRNA correlated significantly when using qmcrA primers. Use of qmcrA primers also revealed linear correlation between *mcrA* DNA copy number and methane yield. Regression analyses based on normalized *mcrA* cDNA abundances revealed no significant linear correlation with methane production per kilogram of dry matter intake. Furthermore, the correlations between normalized *mcrA* DNA abundance and the rumen fluid concentration of acetic and isobutyric acid were positive, whereas the correlations with propionic and lactic acid were negative. These data suggest that the *mcrA* DNA approach based on qmcrA primers could potentially be a molecular proxy for methane yield after further refinement.

Key words: methane, *mcrA* gene, respiration chamber, volatile fatty acids, dairy cow

INTRODUCTION

Enteric methane is an end product of the anaerobic microbial fermentation of feeds generated predominantly within the rumen and, to a lesser extent, in the hindgut of ruminants. Total CH₄ production (L or MJ/d) increases with greater DMI; however, CH₄ production as a proportion of DMI or gross energy intake decreases as DMI increases (Knapp et al., 2014). Loss of CH₄ attributes to a 2 to 12% loss of gross energy intake, resulting in less efficient energy utilization by the animal (Johnson and Johnson, 1995). Conversely, enteric CH₄ generated in the gastrointestinal tract of livestock is the single largest source of anthropogenic CH₄ contributing to greenhouse gas emissions (Knapp et al., 2014). Therefore, reducing CH₄ emission has both ecological and economic importance.

In studies investigating mitigation strategies, various methods to quantify methane emissions from ruminants have been applied (Bhatta et al., 2007). Measurements in respiration chambers are highly precise and accurate, allowing quantitative measurements of methane emissions continuously over a longer period of time (Derno et al., 2013). Conversely, drawbacks of this method include a limited capacity, restricting the number of animals that can be studied at the same time, and the expenses for establishment and maintenance of the chambers. In addition, housing animals in respiration chambers restricts their natural behavior, with the consequence that the results obtained may not simply be transferred to free-ranging animals (Storm et al., 2012). Therefore, an urgent need exists to identify and validate indirect indicator traits that can be used as a proxy to estimate methane production from individual ruminants.

Carbohydrate fermentation in the rumen results in the production of VFA (with acetic, propionic, and butyric acids predominating), CO₂, and CH₄ (Mitsumori and Sun, 2008); this process involves a wide diversity of ruminal microorganisms whose composition may shift due to the type of carbohydrate (Belanche et al., 2012). Among them, a variety of methanogenic archaea, termed methanogens, participate in the final

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stage of carbohydrate degradation converting H₂ and C1 compounds, such as formate, CO₂, or methyl donors, to produce CH₄ (Leahy et al., 2013; Poulsen et al., 2013). The final step of biological methane synthesis is catalyzed by the enzyme methyl-coenzyme M reductase (**MCR**). This enzyme complex is thought to be unique to methanogens (Thauer, 1998). The protein MCR comprises 3 subunits (α , β , and γ). The MCR α -subunit is encoded by the *mcrA* gene, which is highly conserved among all methanogens, suggesting this gene to be a suitable target for quantitative detection in PCR-based methods (Grabarse et al., 2000; Li et al., 2012) and possibly a quantitative indicator for methane yield. The aim of the present study was to assess the relationship between CH₄ emissions of Holstein cows measured in respiration chambers and the quantity and activity of methanogens in the rumen using quantitative PCR detection of genomic DNA and RNA transcripts of the *mcrA* gene relative to the *rrs* gene coding for 16S rRNA.

MATERIALS AND METHODS

Animals, Feeding, Rumen Fluid Sampling, and CH₄ Measurement

All animals were treated in accordance with the guidelines for the use of animals as experimental subjects of the State Government in Mecklenburg-Western Pomerania (Registration No. LALLF M-V/TSD/7221.3–1.1–034/12). During early lactation, 10 German Holstein dairy cows entering the first ($n = 4$) or second lactation ($n = 6$) were fed twice daily at 0700 and 1530 h a TMR with a high concentrate (**HC**) content (Table 1) until 104 DIM. The diet formulation was calculated according to the recommendations of the German Society of Nutrition Physiology (GfE, 2004). Animals were housed in a freestall barn unless they were halter-trained and adapted to the respiration chambers (habituation at least 3 times, eating, drinking, ruminating, laying down).

On 100 DIM, a rumen fluid sample was taken via a rumen tube introduced via the esophagus. One rumen fluid sample from 1 cow fed the HC diet could not be obtained. Subsequently, the body mass was determined and cows were transferred to open-circuit respiration chambers in which gas exchange equilibration was achieved overnight. In the chamber, cows were kept at ambient temperature at 15°C, 60% relative humidity, and a light cycle ranging from 0600 to 1900 h. Cows were milked twice daily at 0700 and 1530 h and had free access to water (Derno et al., 2013). On 101 DIM, a feed sample was taken for analysis (Table 1) and ad

libitum feed intake was recorded every 6 min for 24 h by feed disappearance measured by a scale connected to an electronic registration device. Methane concentration was measured at intervals of 6 min for 24 h by infrared-absorption (Sidor, Sick Maihak, Reute, Germany).

After the stay in the respiration chamber, cows were transferred back to the freestall barn. From 105 DIM onwards (mid lactation), cows received a TMR containing a medium concentrate (**MC**) portion (Table 1) meeting the requirements defined by the German Society of Nutrition Physiology (GfE, 2004). On 138 DIM, when cows were on the MC diet, a rumen fluid sample was taken and cows were transferred to respiration chambers to measure CH₄ production as described above.

Table 1. Ingredients and chemical composition of high-concentrate (HC) and medium-concentrate (MC) diets

Component	Diet	
	HC	MC
Ingredient, g/kg of DM		
Grass silage	217	279
Corn silage	317	177
Alfalfa hay	16	—
Alfalfa silage	5	—
Hay	—	42
Straw	—	83
Dried beet pulp	—	131
Concentrate	368 ¹	214 ²
Extracted soybean meal, 44%XP	55	64
Mineral feed	22 ³	10 ⁴
Chemical analysis		
Starch, g/kg of DM	178	99
Sugar, g/kg of DM	50	61
Utilizable CP, ⁵ g/kg of DM	162	153
Crude fat, g/kg of DM	44	27
Crude fiber, g/kg of DM	170	190
NEL, ⁵ MJ/kg of DM	7.2	6.7
NDF, g/kg of DM	324	349
ADF, g/kg of DM	210	205

¹HaGe, Rendsburg, Germany: 20% sugar beet pulp, 32% rape expeller, 12% wheat bran, 11% rapeseed meal, 10% corn meal, 10% extracted soy meal, 5% molasses.

²MF 2000 (Vollkraft, Güstrow, Germany): 33% extracted soy meal, 20% corn, 17% wheat gluten, 13% wheat, 8% extracted rapeseed meal, 5% sugar beet pulp, 2% sodium hydrogen carbonate, 1.3% calcium carbonate, 0.2% sodium chloride, 8.0 MJ of NE_L/kg of DM, 204 g of utilizable protein/kg of DM.

³ATR R 7256 (ATR, Ratzeburg, Germany): 15.30% calcium, 13.40% sodium, 5.20% magnesium, 0.85% phosphorus, vitamins A, D₃, E, copper sulfate, zinc oxide, sodium selenite, cobalt carbonate, iron sulfate, and calcium iodate.

⁴Rinderstolz 9522 (Salvana, Sparrieshoop, Germany): 92% crude ash, 20% calcium, 5% phosphorus, 6% magnesium, 8% sodium, vitamins A, D₃, E, copper sulfate, zinc oxide, sodium selenite, cobalt carbonate, iron sulfate, and calcium iodate.

⁵Calculated after German Society of Nutrition Physiology (GfE, 2001).

Sample Preparation and Determination of VFA, Lactate, and NH_3

Rumen fluid was sieved through a tea strainer to remove feed particles larger than mesh size (0.3 mm), and the pH was measured with a glass electrode for 5 s (Roth, Karlsruhe, Germany). The whole process was done within 10 min. Thirty milliliters of filtrate was centrifuged (10 min, $4,000 \times g$, 4°C), the supernatant was withdrawn (for metabolite analyses), and the pellet was immediately stored at -80°C for DNA and RNA analyses. Ammonia was determined by microdiffusion and lactate acid was measured colorimetrically, as described previously (Voigt and Steger, 1967). To determine VFA concentration, rumen fluid supernatant (5 mL) was treated with 2 mL of internal standard (43 mM isocaproic acid) and centrifuged again (20 min, $12,000 \times g$, 4°C) to apply 1 μL of the cleared supernatant to an FFA phase column (25 m, 0.25 ID, $0.32 \mu\text{m}$) mounted on a GC-flame ionization detector (Series 17A; Shimadzu, injector temperature: 225°C , oven ramp: 110 – 170°C within 7 min) according to Ryan (1980).

DNA Extraction

Total DNA was isolated and purified from 200-mg pellets using the QIAamp DNA stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions but modifying the temperature of lysis incubation to 95°C . The DNA concentration and purity were assessed using a Nanophotometer TM (Implen GmbH, Munich, Germany). All DNA samples were frozen at -80°C before serving as template in quantitative (q) PCR analyses.

RNA Extraction and Transcription Into cDNA

Frozen pellets were crushed under liquid nitrogen and 100 mg of powder was used for total RNA extraction using TRI reagent (Sigma-Aldrich, Taufkirchen, Germany). Extracts were treated with Baseline-Zero DNase (Epicenter, Madison, WI) and cleaned up using the NucleoSpin RNA XS (Macherey-Nagel, Düren, Germany). The RNA was visualized on 1% agarose gels containing ethidium bromide to examine RNA integrity. The RNA quantity was determined by Nanophotometer (Implen GmbH, Munich, Germany). Absence of DNA contamination was confirmed using the RNA as a template in standard PCR amplifying fragments of the *rrs* gene (Weisburg et al., 1991; Hicks et al., 1992). Due to the poor quality of 2 RNA samples, only 17 of 19 samples were available for analysis. All RNA

samples were stored at -80°C for further analysis. The RNA samples were reverse transcribed into cDNA using M-MLV reverse transcriptase (Promega, Munich, Germany) according to the manufacturer's protocol.

qPCR Analysis of *mcrA* and *rrs* Genes

Total methanogens were targeted with various specific primers for the *mcrA* gene (Mlas-mod, Mlas, *mcrA*). Two *rrs* primers (TBac, SRV3) were used as reference gene to target total bacteria (Table 2). Primers were purchased from Sigma-Aldrich. The qPCR mixtures for both *rrs* and *mcrA* genes contained 5 μL of PCR-grade H_2O , 0.5 μL of forward primer (10 μM), 0.5 μL of reverse primer (10 μM), and 2 μL of LightCycler Fast DNA Master Plus SYBR Green I Reaction Mix (Roche Diagnostics, Mannheim, Germany). LightCycler Master Mix was filled in the LightCycler glass capillaries and 2 μL of extracted DNA (5 ng/ μL) or cDNA (10 ng/ μL) were added as a PCR template (total volume of 10 μL). Nontemplate controls were included in each assay. The qPCR of *rrs* and *mcrA* genes was performed in a LightCycler 2.0 system (Roche Diagnostics). The temperature profiles consisted of initial denaturation at 95°C for 10 min, followed by an amplification protocol (Supplementary Table S1; <http://dx.doi.org/10.3168/jds.2015-9310>) specific for each primer. A melting curve was recorded by measuring the fluorescence between 60 and 95°C with a heating rate of 0.1°C per second. The intra-assay variations were 13.9% for cDNA and 4.3% for DNA, respectively. The interassay variation calculated from 2 different runs on separate days were 19.6% for cDNA and 20.8% for DNA. To determine specificity of primers, *mcrA* and *rrs* PCR products were purified on a 2% agarose gel containing ethidium bromide and amplicon bands were excised and subsequently purified by a High Pure PCR Product Purification kit (Roche Diagnostics). The efficiency of amplification was calculated by using LinRegPCR software (Ruijter et al., 2013) yielding efficiency values between 1.78 and 1.92. Amplicons were sequenced on an ABI 3130 Genetic Analyzer (Life Technologies GmbH, Darmstadt, Germany). The obtained sequences were blasted using the NCBI Blast tool (<http://blast.ncbi.nlm.nih.gov/Blast.cgi>) and findings confirmed sequence identity. The ratio between *mcrA* and *rrs* abundances was calculated using the equation published by Pfaffl (2001):

$$R = \frac{(E_{\text{target}})^{\Delta\text{CP}_{\text{target}} (\text{control} - \text{sample})}}{(E_{\text{ref}})^{\Delta\text{CP}_{\text{ref}} (\text{control} - \text{sample})}},$$

Table 2. Primers used for the quantification of *mcrA*¹ and *rrs*² abundances on DNA and cDNA

Procedure	Target group	Gene	Primer	Product Size bp	Sequence 5'-3'	Reference
qPCR	Bacteria	<i>rrs</i>	TBac-F	110	GTGSTGCAYGGYTGTCTCGTCA	Maeda et al., 2003
			TBac-R		ACGTCRTCCMCACCTTCCTC	
	Methanogen	<i>mcrA</i>	SRV3-F	200	CGGYCCAGACTCCTACGGG	Lee et al., 1996
			SRV3-R		TTACCGCGGCTGCTGGCAC	
			qmcrA-F	140	TTCGGTGGATCDCARAGRC	Denman et al., 2007; Ding et al., 2012
PCR ⁴	Bacteria	<i>rrs</i>	qmcrA-R		GBARGTCGWAWCCGTAGAATCC	Angel et al., 2011 (modified from Steinberg and Regan, 2009)
			Mlas-mod-F ³	460	GGYGGTGTMGDDTTCACMCARTA	
			Mlas-F ³	469	GGTGGTGTMGDDTTCACMCARTA	
			mcrA-R		CGTTCATBGCGTAGTTVGGRTAGT	
			26-F	1,486	AGAGTTTGATCCTGGCTCA	
			1492-R		ACGGYTACCTTGTTACGACTT	Hicks et al., 1992
						Weisburg et al., 1991

¹Gene encoding the subunit α of methyl-coenzyme M reductase.²Gene encoding 16S ribosomal RNA.³To combine with *mcrA*-rev primer.⁴For the detection of DNA contamination in RNA samples.

where R = relative expression ratio of the target gene, E = efficiency, CP_{target} = crossing point of the target gene, and CP_{ref} = crossing point of the reference gene.

Statistical Analysis

Normal distribution of data was assessed by means of univariate procedure in SAS (SAS version 9.3, SAS Institute Inc., Cary, NC). To determine the correlations between relative quantity or activity of methanogens in the rumen, methane production, and VFA, the Spearman correlation was applied by the reg and corr procedures of SAS. To determine differences in DMI, milk yield, methane production, rumen pH, and VFA between different diets, the GLM procedure of SAS was used. Statistical significance of model was accepted at $P < 0.05$.

RESULTS

DMI, Milk Yield, and Rumen Fermentation Characteristics

Dry matter intake and total CH_4 production per day did not differ between HC and MC diets; however, CH_4 yield expressed as liters per kilogram of DMI was significantly lower ($P < 0.05$) when cows received the HC diet (Table 3). As expected, milk yield was lower when cows were offered the MC diet ($P < 0.05$). The ruminal fluid concentration of acetate and butyrate was greater on the MC diet, whereas the propionate concentration

was higher on the HC diet ($P < 0.05$). Accordingly, the $(C_2 + C_4)/C_3$ ratio was greater when cows were fed the MC diet ($P < 0.05$). In addition, ranking of individual cows for methane yield was not equivalent on the HC and MC diet.

Correlations Between CH_4 Yield and *mcrA* Abundance

The abundance of methanogens relative to total bacteria in rumen fluid samples was determined to ex-

Table 3. Dry matter intake, milk yield, and fermentation characteristics of rumen fluid from dairy cows fed a high-concentrate (HC) or a medium-concentrate (MC) diet

Item	Diet		P-value
	HC	MC	
DMI, kg/d	15.6 $\pm$ 4.2	15.2 $\pm$ 4.7	NS
Methane, L/d	494.6 $\pm$ 81.0	550.4 $\pm$ 129.0	NS
Methane yield, L/kg of DMI	31.9 $\pm$ 2.4	37.4 $\pm$ 5.4	0.008
Milk, L/d	32.3 $\pm$ 5.3	24.9 $\pm$ 4.3	0.003
Rumen VFA, mol %			
Acetate	51.8 $\pm$ 2.8	55.0 $\pm$ 2.1	0.0121
Propionate	24.8 $\pm$ 2.7	20.8 $\pm$ 1.0	0.0004
Isobutyrate	1.0 $\pm$ 0.1	1.3 $\pm$ 0.2	0.0122
Butyrate	16.9 $\pm$ 1.9	17.4 $\pm$ 1.2	NS
Isovalerate	1.7 $\pm$ 0.4	2.0 $\pm$ 0.5	NS
Valerate	2.3 $\pm$ 0.5	2.4 $\pm$ 0.3	NS
Caproate	0.8 $\pm$ 0.4	0.8 $\pm$ 0.3	NS
$(C_2 + C_4)/C_3$ ¹	2.8 $\pm$ 0.4	3.6 $\pm$ 0.2	0.0004
Lactate	0.6 $\pm$ 0.5	0.3 $\pm$ 0.37	NS
Rumen pH	6.9 $\pm$ 0.3	7.1 $\pm$ 0.3	NS

¹Sum of acetate and butyrate divided by propionate.

amine whether it reflects methane yield in cows offered diets with different levels of concentrate. To this end, abundance of *mcrA* DNA relative to the DNA abundance of *rrs* was analyzed using discrete combinations of primer sets (Table 3). When using qmcrA primers for the quantification of methanogens, we found significant linear relation between methane yield and relative *mcrA* abundance after normalization with *Tbac* (Figure 1A; $r = 0.47$, $SD = 4.56$, $P < 0.05$) or after normalization with SVR3 (Figure 1B; $r = 0.47$, $SD = 4.72$, $P < 0.05$) primers. There were no significant correlations between methane yield and *mcrA* DNA abundance when Mlas primers were used (Figure 1C and 1D). Moreover, use of qmcrA primers (Figure 2A; $r = 0.58$, $SD = 4.76$, $P < 0.05$) revealed linear correlation between the *mcrA* DNA copy number and methane yield. In addition, when methane production was expressed as liters per day, no significant correlations with *mcrA* DNA or with *mcrA* cDNA were detected (data not shown).

There were no significant correlations between *mcrA* cDNA abundances and methane yield no matter which of the primer sets was used (Supplementary Figure S1; <http://dx.doi.org/10.3168/jds.2015-9310>). Also, no correlation with methane yield was found when *mcrA* transcript abundance was normalized to *mcrA* gene abundance (Supplementary Figure S2), when *mcrA* gene abundance was normalized to *mcrA* transcript abundance (Supplementary Figure S3), or when the *mcrA* cDNA copy number was used (Supplementary Figure S4). When the regression analysis was performed separately for each diet, no significant correlations could be determined for genomic DNA or for cDNA (data not shown).

Correlations Between VFA or Lactate with CH₄ Production and *mcrA* Abundance

We found direct correlations between methane yield and molar percentage of acetate, but also with the $(C_2 + C_4)/C_3$ ratio (Table 4). Inverse correlations were found between methane yield and propionate and lactate, respectively (Table 4). In addition, the *mcrA* DNA abundance correlated significantly with molar percentage of acetate, propionate, (qmcrA/*Tbac* and qmcrA/SRV3 only), but also with isobutyrate, the $(C_2 + C_4)/C_3$ ratio and with lactate (Table 4). When *mcrA* cDNA abundances were used for correlation analyses, significant correlation coefficient values were obtained with isobutyrate, isovalerate, valerate, and lactate (Table 4). When *mcrA* transcript abundance was normalized to *mcrA* gene abundance using qmcrA primers, no correlations with VFA were found (data not shown). Also, there were no significant correlations when VFA concentrations were expressed in millimolar.

DISCUSSION

The aim of the present study was to assess the relationship between CH₄ production from Holstein cows and the quantity and activity of methanogens in the rumen. To approach this aim, we tested 3 different primer sets for the quantification of methanogens, but only the application of qmcrA primers revealed significant correlations with methane yield. The specificity of primers could be the reason for such different results. Whereas the Mlas and Mlas-mod primers designed by Steinberg and Regan (2009) were primarily developed for clone library construction and qPCR analyses in oligotrophic fen and anaerobic digester sludge, the qmcrA primer set has been widely used to quantify methanogens specifically from the ruminant gastrointestinal tract, which harbors a different methanogen population (Denman et al., 2007). First attempts were made to relate rumen methanogens as quantified by qmcrA primers with whole-animal methane production in small ruminants, but the results show some discrepancies (McCartney et al., 2013). Briefly, Ding et al. (2012) reported a highly positive correlation between *mcrA* DNA abundance and methane yield measured in 12 Tibetan sheep, whereas Morgavi et al. (2012) failed to demonstrate a significant relationship for 6 Texel wethers, which could have been due to defaunation applied in the latter study. Our results are in agreement with the results described by Ding et al. (2012), showing significant correlations between *mcrA*/*rrs* DNA abundance and methane yield. The variation of methane yield, and thus the variation of *mcrA* DNA abundance, might be caused by a shift in the microbial community composition. For example, Shi et al. (2014) reported elevated *Methanospira* spp. in the low-CH₄ yield sheep and higher relative abundances of organisms belonging to the *Methanobrevibacter gottschalkii* clade in the high-CH₄ yield sheep. Our results are also comparable to the study by Wallace et al. (2014), who described a linear correlation between methane per kilogram of DMI and the ratio of total archaea normalized to total bacterial abundance. Splitting according to the diet, as in our study, revealed that no significant correlation could be seen between methane emission and the relative abundance of methanogens from animals fed a medium-concentrate diet (Wallace et al., 2014). By contrast, a highly significant correlation between methane emission and the relative abundance of methanogens was found when animals were on a high-concentrate diet (Wallace et al., 2014). The lower number of data obtained from animals on an HC diet might be one reason why we failed to obtain such a diet-specific relationship. Our results are also comparable to findings described for anaerobic digesters, in which the *mcrA* gene copy num-

Figure 1. Linear regression between methane emissions per kilogram of DMI per day and *mcrA* DNA abundance normalized to the *rrs*. The *mcrA* DNA abundance was measured using *qmcrA* (A and B) or *Mlas* (C and D) primers, whereas the *rrs* abundance was measured using *TBac* (A and C) or *SVR3* (B and D) primers. Linear regression equations as well as Spearman correlation coefficients *r* are provided. $\diamond$ = high-concentrate diet; $\bullet$ = medium-concentrate diet.

ber significantly correlated with CH_4 production rates, whereas correlations between *mcrA* transcript number and specific methanogenic activity were not significant (Morris et al., 2014). The *mcrA*-to-*rrs* ratios shown in Figure 1 were calculated by use of the Pfaffl formula (Pfaffl, 2001), which considers the different efficiency of the primers used for *mcrA* and *rrs* quantification. Thus, the *mcrA* DNA-to-*rrs* DNA ratio may be greater than 1. By contrast, Wallace et al. (2014) calculated the archaea-to-bacteria ratio based on copy numbers resulting in values lower than 0.2.

However, it has been suggested that the quantification of methanogens via genomic DNA is not specific to active CH_4 -producing microbes because DNA may also originate from inactive or dead cells (Lettat and Benchaar, 2013). Therefore, cDNA-based analysis is thought to discriminate active microbes from the total microbial community (Lettat and Benchaar, 2013). In support of this assumption, Shi et al. (2014) have recently shown that the transcription of methanogenesis pathway genes was substantially increased in sheep with high methane yield, whereas the abundance of methanogens was not significantly different in high- and low-methane emitters. In the present study, however, we did not observe significant correlations between *mcrA* cDNA abundance (no matter whether expressed as copy number or normalized with *rrs* or *mcrA* DNA) and methane yield. This might be explained by the fact that only 78% of the bacteria detected at DNA and cDNA fingerprints were found to be common to total and active bacterial communities (Lettat and Benchaar, 2013). Moreover, differences between DNA and cDNA fingerprints in total and active bacteria may also affect the *rrs* DNA and *rrs* cDNA abundance, thereby influencing the *mcrA*-to-*rrs* ratio. Another reason may be that the *mcrA* cDNA abundance does not sufficiently reflect the differences in the methane-producing capability per cell. For example, rapidly growing methanogens with low methane-producing activity might explain why the slopes from linear regressions between methane yield and *mcrA* cDNA abundances expressed as copy numbers or relative to *rrs* cDNA or to *mcrA* DNA, respectively, are much lower (despite not significant) than the slopes obtained from the regression with *mcrA* DNA abundances. Absence of correlation between cDNA and methane yield may also be due to the short half-life of prokaryotic mRNA. For example, the half-life of *mcrA* transcripts in *Methanococcus vannielii* at 37°C were found to be 15 min (Hennigan and Reeve, 1994), indicating the interference of RNA based approaches as indicator for methane yield. This might be the reason for the divergent results between the present and the results of Shi et al. (2014), which showed that *mcrA* transcription but not gene abundance corre-

Figure 2. Linear regression between methane emissions per kilogram of DMI per day and copy number of *mcrA* DNA. The *mcrA* DNA abundance was measured using qmcrA (A), Mlas primers (B), or Mlasmod (C) primers. Linear regression equations as well as Spearman correlation coefficients r are provided. $\diamond$ = high-concentrate diet; $\blacklozenge$ = medium-concentrate diet.

sponds to 8 sheep, differing by 38.5% in methane yield. These *mcrA* transcription differences were directly correlated with the retention time of feed particles and the rumen volume of sheep offered the same diet (Shi et al. 2014). Although we did not measure retention time or rumen volume in the present study, methane

production from cows receiving either the HC or MC diet varied only by 29 to 34%, which explains some of the discrepancies between the present and the Shi et al. (2014) study. Moreover, nonobserved association between *mcrA* transcription abundance and methane yield may be due to interindividual differences in the gastrointestinal mucine profile (Hoorens et al., 2011), which is known to affect methanogenesis at least in the colon (Flourié et al., 1991; Gressley et al., 2011).

However, active microbes thought to possess abundant *mcrA* RNA might only have contributed to the very last period of the 24-h methane measurement, whereas all methanogens, regardless of being dead or alive, might have contributed to gas emission many hours before sampling. Thus, it is important to consider dead, resting, or not fully developed methanogens for quantification, because these methanogens contributed or will contribute to methane production before or after rumen fluid sampling. This highlights the importance of a prompt single rumen fluid sampling relative to CH₄ measurement and questions the necessity of long-term methane measurements for the correlation with indicators obtained from a single day of sampling. However, whether data generated from multiple rumen fluid samplings would correlate with multiday methane measurements with a higher regression coefficient needs to be addressed in future studies.

Although the majority of fermentation occurs in the rumen, the hindgut of ruminants contributes to 6 to 14% of the total CH₄ output of cattle (Gressley et al., 2011). We measured whole-animal CH₄ production involving ruminal and hindgut-derived methane, but determined only ruminal *mcrA* abundance. This discrepancy may contribute to a higher standard deviation and lower correlation coefficients values as well. The interception of the regression lines at the y-axis theoretically indicates the amount of methane produced by the hindgut. This would amount to 15 to 30 L of hindgut methane/kg of DMI, which exceeds values known from literature (Gressley et al., 2011). Moreover, Wallace et al. (2014) reported 13 to 14 L for the interception at the methane yield-axis, indicating that our linear regression model has some weaknesses at lower *mcrA*-to-*rrs* ratios. A comparable issue can be seen in the Shi et al. (2014) study, where linear correlation (based on 3 data points) between methane yield and *mcrA* transcription abundance resulted in a negative value for the interception at the methane yield axis.

Even though the relationship between relative quantity of methanogens estimated by *mcrA* DNA-based method and methane emission was significant, the existing standard deviation may refer to the interindividual differences in the rumen methanogen population. Jami and Mizrahi (2012) described the similarities

Table 4. Spearman correlation coefficient between VFA and lactate concentrations (mol %) and the abundance of *mcrA* DNA, *mcrA* cDNA, and methane emission involving rumen fluid samples obtained from dairy cows fed ad libitum a high-concentrate and medium-concentrate TMR

Item	DNA			cDNA			CH ₄
	<i>qmcraA/Tbac</i> ¹	<i>qmcraA/SRV3</i> ¹	<i>qmcraA</i> ²	<i>qmcraA/Tbac</i> ¹	<i>qmcraA/SRV3</i> ¹	<i>qmcraA</i> ²	L/(kg·d)
Acetate	0.626**	0.598**	0.407	0.426	0.506*	−0.197	0.504*
Propionate	−0.628**	−0.572*	−0.402	−0.244	−0.291	0.232	−0.613**
Isobutyrate	0.432	0.370*	0.533*	0.753**	0.750**	0.488	0.418
Butyrate	−0.011	−0.051	−0.168	−0.365	−0.374	−0.188	0.202
Isovalerate	0.142	0.063	0.221	0.685**	0.594*	0.732**	0.121
Valerate	0.000	−0.049	0.177	0.388	0.368	0.559*	0.084
Caproate	0.346	0.296	0.325	0.438	0.491	0.247	0.286
(C ₂ +C ₄)/C ₃ ³	0.637**	0.577*	0.418	0.206	0.259	−0.268	0.630*
Lactate	−0.491*	−0.514*	−0.508*	−0.465	−0.512*	−0.232	−0.539*

¹Primer sequences provided in Table 2, ratio calculated according to Pfaffl (2001).

²Primer sequences provided in Table 2, *mcrA* abundance expressed in unit of copy numbers.

³Sum of acetate and butyrate divided by propionate (mol %).

* $P < 0.05$; ** $P < 0.01$.

and differences of the overall bacterial communities of the rumen ecosystem across individual cows fed the same diet with considerable variations between different phyla and bacteria. Moreover, King et al. (2011) found significant differences in the methanogen population structure between Holstein and Jersey cows that were fed the same diet and maintained under the same environmental conditions, suggesting that these differences may be due to host breed genetics.

Another factor influencing the standard deviation may be the composition of the diet fed to the animals. Yáñez-Ruiz et al. (2010) found a higher abundance of methanogens in the rumen of lambs fed only with grass hay than those fed with concentrate and grass hay. Conversely, Lettat and Benchaar (2013) did not observe a diet effect on methanogenic activity in the rumen of lactating Holstein cows fed either barley silage, a 50:50 mixture of barley silage and corn silage, or corn silage only. In our experiment, animals were fed 2 different diets with different proportions of grass and corn silages, but primarily with different amounts of concentrate providing starch. As mentioned before, the different *mcrA*-to-*rrs* correlations could not be attributed to either of the diets, despite the fact that the contribution of corn starch at the expense of fiber reduces methane production (Jentsch et al., 2007) and causes a different methanogenic species pattern compared with other sources of starch (Beauchemin and McGinn, 2005; Popova et al., 2013).

The molar percentage of VFA has been described to be associated with the amount of methane produced in the rumen. Moss et al. (2000) reported that acetate and butyrate are directly related with methane production, whereas propionate has an inverse relation with methane synthesis as the formation of propionate and methane compete for the nascent hydrogen. Moreover,

Wang et al. (2009) found a reduction of methanogens when the propionic acid concentration in the rumen increased. These findings are consistent with our results presented in Table 4, demonstrating a direct correlation between *mcrA* DNA abundance and acetate, a direct correlation between *mcrA* cDNA abundance and isobutyrate, and an inverse correlation between *mcrA* DNA abundance and propionate.

Lactate, compared with VFA, is a strong acid and produces low pH values that could explain the inhibition of methane production as it has been reported that rumen pH affects ruminal methane production (van Kessel and Russell, 1996). The degradation of lactate under anaerobic conditions produces acetate, which in turn shows a significant positive relation with the abundance of *mcrA* DNA. Furthermore, the production of propionate via lactate reduces the flow of hydrogen to methane (Mitsumori and Sun, 2008), which explains the significantly negative correlation between lactate and methanogens.

CONCLUSIONS

Herein, we report for the first time correlations between the abundance of the archaeal *mcrA* gene and methane emissions from the same animals on 2 different diets. A positive significant relationship was found between ruminal *mcrA* DNA abundance normalized to *rrs* and methane production per kilogram of DMI, in particular when *qmcraA* primers were used for the quantification of methanogens. These results highlight the potential usefulness of *mcrA* DNA as a molecular proxy to estimate methane yield. Nevertheless, reasons for the relatively high standard deviation and low regression coefficients need to be addressed in future studies.

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