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### *Temporal dynamics of the metabolically active rumen bacteria colonising fresh perennial ryegrass*

Huws, Sharon; Edwards, Joan Elizabeth; Creevey, Christopher; Rees Stevens, Pauline; Lin, Wanchang; Girdwood, Susan; Pachebat, Justin; Kingston-Smith, Alison

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1       **Temporal dynamics of the metabolically active rumen**  
2       **bacteria colonising fresh perennial ryegrass**

3       Sharon A. Huws<sup>a\*</sup>, Joan. E. Edwards<sup>a,b\*</sup>, Christopher J. Creevey, Pauline Rees Stevens<sup>a</sup>,  
4       Wanchang Lin<sup>a</sup>, Susan E. Girdwood<sup>a</sup>, Justin A. Pachebat<sup>a</sup>, Alison H. Kingston-Smith<sup>a</sup>

5       <sup>a</sup>Aberystwyth University, Aberystwyth, SY23 3FG, UK; <sup>b</sup>Current address: Wageningen  
6       University, Wageningen, 6703 HB, Netherlands; \*these authors contributed equally to the  
7       study.

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10      **RUNNING TITLE:** Diversity of rumen bacteria attached to perennial ryegrass.

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13      **\*Correspondence:** Sharon A. Huws, Animal and Microbial Sciences, Institute of Biological,  
14      Environmental and Rural Sciences (IBERS), Aberystwyth University, Penglais Campus,  
15      Aberystwyth, SY23 3FG, UK. E-mail: hnh@aber.ac.uk. Tel: +44 1970 823202 and Fax: +44  
16      1970 823155.

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20

21 **ABSTRACT**

22 This study investigated successional colonisation of fresh perennial ryegrass (PRG) by the  
23 rumen microbiota over time. Fresh PRG was incubated *in sacco* in the rumens of three  
24 Holstein x Friesian cows over a period of 8 h, with samples recovered at various times. The  
25 diversity of attached bacteria was assessed using 454 pyrosequencing of 16S rRNA (cDNA).  
26 Results showed that plant epiphytic communities either decreased to low relative abundances  
27 or disappeared following rumen incubation, and that temporal colonisation of the PRG by the  
28 rumen bacteria was biphasic with primary (1 & 2 h) and secondary (4-8 h) events evident  
29 with the transition period being with 2-4 h. A decrease in sequence reads pertaining to  
30 *Succinivibrio* spp. and increases in *Pseudobutyrvibrio*, *Roseburia* and *Ruminococcus* spp.  
31 (the latter all order Clostridiales) were evident during secondary colonisation. Irrespective of  
32 temporal changes, the continually high abundances of *Butyrivibrio*, *Fibrobacter*, *Olsenella*  
33 and *Prevotella* suggest that they play a major role in the degradation of the plant. It is clear  
34 that a temporal understanding of the functional roles of these, and the colonisation specific,  
35 microbiota within the rumen is now required to unravel the role of these bacteria in the  
36 ruminal degradation of fresh perennial ryegrass.

37

## 38 INTRODUCTION

39 Ruminant animals supply much of our meat and nearly all of our milk requirements,  
40 and as such are important to human nutrition. Globally, increased demand coupled with a  
41 growing population means that ruminant products will become increasingly scarce  
42 (Foresight, 2011). Ruminants convert plant biomass to chemical compounds, which are  
43 subsequently metabolised and absorbed by the animal. This process is largely due to the  
44 functional capacity of the rumen microbiome (Mackie, 2002; Edwards *et al.*, 2008a; Brulc *et*  
45 *al.*, 2009; Kingston Smith *et al.*, 2010; Kav *et al.*, 2012). Furthermore, the fermentative  
46 capacity of the rumen microbiota enables microbial breakdown of otherwise undigestible  
47 dietary material and thus defines the amount, quality and composition of the meat and milk  
48 produced (Edwards *et al.*, 2008a; Brulc *et al.*, 2009; Kim *et al.*, 2009; Kingston Smith *et al.*,  
49 2010; Kav *et al.*, 2012; Huws *et al.*, 2014b).

50 The rumen microbiota rapidly colonise ingested feed particles which ultimately  
51 results in the microbial degradation of the plant material, causing release of bioavailable  
52 nutrients (Cheng *et al.*, 1980; Miron *et al.*, 2001; Russell and Rychlik, 2001; Koike *et al.*,  
53 2003; Edwards *et al.*, 2007, 2008b; Huws *et al.*, 2013ab; 2014a). Nonetheless, the process is  
54 relatively inefficient in terms of animal production, with as little as 30% of the ingested  
55 nitrogen being retained by the animal for milk or meat production and the non-incorporated  
56 nitrogen is excreted as urea or ammonia (MacRae and Ulyatt, 1974; Dewhurst *et al.*, 1996;  
57 Kingston-Smith *et al.*, 2008, 2010). This presents a major challenge in terms of increasing  
58 ruminant productivity and consequently in providing a sustainable supply of meat and milk  
59 for the future. As the attachment of rumen microbiota to ingested forage is central for the  
60 availability of nutrients to the ruminant (McAllister *et al.*, 1994; Dewhurst *et al.*, 1996;  
61 Kingston-Smith *et al.*, 2010), understanding rumen plant-microbe interactions is paramount  
62 in order to develop novel methodologies for increasing nutrient use efficiency within

63 ruminants (Leng, 2014). For example, by furthering our fundamental understanding of  
64 temporal plant nutrient breakdown and availability it is possible to define key limitations and  
65 enhance the chemical characteristics of available forages through targeted plant breeding to  
66 circumvent these limitations. Thus, fundamental information on the temporal plant-microbe  
67 interactome can inform plant breeding strategies with the ultimate aim of increasing animal  
68 nutrient use efficiency whilst decreasing environmental impact.

69 We have previously shown using Denaturing Gradient Gel Electrophoresis (DGGE)  
70 that the perennial ryegrass attached microbiome changes in diversity between 2 and 4 h of  
71 incubation, but the capacity of this technique is limited in terms of understanding which  
72 bacteria change in abundance within these primary and secondary colonisation events (Huws  
73 *et al.*, 2013b; Huws *et al.*, 2013a). In this study we used 454 based 16S rRNA (cDNA)  
74 sequencing to characterise time-related changes in the diversity of the rumen bacteria  
75 attaching to fresh perennial ryegrass (PRG). By basing these experiments on RNA rather  
76 than DNA, these data provide an insight specifically into the metabolically active rumen  
77 PRG-attached microbiome.

78

## 79 **MATERIALS AND METHODS**

### 80 **Growth and preparation of plant material**

81 Perennial ryegrass, (*Lolium perenne* cv. AberDart; PRG) was grown from seed in plastic seed  
82 trays (length 38 cm x width 24 cm x depth 5 cm) filled with soil/compost (Levingtons general  
83 purpose). The trays were housed in a greenhouse under natural irradiance with additional  
84 illumination provided during the winter months (minimum 8 h photoperiod). A temperature  
85 of 22/19°C day/night was maintained and plants were watered twice a week. Plants were  
86 harvested after 6 weeks by cutting 3 cm above soil level, before cutting with scissors into 1

87 cm sections just prior to incubation in the rumen as described below. Samples of this  
88 harvested plant material were also snap frozen in dry ice, and stored at -80°C for bacterial  
89 profiling (0 h samples).

90

### 91 ***In sacco* incubations**

92 Three mature, rumen-cannulated, non-lactating Holstein x Friesian cows were used for this  
93 experiment. Experiments were conducted with the authority of Licenses under the United  
94 Kingdom Animal Scientific Procedures Act, 1986. For at least 2 weeks prior to the  
95 experiments, the cows were fed a diet of straw and grass silage *ad libitum* (~6.5 kg dry matter  
96 day<sup>-1</sup>) and were also permitted field grazing on PRG for at least 4 h/day. For the duration of  
97 the experiment animals were fed silage twice daily (07:00 and 16:00). Stitched nylon bags  
98 (10 cm × 20 cm) of 100 µm<sup>2</sup> pore sizes were filled with 15 g (fresh weight) of the processed  
99 plant material and sealed at all perimeters by heating (Impulse sealer, American Int, NI  
100 Electric, AIE, USA). The nylon bag technique was adopted as described previously (Ørskov  
101 *et al.*, 1980; Vanzant *et al.*, 1998). Essentially, bags were connected to a 55-cm, coated  
102 flexible plastic cable with lacing cords and this was placed in the rumen and attached to the  
103 cap of the fistula. Bags were placed simultaneously in the rumen of each cow shortly after  
104 animals were offered the first meal of the morning and removed after 1, 2, 4, 6 and 8 h of  
105 incubation. At each time interval, twelve bags (four from each cow) were withdrawn and the  
106 residual plant material in six of the bags (two for each cow) was processed by washing with  
107 distilled water (500 ml added to plant material within bags and bags gently squeezed  
108 thereafter) to remove loosely attached microbes followed by oven drying for two replicate  
109 samples from each cow and calculation of plant degradation (% dry matter lost). The

110 remaining six bags (two for each cow) were similarly washed with distilled water before  
111 being immediately frozen in dry ice and then stored at -80°C until RNA extraction.

112

### 113 **RNA extraction**

114 Frozen samples were ground to a fine powder under liquid nitrogen before RNA was  
115 extracted using a hot phenol method (Ougham and Davies, 1990). Essentially aquaphenol  
116 (10 mL) was added to the ground sample prior to incubation at 65°C for 1 h. Tubes were  
117 inverted before chloroform was added (5 mL). Tubes were centrifuged (5,000 x g, 30 mins,  
118 20°C) before upper phase was removed then the procedure was repeated by addition of more  
119 chloroform (5 mL) and centrifugation as described. Lithium chloride (2M final  
120 concentration) was then added, to remove any contaminating DNA, and samples stored  
121 overnight at 4°C. Samples were subsequently centrifuged (13,000 x g, 30 mins, 4°C) and  
122 supernatant discarded, then the procedure was repeated from addition of lithium chloride to  
123 ensure all DNA was removed. Once the supernatant was discarded the pellet was  
124 resuspended in ice cold 80% ethanol and centrifuged (13,000 x g, 15 mins, 4°C), this was  
125 repeated twice before the pellet was air dried and resuspended in molecular grade water.  
126 Quality and quantity of retrieved RNA was checked using the Experion automated  
127 electrophoresis system and RNA 'stdsens' chips for standard sensitivity analysis (Bio-rad,  
128 Hemel Hempstead, UK).

129

### 130 **16S rRNA 454 pyrosequencing**

131 RNA (c. 100 ng) was reverse transcribed using the reverse primer R1401  
132 (5'GGGTCTTGTACACACCG 3') and Superscript III reverse transcriptase (Invitrogen Ltd,

133 Paisley, UK) in 20 uL reactions, following the manufacturer's guidelines and as previously  
134 described by Edwards *et al.* (2007) and Huws *et al.* (2011; 2013a). Control reactions were  
135 performed with no reverse transcriptase, and were PCR amplified (as described below) to  
136 confirm that the RNA preparations were free of contaminating DNA. Amplicons of the V6-  
137 V8 variable region of the bacterial 16S rDNA gene were generated in triplicate per cDNA  
138 sample by PCR using the primers 799F2 (5' tagged with Roche B adaptor) and R1401 (5'-  
139 tagged with the Roche A adaptor and MID barcode tags specific for each sample as suggested  
140 by Roche) as described by Edwards *et al.* (2007), except that 30 cycles of amplification was  
141 used. All PCR products were initially verified by electrophoretic fractionation on a 1.0%  
142 agarose gel for 1 h, 120 V, 80 mA in 1% TAE (*Tris* base, acetic acid and EDTA) buffer  
143 before pooling of triplicate amplifications. The pooled PCR products (30 µl each sample)  
144 were subsequently run on a 2.0% agarose gel for 2 h, 120 V, 80 mA in 1% TAE buffer before  
145 bands were viewed and cut on a dark reader transilluminator (Clare Chemical Research Inc.,  
146 Colorado, USA). Amplicons were retrieved from cut bands using the Isolate II PCR and gel  
147 Kit (Bioline, London, UK). Purified amplicons were verified and quantified using the  
148 Agilent High Sensitivity Assay Kit (Agilent Technologies, California, USA) prior to  
149 pyrosequencing using Titanium chemistry on a Roche GS-FLX 454 sequencer (Roche  
150 Diagnostics Ltd, West Sussex, UK) according to the manufacturer's guidelines. These  
151 sequences and associated metadata can be accessed through the NCBI bioproject ID  
152 PRJNA274256.

153

## 154 **Data Analysis**

155 All 16S rRNA sequences with a length less than 400bp were discarded and those remaining  
156 clustering at 97% identity using CD-HIT-OTU (Li *et al.*, 2012) were analysed to identify  
157 Operational Taxonomic Units (OTUs). OTUs with fewer than 5 representatives and those



158 found to be chimeric were removed from subsequent analyses. Abundances of each of the  
159 remaining OTUs were calculated using the “clstr\_sample\_count\_matrix.pl” script from the  
160 CD-HIT-OTU package. These counts were then used as input to the Bioconductor package  
161 DESEQ2 in R (Love *et al.*, 2014) to identify overall changes in the attached microbiota.  
162 Taxonomic identification of the OTUs was carried out using the classifier algorithm from the  
163 RDP database (Cole *et al.*, 2014). Any taxonomic identification below 90% identity to  
164 published sequences was not included in the analysis. Further statistical analyses of changes  
165 at the Phylum, Order, Family and Genus level were carried out by ANOVA in Genstat  
166 (Payne *et al.*, 2007). Only those genera that were present in greater than 1% of the total  
167 microbiome in any time point were included. Dry matter data was also analysed by ANOVA  
168 and Genstat (Payne *et al.*, 2007). PCA plots were generated using the ggplot2 library and  
169 rarefaction curves were drawn using the vegan package in R. Data was transformed into a  
170 heat map using the Heatmap2 package from the Gplots package in R using the summed  
171 abundances of each OTU from the genera indicated.

172

## 173 **RESULTS**

### 174 **16S rRNA sequencing data**

175 Overall, 1,411,847 sequences were generated, of which 1,016,349 (72%) had a length greater  
176 than 400bp (Figure S1). This consisted of an average of 41,646 ( $\pm 4,762$  standard deviation)  
177 sequences/sample pre-filtering and an average of 31,761 ( $\pm 3,523$  standard deviation)  
178 sequences/sample post-filtering (Table S1). After filtering, the average sequence length was  
179 425 bp. Following removal of low abundance and chimeric OTUs 1,201 OTUs remained,  
180 which is consistent with previous reports (Creevey *et al.* 2014). The average number of  
181 sequences per sample, assigned to an OUT, which had a taxonomic classification >90%, was

182 9,093 ( $\pm 1,274$ ) (Table S1). The most abundant OTU (from *Butyrivibrio*) had 29,722  
183 representatives, representing 10% of all sequences found across all time points. The OTU  
184 based rarefaction curve plateaued indicating that a reasonable level of sequencing depth was  
185 obtained (Figure S2).

186

### 187 **Fate of the perennial ryegrass epiphytic microbiota post rumen incubation**

188 16S rRNA pyrosequences showed that the relative abundances of the plant epiphytic  
189 communities decreased substantially to very low levels within the first hour of rumen  
190 incubation with some decreasing to below detection limits (Tables S2-S5 and Figure 1).

191

### 192 **Temporal diversity of the PRG attached microbiota post 1 h of rumen incubation**

193 The PCA plot of OTU abundances showed that the microbiota attached to fresh PRG at 1 & 2  
194 h differed significantly ( $P < 0.05$ ) from those attached during 4-8 h of incubation (Figure 2).  
195 This was in agreement with the results of Denaturing gradient gel electrophoresis (DGGE),  
196 performed as described by Edwards *et al.* (2007) prior to sequencing which indicated  
197 biphasic colonisation, in which the primary (1 & 2 h) and secondary (4-8 h) phases had  
198 approximately 60-75% DGGE profile similarity dependent on cow (data not shown).  
199 Shannon diversity boxplots based on OTU abundance showed a higher bacterial diversity  $\geq 5$   
200 following 1 and 2 h of incubation, which then decreased significantly ( $P < 0.05$ ) between 4-6 h  
201 of incubation (Figure 3). The Shannon diversity at 8 h was not significantly different from  
202 the other time points.

203

### 204 **Phyla level temporal diversity of the attached microbiota post 1 h of rumen incubation**

205 On a phylum level the most abundant attached bacteria were Firmicutes, Bacteroidetes and  
206 Fibrobacteres (on average approx. 75, 17 and 4% of total average normalised reads across  
207 time points respectively) (Table 1), whereas a further 8 phyla were relatively minor (<2% of  
208 total normalised reads/phyla) in comparison. In terms of temporal changes within the more  
209 predominant attached bacterial phyla, Firmicutes changed in abundance significantly over  
210 time, with the greatest abundance observed during secondary colonisation (4-8 h) ( $P<0.05$ )  
211 (Table 1). Bacteroidetes and Fibrobacteres read abundances did not change significantly over  
212 time ( $P>0.05$ ) (Table 1). Despite their lower abundances the reads pertaining to phyla  
213 Actinobacteria, Elusimicrobia, Lentisphaerae and Verrucomicrobia also changed significantly  
214 over time ( $P<0.05$ ) (Table 1). More sequences pertaining to the phyla Actinobacteria were  
215 present at 2 h of rumen incubation compared to all other time points ( $P<0.05$ ) (Table 1).  
216 Elusimicrobia and Verrucomicrobia read abundances decreased significantly post 1 h and 2 h  
217 of rumen incubation respectively ( $P<0.05$ ) (Table 1). Reads pertaining to Lentisphaerae were  
218 maximal at 1 h of incubation ( $P<0.05$ ) (Table 1). Fusobacteria, Proteobacteria, Spirochaetes,  
219 and Tenericutes read abundances did not change significantly over time ( $P>0.05$ ) (Table 1).

220

#### 221 **Order level temporal diversity of the attached microbiome post 1 h of rumen incubation**

222 On an order level the most abundant attached bacteria were Clostridiales, Bacteriodales  
223 Selenomonadales, Fibrobacterales, Coriobacteriales and Spirochaetales (on average approx.  
224 67, 17, 7, 4, 3 and 2% of total average normalised reads across time points respectively),  
225 whereas a further 20 orders were relatively minor (<2% of total normalised reads/phyla) in  
226 comparison (Table 2). The order Clostridiales changed significantly in abundance over time,  
227 with increased abundances present during secondary colonisation events (4-8 h) ( $P<0.05$ )  
228 (Table 2). Bacteroidales, Fibrobacterales and Spirochaetales read abundances did not change

229 significantly over time ( $P>0.05$ ) (Table 2). Read abundances pertaining to the order  
230 Selenomonadales changed significantly over time, with significantly higher abundances  
231 present at 2 and 4 h compared with read abundances at 8 h of rumen incubation ( $P<0.05$ )  
232 (Table 2). The order Coriobacteriales varied at each time interval substantially, with no real  
233 pattern evident ( $P<0.05$ ) (Table 2). Despite their lower abundances the reads pertaining to  
234 orders Aeromonadales, Desulfuromonadales, and Methylophilales also changed significantly  
235 over time ( $P<0.05$ ) (Table 2). More sequences pertaining to the order Aeromondales was  
236 seen at 1 and 2 h of rumen incubation compared with 6 and 8 h of incubation ( $P<0.05$ ) (Table  
237 2). The order Desulfuromonadales varied at each time interval substantially, with no real  
238 pattern evident ( $P<0.05$ ) (Table 2). The order Methylophilales decreased significantly after  
239 the first 1 h of rumen incubation ( $P<0.05$ ) (Table 2). The remaining bacterial orders were  
240 relatively minor and showed no changes in abundance over incubation time ( $P>0.05$ ) (Table  
241 2).

242

### 243 **Family level temporal diversity of the attached microbiome post 1 h of rumen** 244 **incubation**

245 On a family level the most abundant attached bacteria were Lachnospiraceae, Prevotellaceae,  
246 Veillonellaceae, Fibrobacteraceae, Ruminococcaceae and Coriobacteriaceae (on average  
247 approx. 75, 19, 9, 5, 3 and 2% of total average normalised reads across time points  
248 respectively), whereas a further 31 families were relatively minor (<2% of total normalised  
249 reads/phyla) in comparison (Table 3). The family Lachnospiraceae changed significantly in  
250 abundance over time, with increased abundances present during secondary colonisation  
251 events (4-8 h) ( $P<0.05$ ) (Table 3). The family Veillonellaceae changed significantly in  
252 abundance over time, with decreased abundances present after 8 h of incubation ( $P<0.05$ )

253 (Table 3). The families Prevotellaceae and Fibrobacteraceae did not change significantly in  
254 abundance over time ( $P>0.05$ ) (Table 3). The family Ruminococcaceae changed significantly  
255 in abundance over time, with increased abundances present 8 h after incubation ( $P<0.05$ )  
256 (Table 3). The family Coriobacteriaceae changed significantly in abundance over time, with  
257 the highest abundances seen 2 h after incubation ( $P<0.05$ ) (Table 3). Of the other lower  
258 abundance families only Methylophilaceae and Succinivibrionaceae changed significantly in  
259 abundance over time, with both showing decreased abundance after 1 and 2 h of incubation  
260 respectively ( $P<0.05$ ) (Table 3).

261

#### 262 **Genus level temporal diversity of the attached microbiome post 1 h of rumen incubation**

263 On a genus level the most abundant attached bacteria were *Butyrivibrio*, *Pseudobutyrvibrio*,  
264 *Selenomonas*, *Prevotella*, *Fibrobacter*, *Olsenella*, and *Ruminococcus* (approx. 44, 17, 12, 10,  
265 6, 3 and 2% of total average normalised reads across time points respectively), whereas a  
266 further 52 genera were relatively minor ( $<2\%$  of total normalised reads/phyla) in comparison  
267 (Table 4). The number of sequences pertaining to the genera *Butyrivibrio*, *Prevotella*,  
268 *Fibrobacter* and *Olsenella* did not change significantly in abundance over time ( $P>0.05$ )  
269 (Table 4). *Pseudobutyrvibrio* read abundances changed significantly over time, with greater  
270 abundances present from 4-8 h of incubation (secondary colonisation phase) ( $P<0.05$ ) (Table  
271 4). *Selenomonas* read abundances changed significantly over time, but no decipherable  
272 changes in pattern between primary and secondary colonisation could be seen ( $P<0.05$ )  
273 (Table 4). *Ruminococcus* read abundances changed significantly over time, with an increase  
274 in abundance evident after 8 h of incubation within the rumen ( $P<0.05$ ) (Table 4). Despite  
275 their lower abundances the reads pertaining to genera *Rhodanobacter*, *Roseburia*,  
276 *Succinivibrio* and *Murdochiella* also showed temporal variation in abundance ( $P<0.05$ )

277 (Table 4). *Rhodanobacter* and *Murdochiella* abundance was highest at 2 h post incubation  
278 ( $P<0.05$ ) (Table 4), nonetheless even at their highest value they accounted for  $>0.1\%$  of the  
279 attached diversity. *Roseburia* read abundances were significantly higher in the secondary  
280 phase (4-8 h) of rumen incubation ( $P<0.05$ ) (Table 4). Conversely, *Succinivibrio* read  
281 abundances were higher during the primary phase (1 & 2 h) of rumen incubation ( $P<0.05$ )  
282 (Table 4). The remaining bacterial genera were relatively minor and showed no changes in  
283 abundance over incubation time ( $P>0.05$ ) (Table 4).

284

### 285 **Temporal niche specialisation of the perennial ryegrass attached microbiota incubated** 286 **within the rumen**

287 Differences were observed in the dynamics of classified OTUs within some of the dominant  
288 orders within the transition phase (between 2 and 4 h) of PRG incubation within the rumen.  
289 Five OTUs, classified as order Bacteroidales, increased in abundance, whilst 8 decreased in  
290 abundance between 2 and 4 h of PRG incubation within the rumen (Table 5). The variability  
291 in the proportional representation of the order Bacteroidales also decreased post 2 h of  
292 incubation (Figure 4). Conversely, 18 OTUs classified as order Clostridiales increased in  
293 abundance, whilst 11 decreased in abundance between 2 and 4 h of incubation within the  
294 rumen (Table 5). Again, the variability in the proportional representation of the order  
295 Clostridiales also decreased post 2 h of incubation, (Table 5 & Figure 4). Very few changes  
296 in OTU representation were apparent for OTUs within any of the other dominant orders  
297 (Table 5), nonetheless decreases in proportional variability of reads pertaining to  
298 Coriobacterales were seen (Figure 4). Decreases in proportional variability of reads  
299 pertaining to the orders Bacteroidales, Clostridiales and Coriobacterales, alongside the  
300 decrease in Shannon diversity between primary (1 & 2 h) and secondary colonisation (4-8 h),

301 suggest that the attached microbiota show more niche specialisation during secondary  
302 colonisation (Figure 3 & 4). Dry matter (DM) disappearance data showed that a minimal  
303 amount (2.8 %) of PRG was degraded within the primary phase (1 & 2 h) of the incubation,  
304 unlike the transition (2-4 h) between the two phases where 22.2 % was lost (Figure 5). In the  
305 secondary phase between 4 and 8 h of incubation, a further approx. 31.7% of the PRG DM  
306 was degraded (Figure 5).

307

## 308 **DISCUSSION**

309 In this study we characterised the rumen bacteria attached to fresh perennial ryegrass that had  
310 been incubated in the rumen over time in order to enhance our understanding of ruminal  
311 plant-microbe interactions. Within this study we have demonstrated, using 454 based  
312 pyrosequencing of 16S rRNA (cDNA based), that substantial temporal changes occur in the  
313 attached microbiota, resulting in primary (1 & 2 h) and secondary (4-8 h) colonisation events  
314 by rumen bacteria. The change to a secondary phase was mainly associated with decreases in  
315 sequences pertaining to the genera *Succinivibrio* and increases in *Pseudobutyrvibrio*,  
316 *Roseburia* and *Ruminococcus*. *Butyrvibrio*, *Fibrobacter*, *Olsenella* and *Prevotella* also  
317 dominated the attached microbiome irrespective of incubation time.

318 The depth of sequencing and read length obtained within this study is comparative or  
319 higher than those reported in many other published datasets in which 454 technology was  
320 used to probe the rumen microbiome. For example Roggenbuck *et al.* (2014) obtained 1,743  
321 reads/sample with an average read length of 376 bp, Jami *et al.* (2013) obtained an average of  
322 10,938 reads/sample (average read length not specified), Pitta *et al.* (2014) obtained on  
323 average 5,199 reads /sample (average read length not specified), Fouts *et al.* (2012) obtained  
324 23,493 reads and Jami and Mizrahi (2012) reported an average 9,587 reads/sample with an

325 average read length of 338 bp. In this study we obtained on average 31,761 reads/sample, in  
326 the same range as obtained in our previous study (Huws *et al.*, 2014b). Our rarefaction curve  
327 based on OTUs, also demonstrated some plateauing. It was suggested in another study (Kim  
328 *et al* 2011), that to achieve 99.9% coverage at species level, at least 78,218 bacterial 16S  
329 sequences would be needed which equates to approx. 41% of sequences obtained within this  
330 study post-filtering. From the reads generated from rumen incubated samples we identified  
331 11 phyla, 24 orders, 37 families and 59 genera and an average of 9,093 OTUs, which is  
332 similar to that obtained from other previously 454 based rumen microbiome datasets (Fouts *et*  
333 *al.*, 2012; Jami and Mizrahi, 2012; Pope *et al.*, 2012; Jami *et al.*, 2013; Huws *et al.*, 2014b).  
334 Thus whilst it is possible that our coverage doesn't include all the diversity present, the  
335 diversity captured gives a very good indication of the bacterial diversity and temporal  
336 changes, post rumen incubation.

337 Our study shows that the plant epiphytic communities rapidly diminished in  
338 proportional representation when the rumen microbiota begin to colonise. It should be noted  
339 that *Flavobacterium*, *Delftia*, *Cellvibrio* and *Pseudomonas* spp. are still present within the  
340 reads obtained post-rumen incubation. This is likely to be because they were the most  
341 predominant epiphytes found colonising the PRG pre-incubation.

342 The 16S rRNA sequencing information concurred with our previous DGGE based  
343 data showing clear primary (1 & 2 h) and secondary (4-8 h) bacterial colonisation events on  
344 fresh perennial ryegrass within the rumen (Huws *et al.*, 2013b; Huws *et al.*, 2014a).  
345 Interestingly, a recent publication by Kingston-Smith *et al.* (2013) using FT-IR to investigate  
346 the metabolite fingerprint of the interactome (perennial ryegrass coupled with the attached  
347 microbiota) did not demonstrate clear differences between 2 and 4 h although a change from  
348 6 h onwards was noted. This is probably a consequence of the fact that both the perennial  
349 ryegrass and the attached microbiota were analysed together, therefore masking changes



350 occurring in each component separately. A recent DNA based study investigating temporal  
351 colonisation of air dried switchgrass showed changes in the microbiome over time, but the  
352 greatest changes were observed within the initial 30 mins and after 4 h of rumen incubation  
353 (Piao *et al.*, 2014). Nonetheless, previous DGGE analysis of the rumen bacteria attached to  
354 fresh PRG, on both a DNA and RNA basis, found no differences within 30 min of incubation  
355 (Edwards *et al.*, 2007). Sun *et al.* (2008) found using DGGE that temporal changes in the  
356 attached microbiota on Chinese wild rye hay incubated in the rumen occurred between 6 and  
357 12 h of incubation. The likely difference between our study and that of others is due to the  
358 species of the plant material used, and also our plant material was fresh and not conserved.  
359 Furthermore, in this study we also investigated changes on an RNA basis, in order to probe  
360 changes in the truly metabolically active bacterial community, whereas other studies used  
361 DNA (Piao *et al.*, 2014; Sun *et al.*, 2008). Irrespective of this, however, it is clear from these  
362 studies that colonisation events are rapid within the rumen and timings of ecological changes  
363 are dependent on the plant characteristics.

364 The data in this study suggest that primary colonising bacteria are likely to utilise  
365 soluble nutrients, and that the secondary phase colonisers are adept at degrading plant  
366 structural components. This suggestion is based on the fact that only 2.8% plant dry matter  
367 disappearance was seen between 1-2 h (primary colonisation phase) of rumen incubation and  
368 31.7% dry matter disappearance was seen between 4-8 h (secondary colonisation phase) of  
369 rumen incubation. In terms of the temporal changes in the attached microbiota, we observed  
370 that *Succinivibrio* (order Aeromonadales) were more abundant during primary colonisation  
371 events than secondary colonisation events. The reason for increased abundance of  
372 *Succinivibrio* during primary colonisation is unclear as this bacterium is considered to be  
373 predominantly amylolytic. Nonetheless the normalised read abundances of *Succinivibrio* are  
374 low irrespective of time. Conversely, we observed that *Pseudobutyrvibrio*, *Roseburia* and

375 *Ruminococcus* spp. (all order Clostridiales) were less abundant during primary colonisation  
376 events than in secondary colonisation events. Piao *et al.* (2014) also saw increases in  
377 *Pseudobutyrvibrio* and *Ruminococcus* spp. during secondary colonisation events, when  
378 investigating temporal colonisation of switchgrass incubated within the rumen.  
379 *Pseudobutyrvibrio* spp. commonly possess xylanases which randomly cleave the  $\beta$ -1,4  
380 backbone of the complex plant cell wall polysaccharide xylan (Krause, 2003). Likewise,  
381 *Ruminococcus* spp. are well recognised for their fibrolytic capacity due to the possession of  
382 numerous glycosyl hydrolase families (Krause, 2003; Dai *et al.*, 2015). Therefore it is  
383 possible that the increase in these bacteria is at least partially responsible for the 31.7% dry  
384 matter disappearance seen during the secondary phase of colonisation.

385 Irrespective, of temporal changes it was also noted that *Butyrvibrio*, *Fibrobacter*,  
386 *Olsenella* and *Prevotella* spp. read abundances were high irrespective of colonisation phase.  
387 Rumen *Butyrvibrio* spp. are known to have proteolytic, biohydrogenating and plant  
388 hemicellulolytic activity (Hobson and Stewart, 1997; Krause, 2003). *Fibrobacter* spp. are  
389 regarded as being mainly fibrolytic bacteria. Indeed, a recent metatranscriptomic study by  
390 Dai *et al.* (2015) suggested that the bulk of ruminal glycosyl hydrolases, including xylanases  
391 and endoglucanases, are possessed by *Ruminococcus* and *Fibrobacter* spp. *Olsenella*, on the  
392 other hand, is a reasonably newly classified genus (Dewhirst *et al.*, 2001), composed of  
393 bacteria that can ferment carbohydrates to lactic acid (Kraatz *et al.*, 2011). A rumen  
394 *Olsenella* spp. has also been shown to have  $\beta$ -glucosidase activity, showing its capacity to  
395 breakdown glucose (Kraatz *et al.*, 2011). *Prevotella* spp., are mainly known for their starch  
396 degrading and proteolytic capacity, but they also have cellulolytic capacity (Gardner *et al.*,  
397 1995; Krause, 2003). Due to the fact that most of these attached bacteria have many  
398 functions, it is not possible to conclude with absolute certainty what their role is at a given  
399 incubation time in terms of plant degradation without gene expression data.

400

401           Nevertheless, variation in proportional representation of Shannon diversity indices  
402 and significant OTU changes assigned to order level was observed in this study indicating  
403 functional drivers for the succession. It is speculated that the decreased diversity of the  
404 secondary colonisers of the orders Clostridiales and Bacteroidales is due to the fact that these  
405 bacteria play a more focussed role in plant degradation and nutrient assimilation during the  
406 secondary colonisation phase after soluble plant nutrients have been depleted. It has been  
407 shown previously that regardless of the concentration, the rate of release of soluble  
408 carbohydrate from fresh forage is likely to be limiting to the microbiota (Kingston-Smith *et*  
409 *al.*, 2003). This was not apparent in our previous studies (Huws *et al.*, 2013), and is likely to  
410 have been due to methodological limitations of the DGGE technique. The study by Piao *et*  
411 *al.* (2014) showed increases in Shannon's diversity until 1 h of incubation then a plateau.  
412 The reasons for the differences between our findings and those of Piao *et al.* (2014) are  
413 unclear but may be due to the different plants analysed, whether they were conserved or not,  
414 and also the fact that we analysed the adherent bacterial diversity using RNA as opposed to  
415 DNA.

416           In summary, this study demonstrates that fresh perennial ryegrass is rapidly colonised  
417 within the rumen with a substantial decrease in active plant epiphytic communities within 1 h  
418 of incubation, followed by a biphasic temporal change in the ecology of the adherent  
419 bacterial community. These primary (1 & 2 h) and secondary (4-8 h) phases in the attached  
420 microbiota were attributable mainly to decreases in *Succinivibrio* spp. and increases in  
421 *Pseudobutyrvibrio*, *Roseburia* and *Ruminococcus* spp. during secondary colonisation.  
422 Irrespective of temporal changes, the continually high abundances of *Butyrivibrio*,  
423 *Fibrobacter*, *Olsenella* and *Prevotella* suggest that they also play a major role in the  
424 degradation of the plant. It is clear that a temporal understanding of the functional roles of

425 these microbiota within the rumen is now required to understand the plant-microbe  
426 interactome and improve ruminant nutrient use efficiency further. Understanding the plant  
427 degradation limitations encountered by the attached microbiota will lead to novel plant  
428 breeding targets aimed at increasing the potential degradation of PRG within the rumen and  
429 thus increasing animal nutrient use efficiency.

430

#### 431 **SUPPLEMENTARY DATA**

432 Supplementary data is available at FEMSEC online.

433

#### 434 **ACKNOWLEDGEMENTS**

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438

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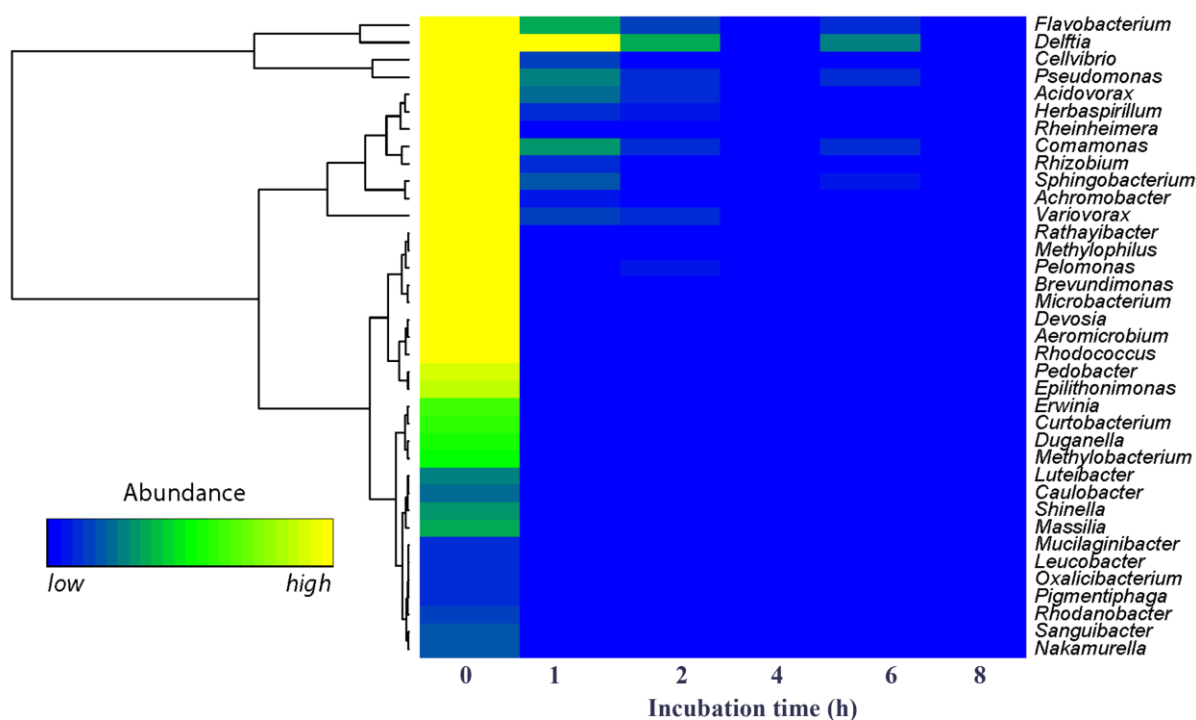
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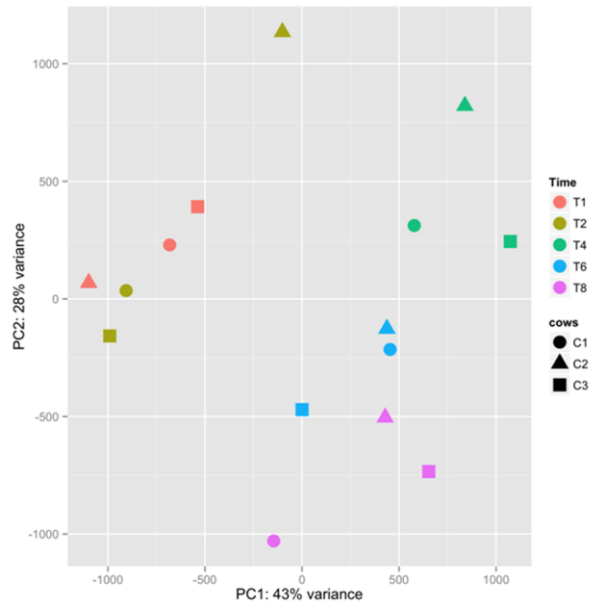
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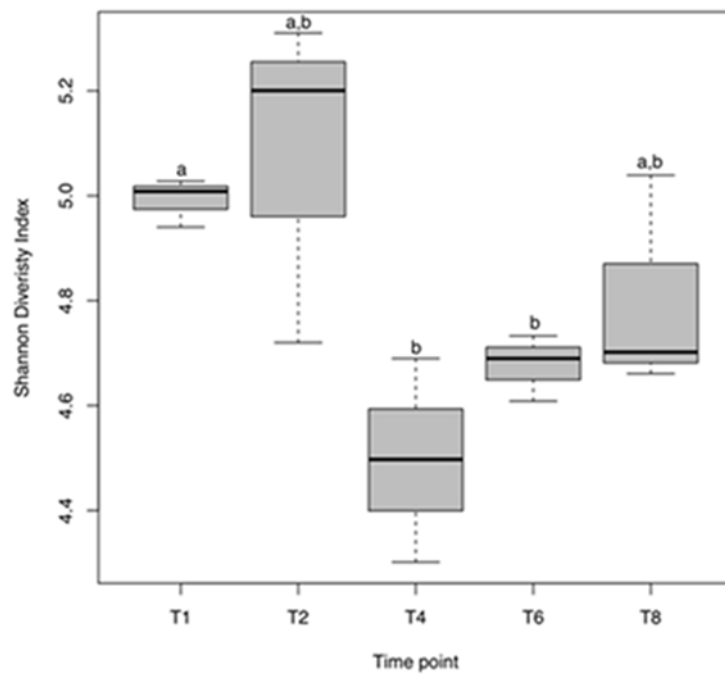
## FIGURE LEGENDS



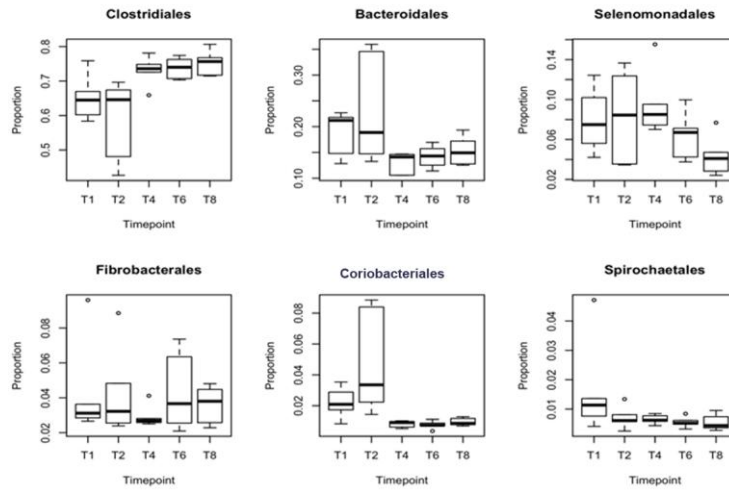
**Figure 1.** Heat map illustrating changes in proportional read abundances of perennial ryegrass epiphytic communities post rumen incubation. Mean data for each time point are shown (n=2 for 0h and n=6 for all other time points).



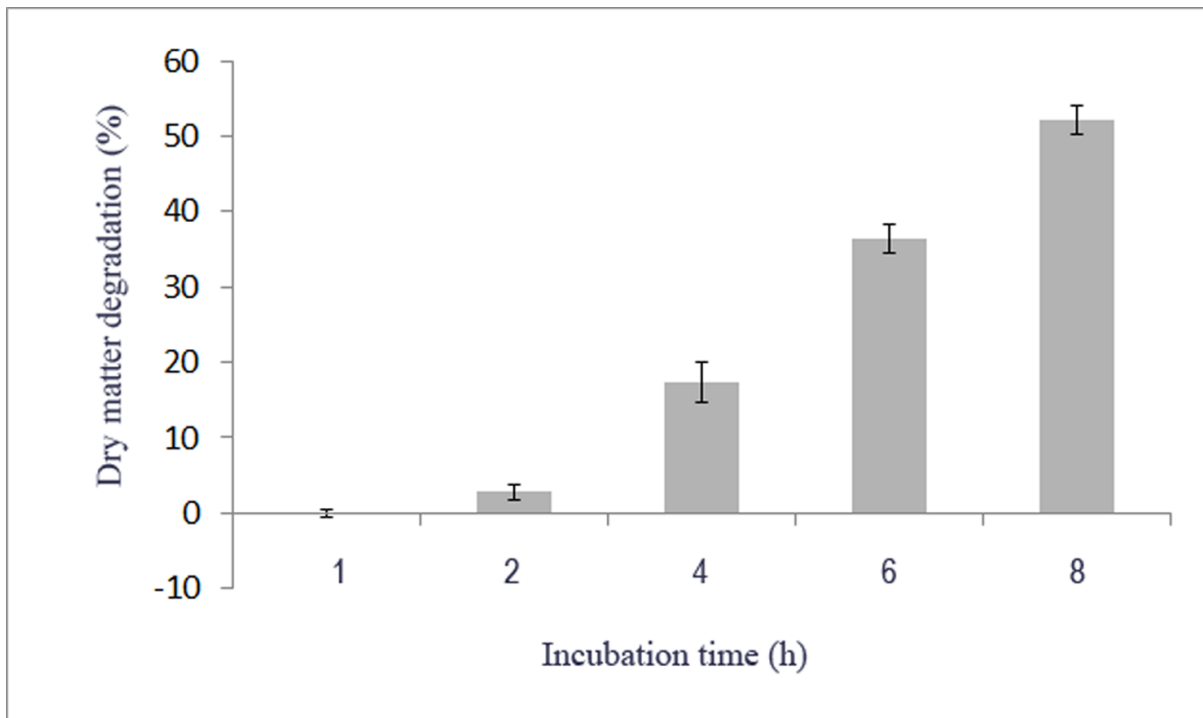
**Figure 2.** PCA plot showing the diversity of rumen bacteria attached to perennial ryegrass over time. Data for post rumen incubation of 1, 2, 4, 6 and 8 h (T1, T2, T4, T6 and T8 respectively) within each cow (C1, C2 and C3 respectively) are shown. Mean data for 2 bags incubated within each cow are shown for each time point.



**Figure 3.** Boxplots of the average Shannon diversity indices at each time point representing each of the three cows sampled in duplicate (n=6). Time points that do not share notations were significantly different ( $P<0.05$ ) according to a t-test. Post rumen incubation time points of 1, 2, 4, 6 and 8 h (T1, T2, T4, T6 and T8 respectively).



**Figure 4.** Proportional changes in the 6 most abundant bacterial orders attached to perennial ryegrass incubated in the rumen over time (n=6). Post rumen incubation time points of 1, 2, 4, 6 and 8 h (T1, T2, T4, T6 and T8 respectively).



**Figure 5.** Perennial ryegrass dry matter disappearance (%) following incubation within the rumen over time. Standard error of the mean for each time point are shown.

**Table 1.** Comparison of the bacterial phyla attached to perennial ryegrass over time within the rumen. Data shown are the average values (n=6) of the normalised reads.

Phylum	Time of incubation (h)					SED	P
	1	2	4	6	8		
<i>Actinobacteria</i>	368.2 <sup>a</sup>	792.6 <sup>b</sup>	146.8 <sup>a</sup>	132.0 <sup>a</sup>	165.6 <sup>a</sup>	162.8	0.016
<i>Bacteroidetes</i>	3356	3954	2341	2528	2726	584.6	NS
<i>Elusimicrobia</i>	11.5 <sup>b</sup>	3.7 <sup>a</sup>	1.3 <sup>a</sup>	0.4 <sup>a</sup>	0.3 <sup>a</sup>	2.6	0.012
<i>Fibrobacteres</i>	675.5	703.2	511.5	744.8	643.4	173.6	NS
<i>Firmicutes</i>	12570 <sup>ab</sup>	11840 <sup>a</sup>	14600 <sup>c</sup>	14151 <sup>bc</sup>	14136 <sup>bc</sup>	819.0	0.040
<i>Fusobacteria</i>	0.9	0.6	0.6	0.3	0.0	0.8	NS
<i>Lentisphaerae</i>	6.2 <sup>c</sup>	5.0 <sup>bc</sup>	1.3 <sup>ab</sup>	1.8 <sup>ab</sup>	0.3 <sup>a</sup>	1.7	0.029
<i>Proteobacteria</i>	515.5	349.9	98.6	160.4	48.7	178.5	NS
<i>Spirochaetes</i>	283.7	122.9	114.7	96.5	95.2	70.9	NS
<i>Tenericutes</i>	0.3	0.6	1.0	1.0	1.6	1.1	NS
<i>Verrucomicrobia</i>	33.0 <sup>ab</sup>	49.3 <sup>b</sup>	7.6 <sup>a</sup>	8.9 <sup>a</sup>	7.7 <sup>a</sup>	10.9	0.015

Values with different superscripts on the same row differed significantly ( $P < 0.05$ ), whereas values that were not significantly (NS) different ( $P > 0.05$ ) have no superscripts in the same row.



**Table 2.** Comparison of the bacterial orders attached to perennial ryegrass over time within the rumen. Data shown are the average values (n=6) of the normalised reads.

Order	Time of incubation (h)					SED	P
	1	2	4	6	8		
<i>Actinomycetales</i>	11.9	27.9	0.6	0.4	0.0	15.0	NS
<i>Aeromonadales</i>	57.4 <sup>b</sup>	64.3 <sup>b</sup>	39.4 <sup>ab</sup>	19.3 <sup>a</sup>	18.8 <sup>a</sup>	11.8	0.013
<i>Anaeroplasmatales</i>	0.3	0.6	1.0	1.0	1.6	1.1	NS
<i>Bacilliales</i>	7.8	2.2	0.0	3.5	0.0	3.5	NS
<i>Bacteroidales</i>	3135	3737	2285	2457	2693	546.1	NS
<i>Burkholderiales</i>	18.7	21.6	13.0	14.2	15.3	3.1	NS
<i>Caulobacterales</i>	6.0	7.0	0.0	3.1	0.7	2.9	NS
<i>Clostridiales</i>	10705 <sup>a</sup>	9871 <sup>a</sup>	12625 <sup>b</sup>	12660 <sup>b</sup>	12943 <sup>b</sup>	749.2	0.011
<i>Coriobacteriales</i>	356.3 <sup>a</sup>	764.4 <sup>b</sup>	145.6 <sup>a</sup>	129.5 <sup>b</sup>	165.0 <sup>a</sup>	152.7	0.014
<i>Desulfuromonadales</i>	0.0 <sup>a</sup>	1.9 <sup>c</sup>	0.3 <sup>ab</sup>	1.1 <sup>bc</sup>	0.7 <sup>ab</sup>	0.4	0.011
<i>Enterobacteriales</i>	2.3	5.4	0.3	0.7	0.0	2.7	NS
<i>Fibrobacterales</i>	675.2	702.9	510.9	744.1	643.4	173.8	NS
<i>Flavobacteriales</i>	50.4	24.5	0.6	17.5	0.0	22.3	NS
<i>Lactobacillales</i>	1.2	25.0	2.6	5.3	4.7	13.5	NS
<i>Methylophilales</i>	3.0 <sup>b</sup>	0.9 <sup>a</sup>	0.0 <sup>a</sup>	0.0 <sup>a</sup>	0.0 <sup>a</sup>	0.7	0.012
<i>Neisseriales</i>	0.9	27.9	0.0	2.1	0.0	14.6	NS
<i>Pseudomonadales</i>	51.4	20.4	0.9	16.4	0.0	25.1	NS
<i>Rhizobiales</i>	17.5	3.5	0.6	3.5	0.3	9.2	NS
<i>Rhodobacterales</i>	0.3	1.6	0.0	0.0	0.0	0.5	NS
<i>Rhodocyclales</i>	1.2	0.0	0.0	0.0	0.0	0.7	NS
<i>Selenomonadales</i>	1357 <sup>ab</sup>	1455 <sup>b</sup>	1663 <sup>b</sup>	1135 <sup>ab</sup>	764 <sup>a</sup>	251.2	0.054
<i>Sphingobacteriales</i>	27.2	6.6	0.0	8.4	0.0	0.2	NS
<i>Spirochaetales</i>	222.5	84.6	81.9	70.7	76.8	76.2	NS
<i>Xanthomonadales</i>	14.3	8.4	0.0	4.9	0.3	5.5	NS

Values with different superscripts on the same row differed significantly ( $P < 0.05$ ), whereas values that were not significantly (NS) different ( $P > 0.05$ ) have no superscripts in the same row.

**Table 3.** Comparison of the bacterial families attached to perennial ryegrass over time within the rumen. Data shown are the average values (n=6) of the normalised reads.

Family	Time of incubation (h)					SED	P
	1	2	4	6	8		
<i>Alcaligenaceae</i>	10.4	4.5	0.3	1.4	0.3	4.8	NS
<i>Anaeroplasmataceae</i>	0.3	0.6	1.0	1.1	1.6	1.1	NS
<i>Beijerinckiaceae</i>	0.3	0.0	0.0	0.0	0.0	0.2	NS
<i>Bradyrhizobiaceae</i>	0.0	0.0	0.6	0.0	0.0	0.4	NS
<i>Burkholderiaceae</i>	0.89	0.0	0.0	0.0	0.0	0.6	NS
<i>Caulobacteraceae</i>	6.0	7.0	0.0	3.1	0.7	2.9	NS
<i>Comamonadaceae</i>	21.1	8.7	0.2	5.1	0.3	9.8	NS
<i>Coriobacteriaceae</i>	356.3 <sup>a</sup>	764.4 <sup>b</sup>	145.6 <sup>a</sup>	129.5 <sup>a</sup>	165.0 <sup>a</sup>	152.7	0.014
<i>Cryomorphaceae</i>	1.2	0.3	0.0	0.0	0.0	0.7	NS
<i>Cytophagaceae</i>	0.3	0.0	0.0	0.0	0.0	0.2	NS
<i>Enterobacteriaceae</i>	0.8	1.8	0.1	0.2	0.0	0.9	NS
<i>Eubacteriaceae</i>	5.9	4.3	4.5	2.9	2.3	2.9	NS
<i>Fibrobacteraceae</i>	675.2	702.9	510.9	744.1	643.4	173.8	NS
<i>Flavobacteriaceae</i>	49.2	24.2	0.6	17.5	0.0	21.7	NS
<i>Hyphomicrobiaceae</i>	3.8	0.0	0.0	0.7	0.3	2.3	NS
<i>Lachnospiraceae</i>	9506 <sup>a</sup>	8631 <sup>a</sup>	11701 <sup>b</sup>	11569 <sup>b</sup>	11458 <sup>b</sup>	814.6	0.016
<i>Methylobacteriaceae</i>	0.0	0.2	0.0	0.0	0.0	0.1	NS
<i>Methylophilaceae</i>	3.1 <sup>a</sup>	0.9 <sup>b</sup>	0.0 <sup>b</sup>	0.0 <sup>b</sup>	0.0 <sup>b</sup>	0.0	0.012
<i>Moraxellaceae</i>	0.5	1.8	0.0	0.0	0.0	1.1	NS
<i>Neisseriaceae</i>	0.9	27.9	0.0	2.1	0.0	14.6	NS
<i>Oxalobacteraceae</i>	18.6	13.5	0.9	0.7	0.4	7.6	NS
<i>Paenibacillaceae</i>	1.6	0.4	0.0	0.7	0.0	0.7	NS
<i>Porphyromonadaceae</i>	9.7	8.0	18.4	16.5	18.1	9.0	NS
<i>Prevotellaceae</i>	2632	3170	1816	1925	2012	550.2	NS
<i>Pseudomonadaceae</i>	50.4	16.8	0.9	16.4	0.0	25.0	NS
<i>Rhizobiaceae</i>	12.9	3.2	0.0	2.8	0.0	6.3	NS
<i>Rhodobacteraceae</i>	0.3	1.6	0.0	0.0	0.0	0.5	NS
<i>Rhodocyclaceae</i>	1.2	0.0	0.0	0.0	0.0	0.7	NS
<i>Ruminococcaceae</i>	445.2 <sup>a</sup>	437.5 <sup>a</sup>	317.0 <sup>a</sup>	400.8 <sup>a</sup>	660.4 <sup>b</sup>	83.0	0.031
<i>Sinobacteraceae</i>	0.3	0.0	0.0	0.0	0.0	0.2	NS
<i>Sphingobacteriaceae</i>	26.9	6.6	0.0	8.4	0.0	15.2	NS
<i>Spirochaetaceae</i>	11.1	4.2	4.1	3.5	3.8	3.8	NS
<i>Streptococcaceae</i>	1.2	25.0	2.6	5.3	4.7	13.5	NS
<i>Succinivibrionaceae</i>	57.0 <sup>a</sup>	62.1 <sup>a</sup>	39.4 <sup>ab</sup>	19.3 <sup>b</sup>	18.8 <sup>b</sup>	11.8	0.016
<i>Sutterellaceae</i>	29.2	39.6	40.3	31.0	8.1	11.5	NS
<i>Veillonellaceae</i>	1357 <sup>ab</sup>	1455 <sup>b</sup>	1663 <sup>b</sup>	1135 <sup>ab</sup>	764 <sup>a</sup>	251.2	0.054
<i>Xanthomonadaceae</i>	14.00	8.54	0.00	4.9	0.33	5.34	NS

Values with different superscripts on the same row differed significantly ( $P < 0.05$ ), whereas values that were not significantly (NS) different ( $P > 0.05$ ) have no superscripts in the same row.

**Table 4.** Comparison of the bacterial genera attached to perennial ryegrass over time within the rumen. Data shown are the average values (n=6) of the normalised reads.

Genus	Time of incubation (h)					SED	P
	1	2	4	6	8		
<i>Acidovorax</i>	27.9	12.2	1.2	3.5	0.3	12.8	NS
<i>Acinetobacter</i>	0.5	1.8	0.0	0.0	0.0	1.1	NS
<i>Advenella</i>	0.3	1.3	0.0	0.0	0.0	0.8	NS
<i>Anaeroplasma</i>	0.3	0.6	1.0	1.0	1.6	1.1	NS
<i>Anaerovibrio</i>	25.4	21.0	16.0	14.9	9.9	7.0	NS
<i>Asticcacaulis</i>	0.0	0.3	0.0	0.4	0.0	0.3	NS
<i>Blautia</i>	1.7	2.5	0.3	0.0	2.0	1.2	NS
<i>Bosea</i>	0.0	0.0	0.6	0.0	0.0	0.4	NS
<i>Brevundimonas</i>	5.1	6.0	0.0	2.8	0.3	2.8	NS
<i>Butyrivibrio</i>	4918	4110	5115	4573	3896	474.3	NS
<i>Camelimonas</i>	0.29 <sup>a</sup>	0.0 <sup>a</sup>	0.0 <sup>a</sup>	0.0 <sup>a</sup>	0.0 <sup>a</sup>	10.9	0.015
<i>Caulobacter</i>	0.9	0.6	0.0	0.0	0.3	0.7	NS
<i>Cellvibrio</i>	18.5	3.8	0.0	3.8	0.0	10.0	NS
<i>Chryseobacterium</i>	24.1	6.3	0.0	4.2	0.3	12.6	NS
<i>Clostridium</i>	114.8	118.8	47.7	45.4	37.9	30.7	NS
<i>Comamonas</i>	38.1	16.9	0.0	14.3	0.0	19.6	NS
<i>Coprococcus</i>	0.0	1.2	0.9	0.7	0.7	0.9	NS
<i>Delftia</i>	148.3	46.8	1.6	36.0	1.0	73.5	NS
<i>Devosia</i>	3.8	0.0	0.0	0.7	0.3	2.3	NS
<i>Duganella</i>	2.5	0.9	0.0	0.0	0.0	1.2	NS
<i>Dyadobacter</i>	0.3	0.0	0.0	0.0	0.0	0.2	NS
<i>Epilithonimonas</i>	0.6	1.6	0.0	0.3	0.0	1.0	NS
<i>Erwinia</i>	0.9	4.2	0.0	0.3	0.0	2.2	NS
<i>Eubacterium</i>	5.9	4.3	4.4	2.9	2.3	2.9	NS
<i>Fibrobacter</i>	675.2	702.9	510.9	744.1	643.4	173.8	NS
<i>Flavobacterium</i>	39.8	17.9	0.6	12.6	0.0	17.3	NS
<i>Helococcus</i>	0.29	1.6	0.0	0.0	0.0	0.7	NS
<i>Herbaspirillum</i>	12.4	8.5	0.9	0.7	0.3	5.5	NS
<i>Howardella</i>	0.9	2.8	0.7	0.0	0.3	0.8	NS
<i>Lachnobacterium</i>	16.3	10.0	61.1	43.9	37.8	22.0	NS
<i>Luteibacter</i>	0.6	1.5	0.0	0.7	0.0	1.1	NS
<i>Methylobacterium</i>	0.0	0.3	0.0	0.0	0.0	0.2	NS
<i>Mogibacterium</i>	2.6	3.1	0.6	0.0	0.7	1.9	NS
<i>Mucilaginibacter</i>	0.6	0.0	0.0	0.0	0.0	0.4	NS
<i>Murdochiella</i>	1.6 <sup>a</sup>	8.0 <sup>b</sup>	1.0 <sup>a</sup>	0.0 <sup>a</sup>	0.3 <sup>a</sup>	4.1	0.014
<i>Olsenella</i>	356.3	765.7	146.5	130.2	165.7	152.2	NS
<i>Oxalicibacterium</i>	0.9	0.0	0.0	0.0	0.0	0.6	NS
<i>Paenibacillus</i>	6.3	1.9	0.0	3.5	0.0	3.4	NS
<i>Pandoraea</i>	0.9	0.0	0.0	0.0	0.0	0.6	NS
<i>Pantoea</i>	0.0	0.6	0.0	0.0	0.0	0.4	NS
<i>Pelomonas</i>	4.2	7.6	0.0	1.4	0.7	2.7	NS
<i>Prevotella</i>	1144	1341	840	939	865	271.3	NS
<i>Propionibacterium</i>	3.5	1.9	0.3	0.4	0.0	1.3	NS
<i>Pseudobutyrvibrio</i>	701 <sup>a</sup>	805 <sup>a</sup>	2500 <sup>b</sup>	2262 <sup>b</sup>	2306 <sup>b</sup>	275.6	<0.001
<i>Pseudomonas</i>	31.9	13.0	0.9	12.6	0.0	15.3	NS

<i>Rheinheimeria</i>	3.8	0.0	0.0	0.0	0.0	2.4	NS
<i>Rhizobium</i>	12.9	3.2	0.0	2.8	0.0	6.3	NS
<i>Rhodanobacter</i>	0.0 <sup>a</sup>	1.3 <sup>b</sup>	0.0 <sup>a</sup>	0.0 <sup>a</sup>	0.0 <sup>a</sup>	0.4	0.046
<i>Roseburia</i>	2.0 <sup>a</sup>	0.9 <sup>a</sup>	9.5 <sup>b</sup>	11.8 <sup>b</sup>	10.4 <sup>b</sup>	2.7	0.010
<i>Ruminococcus</i>	151.4 <sup>a</sup>	155.9 <sup>a</sup>	140.5 <sup>a</sup>	204.1 <sup>ab</sup>	337.4 <sup>b</sup>	59.0	0.050
<i>Saccharofermentans</i>	5.5	5.6	2.6	1.1	7.0	2.0	NS
<i>Selenomonas</i>	1325 <sup>ab</sup>	1426 <sup>b</sup>	1641 <sup>b</sup>	1117 <sup>ab</sup>	747 <sup>a</sup>	244.7	0.050
<i>Shinella</i>	1.17	0.0	0.0	0.0	0.0	0.7	NS
<i>Sphingobacterium</i>	23.2	4.7	0.0	8.4	0.0	13.4	NS
<i>Streptococcus</i>	1.2	25.0	2.6	5.3	4.7	13.47	NS
<i>Succinomonas</i>	0.4	2.2	0.0	0.0	0.0	0.9	NS
<i>Succinivibrio</i>	57.0 <sup>b</sup>	62.1 <sup>b</sup>	39.4 <sup>ab</sup>	19.3 <sup>a</sup>	18.8 <sup>a</sup>	11.8	0.016
<i>Treponema</i>	275.5	169.8	121.0	89.6	94.6	76.7	NS
<i>Variovorax</i>	19.8	13.6	0.0	1.7	0.3	7.2	NS

Values with different superscripts on the same row differed significantly ( $P < 0.05$ ), whereas values that were not significantly (NS) different ( $P > 0.05$ ) have no superscripts in the same row.

**Table 5.** Comparison of OTU changes within the dominant orders of bacteria attached to perennial ryegrass over time

Order	T1 to T2			T2 to T4			T4 to T6			T6 to T8		
	Number of OTUs increased	Number of OTUs decreased	Average overall change in abundance	Number of OTUs increased	Number of OTUs decreased	Average overall change in abundance	Number of OTUs increased	Number of OTUs decreased	Average overall change in abundance	Number of OTUs increased	Number of OTUs decreased	Average overall change in abundance
<i>Bacteroidales</i>	-	-	-	5	8	-321	-	-	-	-	1	-73
<i>Clostridiales</i>	-	-	-	18	11	2746	-	-	-	1	4	-55
<i>Coriobacteriales</i>	-	-	-	-	6	-601	-	-	-	-	-	-
<i>Fibrobacterales</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>Seimonadales</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>Spirochaetales</i>	-	-	-	1	-	15	-	-	-	-	1	-24
Total	0	0		24	25	1824	0	0	0	1	6	-152

T= Time (h)

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