Natural variation in methane emission of sheep fed on a lucerne pellet diet is unrelated to rumen ciliate community type

Sandra Kittelmann,1 Cesar S. Pinares-Patiño,1 Henning Seedorf,1 Michelle R. Kirk,1 John C. McEwan2 and Peter H. Janssen1

Correspondence
Peter H. Janssen
peter.janssen@agresearch.co.nz
1AgResearch Ltd, Grasslands Research Centre, Palmerston North 4442, New Zealand
2AgResearch Ltd, Invermay Agricultural Centre, Mosgiel 9053, New Zealand

INTRODUCTION

Ciliates living in the rumens of ruminant animals have been hypothesized to play an important role in methane (CH4) emissions of their hosts. This is, in part, because hydrogen-producing rumen ciliates represent ideal microhabitats for methanogenic archaea when hydrogen is limiting (Newbold et al., 1995), and close associations between the two groups of micro-organisms have been observed using microscopic as well as molecular methods (Vogels et al., 1980; Stumm et al., 1982; Krumholz et al., 1983; Tokura et al., 1997; Tymensen et al., 2012; Belanche et al., 2014; Xia et al., 2014). It is possible that ciliates allow retention of methanogens in the rumen, which could change methanogen growth kinetics and increase CH4 formation (Janssen, 2010). A number of studies reported that CH4 formation increased in the presence of protozoa, and depletion of ciliate protozoa from the rumen (defaunation) has been suggested as a means to effectively reduce CH4 emissions from animals on diets containing high proportions of grain (Whitelaw et al., 1984; Kreuzer et al., 1986). However, if animals were fed on pasture, defaunation appeared not to be an effective means (Kreuzer et al., 1986; Ranilla et al., 2007; Bird et al., 2008; Hegarty et al., 2008), suggesting that absolute ciliate numbers do not significantly influence CH4 emissions from grazing animals. However, the ciliate community composition may also impact on host CH4 emissions. For example, it appears that some of the larger ciliates are more heavily colonized by intra- and extracellular methanogens than others (Lloyd et al., 1996). Differential associations of certain ciliates with methanogens may facilitate increased or reduced hydrogen formation, and hence more or less CH4 may depend on the composition of the ciliate community.
Ciliate protozoa in the rumen typically occur as mixed microbial communities, so-called community types, each comprising several different genera of ciliates (Eadie, 1962). Type A ciliate communities are characterized by the presence of *Polyplastron multivesiculatum*. B-type communities contain at least one of the indicator species *Epidinium caudatum* and *Eudiplodinium maggi*. The K-type community, characterized by the presence of *Elytroplastron bubali*, appears to be exclusively found in cattle. O-type communities consist of *Entodinium* spp. and members of the holotrich genera *Isotricha* and *Dasytricha*, while any indicator species of A-, B- and K-type communities are absent. So far only a few in *vitro* and even fewer in *vivo* studies have tried to tease apart the roles of individual ciliate genera or ciliate community types in CH$_4$ formation. Newbold et al. (1995) used rumen samples from previously defaunated sheep inoculated with a B-type community (*Epidinium, Eudiplodinium* and *Entodinium*), with a mono-culture of *P. multivesiculatum* (i.e. a partial A-type community), or with *Isotricha prostoma* and *Entodinium* spp. (a partial O-type community), to evaluate differences in CH$_4$ formation between ciliate community types in *vitro*. These authors found that the largest amounts of CH$_4$ were formed by rumen fluid containing the B-type community, less CH$_4$ was produced by defaunated rumen fluid, and the smallest amounts were produced by rumen fluid containing either *P. multivesiculatum* or a mixture of *I. prostoma* and *Entodinium* spp. In line with these observations, a study conducted by Jouany & Toillon (1997) suggested more CH$_4$ production from B-type communities than from *Isotricha* spp. alone, and defaunated rumen content was reported to produce the least CH$_4$. In contrast, Ranilla et al. (2007) found that *Entodinium* spp. increased CH$_4$ formation per unit of substrate fermented by 13% in *in vitro* experiments, compared with the fauna-free treatment, while *Eud. maggi*, *Isotricha intestinalis* or *Metadinium* medium had no effect. Another *in vitro* study examined the fermentation of cellulose and xylan by different ciliate species (Zeit et al., 2013). Of the three different species tested, namely *Entodinium caudatum, Epidinium caudatum* and *Eud. maggi*, only cultures of the latter amended with xylan exhibited higher CH$_4$ formation than the defaunated controls, indicating that different ciliates respond differently to carbohydrates and influence CH$_4$ formation in different ways (Zeit et al., 2013). These studies suggest that different ciliates affect CH$_4$ formation *in vitro*, but there is so far no congruence on the key genera implicated.

To date, only two *in vivo* studies have been carried out, both making use of defaunated animals that were artificially refaunated. They suggest that larger numbers of *Entodinium* spp. are correlated with lower CH$_4$ emission (Jouany et al., 1981), and that holotrich ciliates may be correlated with higher CH$_4$ emission than entodiniomorph ciliates (Belanche et al., 2015). These results are in contrast to findings of the *in vitro* study of Ranilla et al. (2007) and could indicate that *in vitro* studies may not fully reflect the complex processes occurring *in vivo*, thus demonstrating the need for further investigations. The roles of the naturally occurring ciliate community types in CH$_4$ formation have so far not been examined, either *in vitro* or *in vivo*. This may in part be due to the large number of animals that need to be measured for both CH$_4$ emissions and ciliate community composition in order to identify a representative number of animals harbouring each ciliate community type. Recently, validation of next-generation sequencing against microscopy for comparative ciliate community structure analysis has enabled simultaneous analysis of ciliate communities in hundreds of samples (Kittelmann et al., 2015). Using this method, we previously reported that the relative abundances of individual genera of rumen ciliates are not significantly correlated with CH$_4$ yields of sheep feeding on lucerne pellets (Kittelmann et al., 2014). However, it remained to be elucidated whether the analysed samples could be classified into ciliate community types based on relative abundance data stemming from next-generation amplicon sequencing, and if the different ciliate community types that naturally occur *in vivo* were linked to significantly different CH$_4$ yields from sheep feeding on lucerne pellets.

**METHODS**

**Measurement of CH$_4$ yields from sheep.** CH$_4$ measurements were conducted with a total of 340 New Zealand sheep, born in 2009 and separated into four cohorts, over the year 2010 at the New Zealand Ruminant Methane Measurement Centre, AgResearch Ltd, Palmerston North, New Zealand. The animals were fed a pelleted lucerne (alfalfa) diet at 2.0 times their maintenance requirements. The larger experiment of which these animals were a part has been described in detail elsewhere (Pinares-Patiño et al., 2013). Each cohort was measured in two independent measuring rounds. The earlier of the two rounds was labelled Round a, and the later one Round b. Measurements of feed intake were conducted with sheep housed in metabolism crates over 3 days (in a common holding area) and then over 2 days in open-circuit respiration chambers. Two consecutive days of CH$_4$ emissions data were collected for each individual in each of the two measurement rounds. CH$_4$ yields [g CH$_4$ (kg dry matter intake, DMI)$^{-1}$] were calculated by dividing gross CH$_4$ emission (g CH$_4$ day$^{-1}$) as measured in the respiration chambers by the measured daily feed intake (dry matter basis), using the mean of both days in a round to give the yield for each animal in each round (Pinares-Patiño et al., 2013).

**Rumen sampling, nucleic acid extraction, and pyrosequencing of rumen ciliate 18S rRNA genes.** At the end of each of the two CH$_4$ measurement rounds, a representative rumen sample (~50 g wet weight) was collected by stomach tubing and immediately stored at ~20 °C (Henderson et al., 2013). In this way, a total of 680 rumen samples were collected (two samples per animal). The highest and lowest CH$_4$-emitting animals within each separate cohort, based on their 4 day mean CH$_4$ yields (i.e. the mean of both rounds), were identified and a total of 236 samples from 118 animals were analysed for rumen ciliate community structure as described previously (Kittelmann et al., 2014). Briefly, nucleic acids were extracted from 30 mg freeze-dried and homogenized rumen content samples using a combined bead-beating, phenol/chloroform and column purification protocol (Rijs et al., 2012). Rumen ciliate 18S rRNA genes were amplified using barcoded primers RP841F (Kittelmann & Janssen, 2011)
and Reg1302R (Regensbogenova et al., 2004) and prepared for 454 Titanium pyrosequencing at the Center for Genome Sciences and Systems Biology, Washington University of St Louis, USA, according to Kittelmann et al. (2014). Sequence data have been deposited in the EMBL database under the study accession number ERP003772.

### Analysis of pyrosequencing data

Sequence data were processed and analysed following the procedure described by Caporaso et al. (2010). Sequence reads were assigned to corresponding samples by examining the 12 bp error-correcting Golay barcodes with default QIME v1.5 parameters as described previously (Kittelmann et al., 2014). Briefly, sequences that were > 200 bp in length were truncated to variable lengths so that the mean quality score was > 25 and only sequences without ambiguous characters were included in the analysis. The prefix/suffix method was used for operational taxonomic unit clustering as previously described (Kittelmann et al., 2013). Sequence data were assigned to named taxa using BLAST (Altschul et al., 2000) against a rumen-specific, in-house database derived from an earlier study (Kittelmann & Jansen, 2011). The resulting abundance table was summarized at the genus-level for subsequent statistical analysis.

### Definition of rumen ciliate community types

To classify ciliate communities in the analysed rumen samples into types based on relative abundances from sequence data, the following rules were applied. Ciliate communities with a relative abundance of ≥1% of *Polyplastron* spp. were defined as A-type communities. If the relative abundances of *Epidinium* and *Eudiplodinium* spp. added up to ≥10%, the community was defined as B-type. If both criteria were applicable, the community was defined as a mixed AB-type, and if none of the rules applied, and *Entodinium* and holotrich protozoa (*Dasytricha* and *Isotricha* spp.) together represented ≥90% of the community, the composition was determined as O-type.

### Statistical analysis

Principal coordinate analysis was carried out using the Bray–Curtis dissimilarity metric (Bray & Curtis, 1957) in QIIME, and principal coordinates were visualized in SigmaPlot v13.0.

Wilcoxon rank sum tests were performed in R (R Core Team, 2014) to test for statistical significance of differences between CH₄ yields associated with the rumen samples of the 60 high emitters and 58 low emitters and with rumen samples from sheep harbouring different ciliate community types. To account for potential measuring round or cohort effects, Wilcoxon rank sum tests for significance were also performed using a rank-based approach, in which the rumen sample associated with the highest CH₄ yield within each measuring round was given a rank score of 30, and the rumen sample associated with the lowest CH₄ yield within each measuring round was given a rank score of 1. For rounds in which only 28 animals were measured, ranks 15 and 16 were omitted.

To evaluate which taxa, if any, were significantly correlated with CH₄ rank, relative abundances of ciliate taxa were compared with CH₄ rank using Pearson correlation. Pearson correlation coefficients were calculated in Microsoft Excel. Correlations between 0.3 and −0.3 were regarded as negligible (Hinkle et al., 2003).

In addition to community typing based on the definition by Eadie (1962), we performed unbiased hierarchical clustering on ciliate 18S rRNA gene community profiles. Wilcoxon rank sum tests were carried out between the four groups defined based on hierarchical clustering to test for statistical significance of differences between their associated CH₄ rank scores.

### RESULTS AND DISCUSSION

A total of 236 rumen samples derived from the highest and lowest CH₄-emitting animals in each cohort, which were identified on the basis of the animals’ mean CH₄ yields across both measuring rounds and analysed for rumen ciliate community structure based on 18S rRNA marker genes (Kittelmann et al., 2014). Pyrosequencing resulted in a total of 323 463 reads, with a minimum of 368 reads and a maximum of 5130 reads per sample (mean ± SD 1376 ± 609). One sample did not yield any sequencing reads, so further analyses were based on the data obtained from the remaining 235 samples. Each of these samples had an associated CH₄ yield and a ranking based on the CH₄ yield within the measuring round. The abundance of none of the taxa was correlated with CH₄ emissions ranking. In total, seven different ciliate genera were present at ≥1% relative abundance in at least one sample. These were, in order of mean relative abundance (±SD; Pearson’s correlation with CH₄ rank), *Epidinium* (49.1 ± 30.7%; −0.166), *Entodinium* (34.5 ± 23.6%; 0.103), *Dasytricha* (5.0 ± 10.1%; 0.028), *Eudiopolidinium* (4.8 ± 6.6%; 0.164), *Polyplastron* (3.9 ± 11.4%; 0.038), *Isotricha* (2.3 ± 8.0%; 0.126), and *Anoplopidinium–Diplodinium* (0.4 ± 2.4%; −0.063). Following Eadie (1962), we distinguished between three distinct ciliate community types, the A-, B- and O-type communities, and additionally identified the mixed AB-type (Fig. 1; Eadie, 1962; Göçmen et al., 2003; Towne et al., 1988).

Although different species of rumen ciliates can vary significantly in their total numbers of 18S rRNA gene copies, pyrosequencing can be used to reveal trends in relative abundances of genera and species between different samples processed in the same way (Kittelmann et al., 2015). Therefore, each rumen sample was classified into one of three rumen ciliate community types or the mixed AB-type. Of the 235 samples analysed, 181 harboured B-type ciliate communities, 28 harboured A-type ciliate communities, 16 harboured O-type ciliate communities and 10 harboured mixed AB-type ciliate communities (Table 1). The prevalence of B-type ciliate communities in sheep feeding on lucerne pellets in our study was not surprising, given that the type species present in these, *Epidinium caudatum* and *Eud. maggi*, harbour high cellulytic activities (Coleman, 1986). Moreover, it appears that *Epidinium* spp. are unique amongst the entodiniomorphid protozoa in that they actively attach to and attack plant material (Bauchop & Clarke, 1976; Bauchop, 1979).

Principal coordinate analysis was carried out to visualize clustering of samples based on ciliate community composition (Fig. 2a, b). Samples harbouring A-type ciliate communities clustered separately from those containing B-type communities, while individual samples containing an AB-mixed community clustered with either the A-type samples or B-type samples, depending on whether diagnostic taxa of A-type (*Polyplastron*) or B-type (*Epidinium* and *Eudiopolidinium*) communities dominated in them. Thirteen samples containing almost exclusively *Entodinium* spp.
(≥90%; O-type) clustered separately from samples showing the A- or B-type; however, the remaining three samples classified as O-type in this study clustered along the line of separation between samples with A- and B-type ciliate communities. This is explained by the fact that these samples were not clearly dominated by the genus Entodinium, but instead contained similar relative abundances of both Entodinium spp. (48.6 ± 20.1%) and holotrichs, such as Isotricha and Dasytricha (46.5 ± 18.6%), and less than 10% Epidinium and Eudiplodinium (4.7 ± 1.4%). Future studies involving more animals will reveal whether it is warranted to define a novel community type for such a holotrich-enriched rumen ciliate community. Independent hierarchical clustering of samples also classified the samples into four groups, with one group resembling Eadie’s A-type community, one group resembling the O-type community and two groups resembling the B- and AB-type communities. Interestingly, we observed that the ciliate community types in the rumens of some animals changed over time, appearing to pass through a succession from O to B, to AB, and to A (Fig. 3). This was perhaps driven by the continued feeding of lucerne pellets or potential infection of B-type animals with A-type ciliate species from other animals over time. Changes in ciliate communities by invasion of one type by another have been reported previously (Eadie, 1962, 1967). There appear to be ciliate communities that represent transitions between the idealized types, and the nature, frequency, and drivers of these transitions remain to be understood. If ciliate communities cycle through different types in a predictable sequence, then understanding the causes of the transition from one type to the next is important for interpreting differences in rumen ciliate community structure between animals and within animals over time or with changing treatments.

We hypothesized that, if the naturally occurring ciliate community types significantly impacted on CH4 yield, then rumen samples harbouring a particular ciliate community type would be associated with significantly higher or lower CH4 yields. There were significant differences (P < 2.2 × 10−16) in the CH4 yields (±SD) from the sheep that were ranked as high [16.7 ± 1.5 g CH4 (kg DMI)−1] and low [13.3 ± 1.5 g CH4 (kg DMI)−1] emitters. However, Wilcoxon rank sum tests showed that the different, naturally occurring types of ciliate communities were not correlated with significantly higher or lower CH4 yields (Fig. 4a). The mean CH4 yields for sheep with A- and B-type ciliate communities were 14.9 ± 1.7 and 15.0 ± 2.3 g CH4 (kg DMI)−1, respectively (P=0.75). The mixed AB-type had a mean CH4 yield of 15.5 ± 2.1 g CH4 (kg DMI)−1 and O-type ciliate communities produced a mean of 15.6 ± 2.8 g CH4 (kg DMI)−1. Hence, CH4 yields of animals with O-type ciliate communities did not

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**Table 1.** Distribution of different ciliate community types in rumen samples obtained from sheep measured in four cohorts.

The numbers of samples analysed per cohort and in total are given.

<table>
<thead>
<tr>
<th>Community type</th>
<th>Cohort 1</th>
<th>Cohort 2</th>
<th>Cohort 3</th>
<th>Cohort 4</th>
<th>No. rumen samples</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>24</td>
<td>28</td>
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<tr>
<td>AB</td>
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<td>4</td>
<td>6</td>
<td>10</td>
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<tr>
<td>B</td>
<td>53</td>
<td>49</td>
<td>51</td>
<td>28</td>
<td>181</td>
</tr>
<tr>
<td>O</td>
<td>3</td>
<td>11</td>
<td>1</td>
<td>1</td>
<td>16</td>
</tr>
<tr>
<td>Total</td>
<td>56</td>
<td>60</td>
<td>60</td>
<td>59</td>
<td>235</td>
</tr>
</tbody>
</table>

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**Fig. 1.** Mean relative abundances of ciliate genera present in 235 rumen samples classified into the four ciliate community types defined by Eadie (1962). Error bars indicate SEM.
significantly differ from animals harbouring A- (P=0.26), B- (P=0.32), or AB-type ciliate communities (P=0.86), nor were any of the remaining comparisons significant (P>0.05). Similarly, we detected no significant differences between any two of the four different ciliate community types, when using the rank-based approach (P>0.05 for all comparisons, Figs 2c, d and 4b). These results were confirmed by the independent hierarchical clustering approach, which showed no significant differences between CH4 rank scores of the four clusters. These findings suggest that the naturally assembling A-, B-, AB- and O-type ciliate communities do not result in significant overall differences in CH4 yields of sheep feeding on lucerne pellets. Based on our data, selection for animals with a certain natural ciliate community type is not likely to achieve a reduction in ruminant CH4 emissions. However, these findings do not preclude an impact of ciliates on CH4 at a lower taxonomic or transcriptional level (Kittelmann et al., 2014), or that ciliate community composition may play an important role in CH4 emissions of sheep feeding on less fibrous diets than the one we used in our study. Future research

<table>
<thead>
<tr>
<th>Type</th>
<th>O</th>
<th>B</th>
<th>AB</th>
<th>A</th>
</tr>
</thead>
<tbody>
<tr>
<td>Round a</td>
<td>16</td>
<td>88</td>
<td>4</td>
<td>9</td>
</tr>
<tr>
<td>Changes</td>
<td>15</td>
<td>76</td>
<td>6</td>
<td>4</td>
</tr>
<tr>
<td>Round b</td>
<td>0</td>
<td>92</td>
<td>6</td>
<td>19</td>
</tr>
</tbody>
</table>

Fig. 3. Schematic representation of succession of ciliate community types in the rumens of 117 sheep across two sampling time points (Round a and Round b, which took place 2 to 4 weeks apart). The changes indicate the number of animals in which a change in community type was observed between the two time points, and thick grey arrows indicate the direction of the community shift. A thin-line arrow represents a shift in a single animal. The sheep from which data were obtained for only one of the two rounds was excluded from this analysis.
CONCLUSIONS

Our data show that, while CH4 emission rankings of sheep were repeatable, rumen ciliate community types shifted over time. Thus, we found no correlation between naturally assembling ciliate community types and CH4 yields in sheep fed on a pelleted lucerne diet. In summary, it appears that the naturally occurring ciliate community types are much alike in terms of their roles in enteric CH4 formation in vivo, ruling out ciliate community typing as a simple means for classification of ruminants into high and low CH4 emitters.

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