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

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

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ABSTRACT

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

INTRODUCTION

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

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

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

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

MATERIALS AND METHODS

Growth and preparation of plant material

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

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

In sacco incubations

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

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

RNA extraction

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

16S rRNA 454 pyrosequencing

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

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

Data Analysis

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

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

RESULTS

16S rRNA sequencing data

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

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

Fate of the perennial ryegrass epiphytic microbiota post rumen incubation

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

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

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

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

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

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

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

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

Family level temporal diversity of the attached microbiome post 1 h of rumen incubation

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

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

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

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

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

Temporal niche specialisation of the perennial ryegrass attached microbiota incubated within the rumen

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

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

DISCUSSION

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

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

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

The data in this study suggest that primary colonising bacteria are likely to utilise soluble nutrients, and that the secondary phase colonisers are adept at degrading plant structural components. This suggestion is based on the fact that only 2.8% plant dry matter disappearance was seen between 1-2 h (primary colonisation phase) of rumen incubation and 31.7% dry matter disappearance was seen between 4-8 h (secondary colonisation phase) of rumen incubation. In terms of the temporal changes in the attached microbiota, we observed that *Succinivibrio* (order Aeromonadales) were more abundant during primary colonisation events than secondary colonisation events. The reason for increased abundance of *Succinivibrio* during primary colonisation is unclear as this bacterium is considered to be predominantly amylolytic. Nonetheless the normalised read abundances of *Succinivibrio* are low irrespective of time. Conversely, we observed that *Pseudobutyrivibrio*, *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 *Pseudobutyrivibrio* and *Ruminococcus* spp. during secondary colonisation events, when
378 investigating temporal colonisation of switchgrass incubated within the rumen.
379 *Pseudobutyrivibrio* spp. commonly possess xylanases which randomly cleave the β-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 *Butyrivibrio*, *Fibrobacter*,
386 *Olsenella* and *Prevotella* spp. read abundances were high irrespective of colonisation phase.
387 Rumen *Butyrivibrio* 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 xylanses
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 β-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.

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

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

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

SUPPLEMENTARY DATA

Supplementary data is available at FEMSEC online.

ACKNOWLEDGEMENTS

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FIGURE LEGENDS

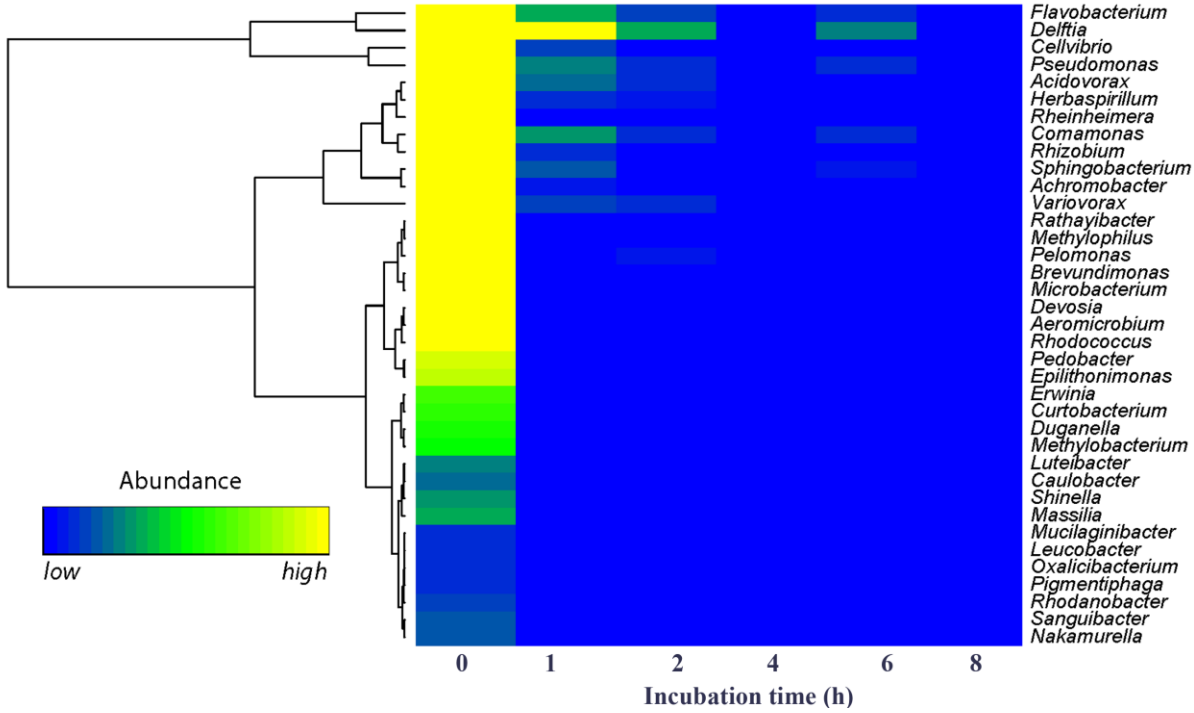

Figure 1. Heat map illustrating changes in proportional read abundances of perennial ryegrass epiphytic communities post rumen incubation. Mean data for each time point are shown (n=2 for 0h and n=6 for all other time points).

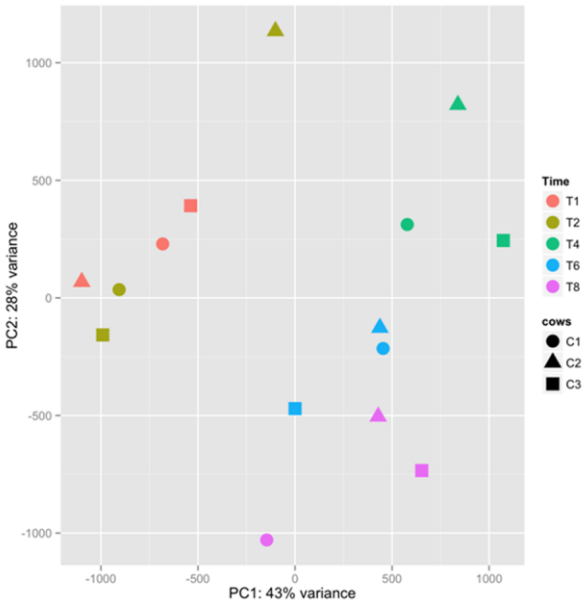

Figure 2. PCA plot showing the diversity of rumen bacteria attached to perennial ryegrass over time. Data for post rumen incubation of 1, 2, 4, 6 and 8 h (T1, T2, T4, T6 and T8 respectively) within each cow (C1, C2 and C3 respectively) are shown. Mean data for 2 bags incubated within each cow are shown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

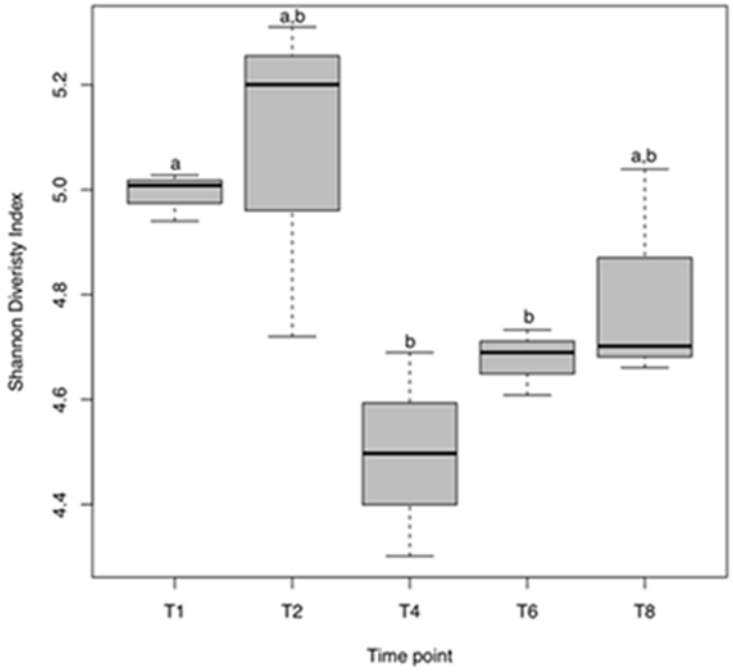

Figure 3. Boxplots of the average Shannon diversity indices at each time point representing each of the three cows sampled in duplicate (n=6). Time points that do not share notations were significantly different ($P<0.05$) according to a t-test. Post rumen incubation time points of 1, 2, 4, 6 and 8 h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

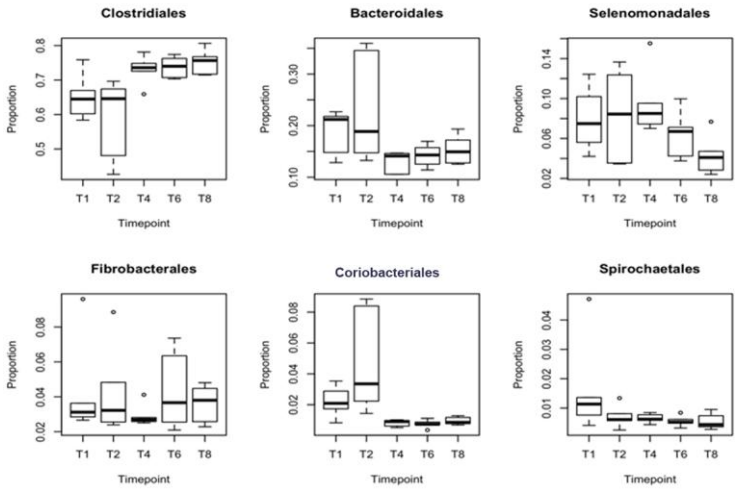

Figure 4. Proportional changes in the 6 most abundant bacterial orders attached to perennial ryegrass incubated in the rumen over time (n=6). Post rumen incubation time points of 1, 2, 4, 6 and 8 h (T1, T2, T4, T6 and T8 respectively).

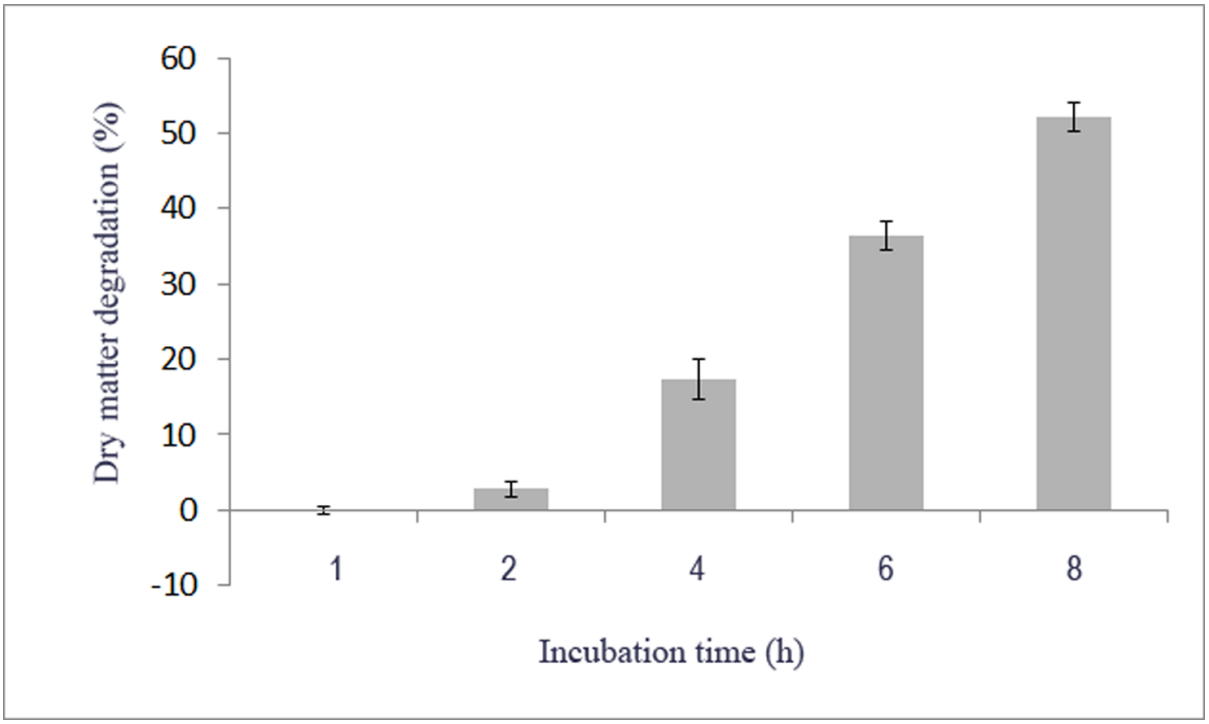

Figure 5. Perennial ryegrass dry matter disappearance (%) following incubation within the rumen over time. Standard error of the mean for each time point are shown.

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.

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

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

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

	Time of incubation (h)						
Genus	1	2	4	6	8	SED	*P*
Acidovorax	27.9	12.2	1.2	3.5	0.3	12.8	NS
Acinetobacter	0.5	1.8	0.0	0.0	0.0	1.1	NS
Advenella	0.3	1.3	0.0	0.0	0.0	0.8	NS
Anaeroplasma	0.3	0.6	1.0	1.0	1.6	1.1	NS
Anaerovibrio	25.4	21.0	16.0	14.9	9.9	7.0	NS
Asticcacaulis	0.0	0.3	0.0	0.4	0.0	0.3	NS
Blautia	1.7	2.5	0.3	0.0	2.0	1.2	NS
Bosea	0.0	0.0	0.6	0.0	0.0	0.4	NS
Brevundimonas	5.1	6.0	0.0	2.8	0.3	2.8	NS
Butyrivibrio	4918	4110	5115	4573	3896	474.3	NS
Camelimonas	0.29[a]	0.0[a]	0.0[a]	0.0[a]	0.0[a]	10.9	0.015
Caulobacter	0.9	0.6	0.0	0.0	0.3	0.7	NS
Cellvibrio	18.5	3.8	0.0	3.8	0.0	10.0	NS
Chryseobacterium	24.1	6.3	0.0	4.2	0.3	12.6	NS
Clostridium	114.8	118.8	47.7	45.4	37.9	30.7	NS
Comamonas	38.1	16.9	0.0	14.3	0.0	19.6	NS
Coprococcus	0.0	1.2	0.9	0.7	0.7	0.9	NS
Delftia	148.3	46.8	1.6	36.0	1.0	73.5	NS
Devosia	3.8	0.0	0.0	0.7	0.3	2.3	NS
Duganella	2.5	0.9	0.0	0.0	0.0	1.2	NS
Dyadobacter	0.3	0.0	0.0	0.0	0.0	0.2	NS
Epilithonimonas	0.6	1.6	0.0	0.3	0.0	1.0	NS
Erwinia	0.9	4.2	0.0	0.3	0.0	2.2	NS
Eubacterium	5.9	4.3	4.4	2.9	2.3	2.9	NS
Fibrobacter	675.2	702.9	510.9	744.1	643.4	173.8	NS
Flavobacterium	39.8	17.9	0.6	12.6	0.0	17.3	NS
Helococcus	0.29	1.6	0.0	0.0	0.0	0.7	NS
Herbaspirillum	12.4	8.5	0.9	0.7	0.3	5.5	NS
Howardella	0.9	2.8	0.7	0.0	0.3	0.8	NS
Lachnobacterium	16.3	10.0	61.1	43.9	37.8	22.0	NS
Luteibacter	0.6	1.5	0.0	0.7	0.0	1.1	NS
Methylobacterium	0.0	0.3	0.0	0.0	0.0	0.2	NS
Mogibacterium	2.6	3.1	0.6	0.0	0.7	1.9	NS
Mucilaginibacter	0.6	0.0	0.0	0.0	0.0	0.4	NS
Murdochiella	1.6[a]	8.0[b]	1.0[a]	0.0[a]	0.3[a]	4.1	0.014
Olsenella	356.3	765.7	146.5	130.2	165.7	152.2	NS
Oxalicibacterium	0.9	0.0	0.0	0.0	0.0	0.6	NS
Paenibacillus	6.3	1.9	0.0	3.5	0.0	3.4	NS
Pandoraea	0. 9	0.0	0.0	0.0	0.0	0.6	NS
Pantoea	0.0	0.6	0.0	0.0	0.0	0.4	NS
Pelomonas	4.2	7.6	0.0	1.4	0.7	2.7	NS
Prevotella	1144	1341	840	939	865	271.3	NS
Proprionibacterium	3.5	1.9	0.3	0.4	0.0	1.3	NS
Pseudobutyrivibrio	701[a]	805[a]	2500[b]	2262[b]	2306[b]	275.6	<0.001
Pseudomonas	31.9	13.0	0.9	12.6	0.0	15.3	NS

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

	T1 to T2			T2 to T4			T4 to T6			T6 to T8		
Order	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**
Bacteroidales	-	-	-	5	8	-321	-	-	-	-	1	-73
Clostridiales	-	-	-	18	11	2746	-	-	-	1	4	-55
Coriobacteriales	-	-	-	-	6	-601	-	-	-	-	-	-
Fibrobacterales	-	-	-	-	-	-	-	-	-	-	-	-
Selemonadales	-	-	-	-	-	-	-	-	-	-	-	-
Spirochaetales	-	-	-	1	-	15	-	-	-	-	1	-24
Total	0	0		24	25	1824	0	0	0	1	6	-152

T= Time (h)