Temporal Metagenomic and Metabolomic Characterization of Fresh Perennial Ryegrass Degradation by Rumen Bacteria

Mayorga, O. L., Kingston-Smith, A. H., Kim, E. J., Allison, G. G., Wilkinson, T. J., Hegarty, M. J., Theodorou, M. K., Newbold, C. J., & Huws, S. A. (2016). Temporal Metagenomic and Metabolomic Characterization of Fresh Perennial Ryegrass Degradation by Rumen Bacteria. Frontiers in Microbiology, 7, 1854. https://doi.org/10.3389/fmicb.2016.01854

Published in:
Frontiers in Microbiology

Document Version:
Publisher's PDF, also known as Version of record

Queen's University Belfast - Research Portal:
Link to publication record in Queen's University Belfast Research Portal

Publisher rights
Copyright 2017 the authors. This is an open access article published under a Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution and reproduction in any medium, provided the author and source are cited.

General rights
Copyright for the publications made accessible via the Queen's University Belfast Research Portal is retained by the author(s) and / or other copyright owners and it is a condition of accessing these publications that users recognise and abide by the legal requirements associated with these rights.

Take down policy
The Research Portal is Queen's institutional repository that provides access to Queen's research output. Every effort has been made to ensure that content in the Research Portal does not infringe any person's rights, or applicable UK laws. If you discover content in the Research Portal that you believe breaches copyright or violates any law, please contact openaccess@qub.ac.uk.

Open Access
This research has been made openly available by Queen's academics and its Open Research team. We would love to hear how access to this research benefits you. – Share your feedback with us: http://go.qub.ac.uk/oa-feedback
Temporal Metagenomic and Metabolomic Characterization of Fresh Perennial Ryegrass Degradation by Rumen Bacteria

Olga L. Mayorga1†, Alison H. Kingston-Smith1, Eun J. Kim2, Gordon G. Allison1, Toby J. Wilkinson1, Matthew J. Hegarty1, Michael K. Theodorou3, Charles J. Newbold1 and Sharon A. Huws1*†

1Institute of Biological, Environmental and Rural Sciences, Aberystwyth University, Aberystwyth, UK, 2Department of Animal Science, Kyungpook National University, Sangju, Korea, 3Department of Animal Production, Welfare and Veterinary Sciences, Harper Adams University, Newport, UK

Understanding the relationship between ingested plant material and the attached microbiome is essential for developing methodologies to improve ruminant nutrient use efficiency. We have previously shown that perennial ryegrass (PRG) rumen bacterial colonization events follow a primary (up to 4 h) and secondary (after 4 h) pattern based on the differences in diversity of the attached bacteria. In this study, we investigated temporal niche specialization of primary and secondary populations of attached rumen microbiota using metagenomic shotgun sequencing as well as monitoring changes in the plant chemistry using mid-infrared spectroscopy (FT-IR). Metagenomic Rapid Annotation using Subsystem Technology (MG-RAST) taxonomical analysis of shotgun metagenomic sequences showed that the genera Butyrivibrio, Clostridium, Eubacterium, Prevotella, and Selenomonas dominated the attached microbiome irrespective of time. MG-RAST also showed that Acidaminococcus, Bacillus, Butyrivibrio, and Prevotella rDNA increased in read abundance during secondary colonization, whilst Blautia decreased in read abundance. MG-RAST Clusters of Orthologous Groups (COG) functional analysis also showed that the primary function of the attached microbiome was categorized broadly within “metabolism;” predominantly amino acid, carbohydrate, and lipid metabolism and transport. Most sequence read abundances (51.6, 43.8, and 50.0% of COG families pertaining to amino acid, carbohydrate and lipid metabolism, respectively) within these categories were higher in abundance during secondary colonization. Kyoto encyclopedia of genes and genomes (KEGG) pathways analysis confirmed that the PRG-attached microbiota present at 1 and 4 h of rumen incubation possess a similar functional capacity, with only a few pathways being uniquely found in only one incubation time point only. FT-IR data for the plant residues also showed that the main changes in plant chemistry between primary and secondary colonization was due to increased carbohydrate, amino acid, and lipid metabolism. This study confirmed primary and secondary colonization events and supported the hypothesis that functional changes occurred as a consequence of taxonomical changes. Sequences within the carbohydrate metabolism COG families contained only 3.2% of cellulose activities, on average across
INTRODUCTION

Due to a growing population and increased demand for livestock products by developing countries, current projections estimate that global demand for meat and milk will have doubled by 2050 compared to the start of the twenty-first century (Foresight, 2011). Ruminants supply much of our red meat and nearly all our milk supplies globally. Therefore, there is a real challenge to ensure sustainability and efficiency of ruminant production given that land is also at a premium due to increasing bioenergy crop production. A major hurdle in increasing ruminant productivity is that the conversion of plant to microbial protein is inefficient. As little as 30% of the ingested nitrogen is utilized by ruminants for milk or meat production, and the non-incorporated nitrogen is excreted to the environment as urea or ammonia (MacRae and Ulyatt, 1974; Dewhurst et al., 1996; Kingston-Smith et al., 2008, 2010).

The rumen, via its complex microbiome is responsible for the breakdown of plant material and the functional capacity of the microbiome defines the amount, quality, and composition of meat and milk produced, whilst also defining the release of nitrogen and greenhouse gases to the environment (Edwards et al., 2008a; Kim et al., 2009; Kingston-Smith et al., 2010; Brown Kav et al., 2012; Huws et al., 2014a). The process of colonizing ingested plant material by the rumen microbiome is rapid (Cheng et al., 1980; Miron et al., 2001; Russell and Rychlik, 2001; Koike et al., 2003a; Edwards et al., 2007, 2008b; Huws et al., 2013, 2014b, 2016), and eventually these populations form mature biofilms encompassed in self-produced polymeric substances (EPS) (Akin, 1976; Cheng et al., 1980, 1981; McAllister et al., 1994; Huws et al., 2013; Leng, 2014). We have previously shown that bacterial diversity attached to fresh perennial ryegrass (PRG) incubated within the rumen is different prior to 4 h and post 4 h of incubation, thus demonstrating that colonization undergoes primary (up to 4 h) and secondary (after 4 h) events, respectively (Huws et al., 2013, 2014b, 2016). Nonetheless, the functionality of the primary and secondary colonizers in terms of plant degradation and availability of nutrients to the host remains unclear.

In this study, we investigated the diversity and function of the attached microbiota using metagenomic based shotgun Illumina sequencing to gain insight into their function and importance in terms of plant degradation and subsequent nutrient availability to the microbes and ultimately the ruminant. Temporal changes in plant chemistry were also monitored using Fourier transform infrared spectroscopy (FT-IR) to confirm that microbial gene abundances were related to substrate changes within the plant itself. Understanding temporal bacterial-driven plant degradation and factors controlling these events will promote the development of novel strategies to increase ruminant production in order to meet the increasing demand for meat and milk.

MATERIALS AND METHODS

Growth and Preparation of Plant Material
PRG (Lolium perenne cv. AberDart) was grown from seed in plastic seed trays (length 38 cm × width 24 cm × depth 5 cm) filled with 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 and cut 3 cm above soil level, before washing in cold distilled water and cutting with scissors into 1 cm sections. Sub-samples of plant material were freeze-dried and stored at −20°C for metagenomic sequencing, plant dry matter (DM) and Fourier transform mid-infrared spectroscopy (FT-IR) analysis (0 h samples).

In vitro Incubations
Cut PRG (7.5 g) was added to Duran bottles (250 mL) together with anaerobic incubation buffer (135 mL pre-warmed to 39°C; Van Soest, 1967) and rumen fluid inoculum (15 mL, strained through two layers of muslin and held under CO₂ at 39°C; rumen fluid was taken from three cannulated cows grazed mainly on fresh forage and pooled before inoculation). Rumen fluid was obtained from cannulated cows under the authority of Licenses under the UK Animal Scientific Procedures Act, 1986. Bottles were incubated in a horizontally rotating rack at 100 rpm and 39°C (Incubator-shaker, LA Engineering, UK). Bottle contents were harvested at 0.25, 0.5, 1, 2, 4, 8, and 24 h. At each time interval bottle contents were harvested by vacuum filtration through filter paper (11 µm² pore size; E Quốc L100, Fisher Scientific, Leicestershire, UK). Retained plant material was washed with phosphate buffered saline (PBS; 50 mL) to remove loosely attached bacteria, before attached bacteria were removed by incubation overnight in gluteraldehyde (3 % w/v in PBS) at 4°C (Azeredo et al., 1999). The remaining plant material from which colonizing bacteria had been removed was retrieved by squeezing contents of overnight gluteraldehyde incubations through one layer of muslin. Plant residues within the muslin were then freeze-dried and weighed to allow calculation of percentage plant degradation. Absence of remaining attached bacteria was also checked using Quantitative PCR (QPCR), as described below, to validate the method of detachment.
of attached microbes. The plant material was subsequently finely ground with the aid of a reciprocal shaking system in the presence of liquid nitrogen (particle size <0.5 mm) for Fourier transform infrared spectroscopy (FT-IR) analyses. The suspension of previously attached bacteria (supernatant retrieved post squeezing of overnight gluteraldehyde incubations) was centrifuged (10,000 x g, 10 min), before the pellet was freeze-dried for subsequent QPCR and metagenomic sequencing. The experiment was repeated on three separate occasions (n = 3) within the same week.

**DNA Extraction, QPCR and Metagenomic Sequencing**

DNA extraction and total bacterial 16S rDNA QPCR were completed as described by Huws et al. (2013) and Huws et al. (2016) using the primers 5′-GTG STG CAY GGY TGT CGT

### TABLE 1 | Plant dry matter degradation and concentration of attached bacterial 16S rDNA following incubation of fresh perennial ryegrass in the presence of rumen fluid.

| Incubation time (h) | 0.0 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 24 | SED | P  |
|---------------------|-----|------|-----|---|---|---|---|----|-----|----|
| Dry matter degradation (%) (g DM lost from 100 g of initial DM) | 0.0<sup>a</sup> | 1.6<sup>a</sup> | 2.3<sup>a</sup> | 4.2<sup>a</sup> | 7.9<sup>ab</sup> | 13.8<sup>b</sup> | 35.2<sup>c</sup> | 76.2<sup>d</sup> | 3.52 | <0.001 |
| Solid-associated bacteria [SAB] (Log<sub>10</sub> bacterial DNA concentration [ng g<sup>−1</sup> RDM]) | 1.8<sup>a</sup> | 2.3<sup>ab</sup> | 2.3<sup>ab</sup> | 2.4<sup>ab</sup> | 2.7<sup>b</sup> | 3.4<sup>c</sup> | 4.4<sup>dc</sup> | 4.7<sup>d</sup> | 0.2 | <0.001 |

One-way ANOVA was conducted on effect of incubation time. The results are the mean values of triplicate data sets. RDM, remaining dry matter; SED, standard errors of differences of means. Values within the same row with different superscripts were significantly different (P < 0.05).

**FIGURE 1 |** Principal coordinates analysis (PCoA) analysis of taxonomical classifications of attached bacteria present after 1 and 4 h of rumen incubation. Rep: Replicate.
CA-3’ (Forward) and 5’-GAG GAA GGTGKG GAY GAC GT-3’ (Reverse) (Maeda et al., 2003). The presence of primary and secondary bacterial colonizing events were initially corroborated using denaturing gradient gel electrophoresis (DGGE) as described by Huws et al. (2013) before taxonomy and function of the primary and secondary attached microbiome was further investigated by sequencing. Essentially, 1 (primary colonizers) and 4 h (secondary colonizers) DNA samples from the attached microbiome were sequenced using Illumina HiSeq DNA was normalized to 0.2 ng/µL and 1 ng used to create metagenomic libraries using the Nextera® XT DNA kit (Invitrogen, San Diego, USA) following manufacturer guidelines. Sample libraries were sequenced at 2 × 151 bp using an Illumina HiSeq 2500 rapid run, following standard manufacturer’s instructions at the IBERS Aberystwyth Translational Genomics Facility. Reads of 126 bases were merged and first trimmed to remove the Nextera library adapters then trimmed at the 3’-end when a sliding window over four bases fell below an average Phred Q score of 30. Sequences are deposited and publically available within Metagenomic Rapid Annotation using Subsystem Technology (MG-RAST) [Identification numbers 4653312.3 (1 h), 4653313.3 (1 h), 4653314.3 (1 h), 4653315.3 (4 h), 4653316.3 (4 h), and 4653317.3 (4 h)].

Metagenomic Sequence Analysis

Sequencing files containing the merged, quality trimmed reads, were uploaded to MG-RAST (Meyer et al., 2008) as FASTQ files. The MG-RAST best hit organism abundance function was employed against the RDP comparison pipeline, using constraints of 97% sequence similarity and a maximum e-value of 1 × 10^-5 and minimum sequence alignment of 15, to assign taxonomy to sequences. The MG-RAST functional abundance hierarchical abundance function was employed against the COG database to assign function, based on a maximum e-value of 1 × 10^-5, minimum identity cut-off of 60% and minimum sequence alignment of 15. Normalized taxonomic and functional abundance data were exported as excel files for statistical analysis. Heatmap and bar chart visualization of gene function data was completed within MG-RAST. Eukaryotic rRNA sequences were removed from the analysis (these made up on average 51.6% of the reads obtained and were mainly 18S rDNA from PRG); the COG database does not annotate eukaryote sequences allowing an analysis of taxonomy and function of the rumen prokaryotic microbiota only (Li et al., 2016).

Fourier Transform Infrared Spectroscopy (FT-IR)

Mid infrared spectra reflecting plant chemical composition were obtained at each time point by attenuated total reflectance (ATR) FT-IR analysis using a Bruker Equinox 55 spectrometer (Bruker Optics Ltd., Coventry, UK) equipped with a deuterated tryglycine sulfate detector and a Golden Gate ATR accessory (Specac Ltd., Orpington, UK). Spectra were acquired over the range 4000 to 500 cm^-1 as a mean of 32 scans and at a spectral resolution of 4 cm^-1 using OPUS software (version 4.2, Bruker Optics Ltd., Coventry, UK).

Statistical Analysis

For plant degradation, QPCR data, taxonomy, and COG gene abundance data, significant differences between groups was determined using one-way analysis of variance (ANOVA) followed by Duncan’s multiple range tests to detect significant differences between groups where appropriate (Duncan, 1955) using the GenStat program (Tenth Edition, VSN International Ltd., Hemel Hemstead, UK; Payne et al., 2007). Taxonomy and function based PCoA plots, function based-bar charts, heatmaps, and KEGG pathways were generated in MG-RAST. T-test were conducted within MG-RAST using level 2 COG based bar chart

| Phylum                | Time (h) | SED | P     |
|-----------------------|----------|-----|-------|
| Actinobacteria        | 71       | 116 | 34.4  | NS    |
| Bacteroidetes         | 453      | 571 | 48.5  | NS    |
| Fibrobacteria         | 20.7     | 10.7| 9.57  | NS    |
| Firmicutes            | 1536     | 2748| 676.3 | NS    |
| Proteobacteria        | 37       | 55  | 26.2  | NS    |
| Spirochaetes          | 36       | 53  | 32.7  | NS    |
| Tenericutes           | 32.3     | 41.7| 18.84 | NS    |
| Verrucomicrobia       | 2        | 1   | 1     | NS    |
| Unclassified          | 132      | 233 | 64.4  | NS    |

Data shown are for normalized reads. NS: Not Significant.
RESULTS

Plant Dry Matter Disappearance and Attached Bacterial 16S rDNA Abundance

Residual plant digestibility data (with microbes removed) showed that by 4 h 13.6% of the plant material had been degraded with this increasing to 76.2% by 24 h (Table 1). Post incubation of PRG under rumen-like in vitro conditions, rumen bacteria attached quickly to the plant material, and bacterial 16S rDNA abundance increased rapidly and continued to rise for the duration of the experiment; by 4 h attached bacterial 16S rDNA concentration had increased by 1.9X, and by 24 h by 2.6X compared with 0 h concentrations (Table 1).

**Table 4** | Comparison of the primary (1 h) and secondary (4 h) colonizing bacterial families attached to perennial ryegrass.

| Family               | Time (h) | 1     | 4     | SED   | P     |
|----------------------|----------|-------|-------|-------|-------|
| Acidaminococcaceae   |          | 11    | 21    | 7.16  | NS    |
| Anaeroplasmataceae   |          | 5.3   | 5.7   | 2.62  | NS    |
| Bacillaceae          |          | 18.7  | 51.7  | 8.1   | 0.015 |
| Bacteroidaceae       |          | 73    | 93    | 29.1  | NS    |
| Clostridiaceae       |          | 105   | 235   | 50.2  | NS    |
| Coryobacteriaceae    |          | 34.7  | 50.7  | 14.6  | NS    |
| Erysipelotrichaceae  |          | 4.3   | 15.3  | 11.5  | NS    |
| Eubacteriaceae       |          | 215   | 169   | 166.6 | NS    |
| Fibrobacteraceae     |          | 20.7  | 10.7  | 9.6   | NS    |
| Flavobacteriaceae    |          | 24    | 9.7   | 13.9  | NS    |
| Lachnospiraceae      |          | 556   | 1166  | 151.9 | 0.016 |
| Lactobacillaceae     |          | 8     | 10    | 5.1   | NS    |
| Mycoplasmataceae     |          | 11.7  | 7.7   | 6.5   | NS    |
| Paenibacillaceae     |          | 8     | 82    | 76    | NS    |
| Peptococcaceae       |          | 11.7  | 38.7  | 17.6  | NS    |
| Peptostreptococcaceae|          | 19    | 20.3  | 10.6  | NS    |
| Porphyromonadaceae   |          | 16.7  | 47.7  | 5.4   | 0.006 |
| Prevotellaceae       |          | 273   | 378   | 31.1  | 0.028 |
| Ruminococcaceae      |          | 80    | 112   | 38.2  | NS    |
| Sphingobacteriaceae  |          | 8     | 5.3   | 3.5   | NS    |
| Spirochaetaceae      |          | 36    | 52    | 32.3  | NS    |
| Streptococcaceae     |          | 18    | 17.5  | 9.6   | NS    |
| Streptomycesaceae    |          | 12.7  | 19.5  | 10    | NS    |
| Succinivibrionaceae  |          | 10    | 8.3   | 9.9   | NS    |
| Thermoanaerobacteriaceae|       | 8.3   | 13.7  | 5.2   | NS    |
| Veillonellaceae      |          | 400   | 638   | 238.7 | NS    |
| Unclassified         |          | 230   | 287   | 114   | NS    |

Data shown are for normalized reads. NS: Not Significant.

**Table 5** | Comparison of the primary (1 h) and secondary (4 h) colonizing bacterial genera attached to perennial ryegrass.

| Genus                 | Time (h) | 1     | 4     | SED   | P     |
|-----------------------|----------|-------|-------|-------|-------|
| Acidaminococcus       |          | 4     | 10.7  | 2.03  | 0.03  |
| Anaeroplasma          |          | 5.3   | 5.7   | 2.62  | NS    |
| Anaerobacterium       |          | 3     | 3.3   | 2.24  | NS    |
| Atopobium             |          | 12    | 17    | 7.96  | NS    |
| Bacillus              |          | 17    | 49.7  | 7.22  | 0.011 |
| Bacteroides           |          | 73    | 93    | 29.1  | NS    |
| Blautia               |          | 16    | 60.7  | 12.21 | 0.022 |
| Butyrivibrio          |          | 429   | 800   | 107.6 | 0.026 |
| Caudobacteriaceae     |          | 5.7   | 12.7  | 4.38  | NS    |
| Cellulosiicumin       |          | 2.7   | 5.7   | 3.77  | NS    |
| Clostridiurn          |          | 102   | 225   | 47.5  | NS    |
| Clostrinella          |          | 5     | 2     | 2.08  | NS    |
| Clostridium           |          | 1214  | 168   | 166.4 | NS    |
| Faecalibacterium      |          | 14.3  | 21.7  | 11.6  | NS    |
| Fibrobacter           |          | 20.7  | 10.7  | 9.6   | NS    |
| Flavobacterium        |          | 4.5   | 5     | 2.48  | NS    |
| Flavonibacter         |          | 8     | 10.3  | 3.23  | NS    |
| Gordonibacter         |          | 2.7   | 2.3   | 0.75  | NS    |
| Halothrombrix         |          | 4.3   | 5.7   | 1.8   | NS    |
| Lactobacillus         |          | 8     | 10    | 5.1   | NS    |
| Lactobacillus         |          | 1.3   | 5.7   | 2.75  | NS    |
| Peaibacillus          |          | 7     | 82    | 76    | NS    |
| Parabacteroides       |          | 5     | 16.7  | 6.84  | NS    |
| Parvococctibacterium  |          | 7     | 7     | 3.32  | NS    |
| Pharynibacter         |          | 9.7   | 18.3  | 11.7  | NS    |
| Prevotella            |          | 270   | 358   | 25.8  | 0.027 |
| Pseudobutyribrio      |          | 74    | 211   | 72    | NS    |
| Roseburia             |          | 11.7  | 23.7  | 6.5   | NS    |
| Ruminococcus          |          | 63    | 89    | 26.3  | NS    |
| Selenomonos           |          | 386   | 616   | 231.8 | NS    |
| Slackia               |          | 4.67  | 7     | 1.86  | NS    |
| Streptococcus         |          | 13.7  | 15.7  | 7.97  | NS    |
| Streptomyces          |          | 17.5  | 14    | 10.44 | NS    |
| Tissierella           |          | 9     | 9.7   | 9.48  | NS    |
| Treponema             |          | 31    | 40    | 31.5  | NS    |
| Unclassified          |          | 211   | 401   | 83.5  | NS    |

Data shown are for normalized reads. NS: Not Significant.

Seqeuencing Data

Post quality control of sequences we obtained on average 0.9 GB/sample, with a mean sequence length of 163 bp...
Mayorga et al. Perennial Ryegrass Attached Rumen Bacteria

(Supplementary Table 1). Rarefaction curves showed that all samples approached a plateau, suggesting reasonable sequence coverage (Supplementary Figure 2).

**Taxonomy of the Primary and Secondary Attached Microbiota**

Principal coordinate axis (PCoA) plots showed that bacterial diversity differed between 1 and 4 h of colonization (Figure 1). DGGE was also undertaken on samples for all time points and confirmed the presence of primary (up to 4 h) and secondary (post 4 h) bacterial colonization events (Supplementary Figure 1). Phyla level taxonomy showed that the most abundant attached phyla were Firmicutes (66–72% of total read abundances) and Bacteroidetes (15–20% of total read abundances) (Table 2). No significant changes ($P > 0.05$) in read abundances were evident at a phyla level between primary (1 h) and secondary (4 h) colonization events (Table 2). On an order level, Clostridiales (46–51% of total read abundances), Selmonadales (18% of total read abundances), and Bacteroidales (14–15% of total read abundances) were the most abundant, with the remaining orders representing <3% on average of the total read abundances (Table 3). No significant changes ($P > 0.05$) in read abundances were evident at an order level between primary (1 h) and secondary (4 h) colonization events (Table 3). Family level taxonomy showed that the most abundant classified families were Lachnospiraceae (25–33% of total read abundances), Veillonellaceae (18% of total read abundances), Prevotellaceae (11–12% of total read abundances), Eubacteriaceae (5–10% of total read abundances), Clostridiaceae (5–7% of total read abundances), and Ruminococcaceae (3–4% of total read abundances), with the remaining families representing <3% on average of total read abundances (Table 4). Significant ($P < 0.05$) increases in Bacillaceae, Lachnospiraceae, Porphyromonadaceae, and Prevotellaceae were seen during secondary colonization events compared with their rDNA gene abundances present during primary colonization (Table 4). On a genera level, Butyrivibrio (20–23% of total read abundances), Selenomonas (17–18%), Prevotella (10–13%), Eubacterium (5–10%), Pseudobutyrivibrio (4–6%), and Ruminococcus (3%) were the most abundant, with the remaining genera representing

![FIGURE 2 | Principal coordinates analysis (PCoA) analysis of functional classifications of attached bacteria present after 1 and 4 h of rumen incubation. Rep: Replicate.](image-url)
<3% on average of total read abundances (Table 5). Significant (P < 0.05) increases in Acidaminococcus, Bacillus, Blautia, Butyrivibrio, and Prevotella, were also seen during secondary colonization events compared with their rDNA gene abundances present during primary colonization (Table 5).

Functionality of the Primary and Secondary Attached Microbiota

When assessing bacterial functional gene abundance at 1 and 4 h post rumen incubation, PCoA plots showed that bacterial function differed between 1 and 4 h of colonization (Figure 2). Bar charts generated within MG-RAST based on hierarchical functional categories showed that genes attributed broadly within metabolism, information, storage, and processing and cellular processes and signaling were significantly (P > 0.05) more abundant within secondary colonizing bacteria than they were within the population of primary colonizers (Figure 3A). The COG level 2 heatmap corroborated the difference in functionality seen within the primary and secondary colonizing bacteria (Figure 3B). The heatmap also illustrated that the primary functions of the attached microbiota were amino acid transport and metabolism, carbohydrate transport and metabolism, general function, lipid transport, and metabolism (Figure 3B). Further prospecting of amino acid transport and metabolism COG families showed that 51.6% of the COG families showed significant (P < 0.05) increases in abundance from primary (1 h) to secondary (4 h) colonization events (Table 6). No COG families decreased in significantly in their abundance from primary (1 h) to secondary (4 h) colonization events (Table 6). Further prospecting of carbohydrate transport and metabolism COG families showed that 43.8% of the COG families showed significant (P < 0.05) increases in abundance, whilst only 0.01% significant (P < 0.05) decreased in abundance between primary (1 h) to secondary (4 h) colonization events (Table 7). Only 3.2% of total reads pertained to cellulases (Table 7). Further prospecting of lipid transport and metabolism COG families showed that 50.0% of the COG families showed significant (P > 0.05) increases from primary (1 h) to secondary (4 h) colonization events (Table 8). No COG families decreased significantly in abundance from primary (1 h) to secondary (4 h) colonization events (Table 8). COG families with a low abundance (<1) were
| COG number | Function                                                                 | Average COG normalized read abundance |
|------------|--------------------------------------------------------------------------|----------------------------------------|
|            |                                                                          | 1 h          | 4 h          | SED               | P      |
| 2          | Acetylglutamate semialdehyde dehydrogenase                                | 38           | 94.3         | 16.2              | 0.026  |
| 6          | Xaa-Pro aminopeptidase                                                   | 87           | 148          | 21.9              | 0.049  |
| 10         | Arginase/Agmatinase/formiminoglutamate hydrolase, arginase family        | 11.7         | 14           | 4.48              | NS     |
| 14         | Gamma-glutamyl phosphate reductase                                       | 77.3         | 163          | 18.39             | 0.01   |
| 19         | Diaminopimelate decarboxylase                                            | 115          | 221          | 32                | 0.029  |
| 31         | Cysteine synthase                                                        | 93           | 166          | 23.6              | 0.038  |
| 40         | ATP phosphoribosyltransferase                                            | 39.3         | 64.7         | 6.45              | 0.017  |
| 65         | 3-Isopropylmalate dehydratase large subunit                              | 68           | 133          | 21.2              | 0.036  |
| 69         | Glutamate synthase domain 2                                              | 206          | 384          | 59.4              | 0.04   |
| 70         | Glutamate synthase domain 3                                              | 182          | 345          | 47.9              | 0.027  |
| 75         | Serine-pyruvate aminotransferase/archaeal aspartate aminotransferase     | 35           | 62.7         | 9.74              | 0.047  |
| 76         | Glutamate decarboxylase and related PLP-dependent proteins               | 21           | 28.3         | 7.64              | NS     |
| 77         | Prephenate dehydratase                                                   | 34.7         | 61           | 11.54             | NS     |
| 78         | Ornithine carbamoyltransferase                                           | 76           | 158          | 29.3              | 0.05   |
| 79         | Histidinol-phosphate/aromatic aminotransfer and cobyric acid decarboxylase | 7.3          | 13           | 0.8               | 0.003  |
| 82         | Chorismate synthase                                                      | 45.7         | 92.3         | 17.52             | NS     |
| 83         | Homoserine kinase                                                        | 51           | 88.3         | 4.4               | 0.001  |
| 106        | Phosphoribosylformimino-5-aminimidazole carboxamide ribonucleotide (ProFAR) | 41           | 70.3         | 11.32             | NS     |
| 107        | Isomerase-3-isopropylmalate-phosphate synthase                           | 74           | 160          | 23.5              | 0.022  |
| 111        | Phosphoglycerate dehydrogenase and related dehydrogenases                | 107          | 206          | 33.6              | 0.05   |
| 112        | Glycine/Serine hydroxymethyltransferase                                  | 43           | 77.7         | 7.6               | 0.01   |
| 118        | Glutamine amidotransferase                                               | 159          | 308          | 36                | 0.014  |
| 119        | Isopropylmalate/Homocitrate/Citramalate synthases                        | 70.3         | 130.3        | 9.4               | 0.003  |
| 128        | Tryptophan synthase beta chain                                           | 49           | 89           | 17.87             | NS     |
| 131        | Imidazoleglycerol-phosphate dehydratase                                  | 58.7         | 97.7         | 16.7              | NS     |
| 133        | Tryptophan synthase beta chain                                           | 23           | 40.7         | 4.7               | 0.019  |
| 134        | Phosphoribosylanthranilate isomerase                                     | 27.7         | 49.7         | 8.6               | NS     |
| 135        | Phosphoribosylanthranilate isomerase                                     | 59.7         | 121.3        | 17.6              | 0.025  |
| 136        | Aspartate-semialdehyde dehydrogenase                                     | 62.3         | 131          | 14.4              | 0.009  |
| 137        | Argininosuccinate synthase                                               | 27.3         | 46.3         | 6.8               | 0.049  |
| 139        | Phosphoribosyl-AMP cyclohydrolase                                        | 31           | 54           | 8.64              | NS     |
| 140        | Phosphoribosyl-ATP pyrophosphohydrolase                                  | 69.7         | 131.7        | 18.8              | 0.03   |
| 141        | Histidinol dehydrogenase                                                 | 86           | 173          | 27.4              | 0.034  |
| 159        | Tryptophan synthase alpha chain                                          | 31.7         | 49.3         | 6.92              | NS     |
| 165        | Argininosuccinate lyase                                                  | 52.7         | 86.7         | 8.3               | 0.015  |
| 169        | Shikimate 5-dehydrogenase                                                | 27.3         | 51           | 10.48             | NS     |
| 174        | Glutamine synthetase                                                     | 108          | 218          | 41.9              | NS     |
| 241        | Histidinol phosphatase and related phosphatases                           | 36.3         | 55.3         | 8.69              | NS     |
| 253        | Diaminopimelate epimerase                                                | 12           | 25           | 3.3               | 0.016  |
| 260        | Leucyl aminopeptidase                                                    | 8.3          | 9.3          | 4.03              | NS     |
| 263        | Glutamate 5-kinase                                                       | 45.7         | 81.7         | 15.28             | NS     |
| 287        | Prephenate dehydrogenase                                                 | 23           | 48.3         | 6.9               | 0.021  |
| 289        | Dihydronicolinate reductase                                              | 145          | 263          | 27.8              | 0.013  |
| 308        | Aminopeptidase N                                                         | 42           | 45.7         | 11.22             | NS     |
| 334        | Glutamate dehydrogenase/Leucine dehydrogenase                           | 25.7         | 54.3         | 8                 | 0.023  |
| 337        | 3-Dehydroquininate synthetase                                           | 15           | 37.3         | 5.7               | 0.017  |
| 339        | Zn-dependent oligopeptidases                                             | 26.3         | 52.3         | 8.5               | 0.038  |
| 345        | Pyrroline-5-carboxylate reductase                                        | 25.3         | 43           | 5.1               | 0.026  |

(Continued)
| COG number | Function | Average COG normalized read abundance |
|------------|----------|--------------------------------------|
| 346        | Lactoylglutathione lyase and related lyases | 51 85 20.8 NS |
| 347        | Nitrogen regulatory protein Pil | 147 283 22.9 0.004 |
| 367        | Asparagine synthase (glutamine-hydrolyzing) | 85 185 29.9 0.028 |
| 403        | Glycine cleavage system protein P (pyridoxal-binding), N-terminal domain | 18 34.7 7.9 NS |
| 404        | Glycine cleavage system T protein (aminomethyltransferase) | 13 23.7 6.57 NS |
| 405        | Gamma-glutamyltransferase | 12.7 18.3 6.55 NS |
| 410        | ABC-type branched-chain amino acid transport systems, ATPase component | 56.7 105.7 13.7 0.023 |
| 421        | Spermidine synthase | 350 651 84 0.023 |
| 436        | Aspartate/Tyrosine/Aromatic aminotransferase | 73.7 126.7 19 0.05 |
| 440        | Acetolactate synthase, small (regulatory) subunit | 26 46.3 13.82 NS |
| 460        | Homoserine dehydrogenase | 115 187 24.2 0.04 |
| 462        | Phosphoribosylpyrophosphate synthetase | 38.7 80.7 13.82 NS |
| 473        | Isocitrate/isopropylmalate dehydrogenase | 69 126 25 NS |
| 498        | Threonine synthase | 97 166 40.1 NS |
| 506        | Proline dehydrogenase | 4.3 12.3 2.98 NS |
| 509        | Glycine cleavage system H protein (lipoate-binding) | 5.35 8 1.563 NS |
| 520        | Selenocysteine lyase | 83.3 159.3 19.9 0.019 |
| 527        | Aspartokinases | 51.3 85 11.5 0.043 |
| 547        | Anthranilate phosphoribosyltransferase | 29.7 40.7 9.91 NS |
| 548        | Acetylglutamate kinase | 15.3 37 5.9 0.021 |
| 549        | Carbamate kinase | 17.7 35 5.5 0.034 |
| 559        | Branched-chain amino acid ABC-type transport system, permease components | 37.3 80.7 19.15 NS |
| 560        | Phosphoserine phosphatase | 17.7 36.7 6.3 0.044 |
| 620        | Methionine synthase II (cobalamin-independent) | 26.3 64.7 9.4 0.015 |
| 624        | Acetylornithine deacetylase/Succinyl-diaminopimelate desuccinylase and related deacylases | 57 109 21.4 NS |
| 626        | Cystathionine beta-lyases/cystathionine gamma-synthases | 70 118 27.4 NS |
| 646        | Methionine synthase I (cobalamin-dependent), methyltransferase domain | 127 206 36 NS |
| 655        | Glycine/D-amino acid oxidases (deaminating) | 7 8.3 2.19 NS |
| 683        | ABC-type branched-chain amino acid transport systems, periplasmic component | 26.3 56.7 8 0.019 |
| 685        | 5,10-Methylenetetrahydrofolate reductase | 46.3 77.7 10.8 0.043 |
| 686        | Alanine dehydrogenase | 4.7 12.7 2.8 0.044 |
| 687        | Spermidine/Putrescine-binding periplasmic protein | 15.7 38.3 9.07 NS |
| 703        | Shikimate kinase | 13 18 2.31 NS |
| 710        | 3-Dehydroquinate dehydratase | 57 110.3 19.3 0.051 |
| 722        | 3-Deoxy-D-arabino-heptulosonate 7-phosphate (DAHP) synthase | 44.7 94.7 12.6 0.017 |
| 747        | ABC-type dipeptide transport system, periplasmic component | 120.3 236 17.7 0.003 |
| 757        | 3-Dehydroquinate dehydratase II | 32 49.7 6.98 NS |
| 765        | ABC-type amino acid transport system, permease component | 49.3 90.3 9.5 0.013 |
| 786        | Na+/Glutamate symporter | 7.3 13.3 4.11 NS |
| 814        | Amino acid permeases | 13 14 5.32 NS |
| 1003       | Glycine cleavage system protein P (pyridoxal-binding), C-terminal domain | 29.3 40.3 6.72 NS |
| 1027       | Aspartate ammonia-lyase | 18.7 30 7.49 NS |
| 1045       | Serine acetyltransferase | 77 189 29.4 0.019 |
| 1104       | Cysteine sulfinate desulfinase/Cysteine desulfurase and related enzymes | 12 22.7 2.96 0.023 |
| 1113       | Gamma-aminobutyrate permease and related permeases | 24.7 31.7 11.54 NS |
| 1114       | Branched-chain amino acid permeases | 210 396 58.6 0.034 |
| 1115       | Na+/Alanine symporter | 139 276 57.8 NS |
| 1125       | ABC-type proline/glycine betaine transport systems, ATPase components | 4.3 6.7 3.35 NS |

(Continued)
| COG number | Function                                                                 | Average COG normalized read abundance |
|-----------|--------------------------------------------------------------------------|---------------------------------------|
| 1126      | ABC-type polar amino acid transport system, ATPase component             | 62 (1 h), 173 (4 h), SED 24.1, P 0.01 |
| 1164      | Oligoendopeptidase F                                                     | 18.7 (1 h), 33 (4 h), SED 5.1, P 0.049  |
| 1166      | Arginine decarboxylase (sperrmidine biosynthesis)                        | 54.3 (1 h), 108.3 (4 h), SED 18.2, P 0.041  |
| 1168      | Bifunctional PLP-dependent enzyme with beta-cystathionase and maltose regulon repressor activities | 18.3 (1 h), 38.3 (4 h), SED 6.9, P 0.044  |
| 1171      | Threonine dehydratase                                                    | 34.3 (1 h), 76.3 (4 h), SED 17.09, NS   |
| 1174      | ABC-type proline/glycine betaine transport systems, permease component  | 2 (1 h), 4.67 (4 h), SED 1.86, NS       |
| 1176      | ABC-type spermidine/putrescine transport system, permease component I    | 15.7 (1 h), 41.7 (4 h), SED 9.84, NS     |
| 1177      | ABC-type spermidine/putrescine transport system, permease component II   | 7.7 (1 h), 16 (4 h), SED 2.3, P 0.021    |
| 1280      | Putative threonine efflux protein                                        | 4.3 (1 h), 6.7 (4 h), SED 2.49, NS      |
| 1296      | Predicted branched-chain amino acid permease (azaleucine resistance)     | 9.3 (1 h), 49 (4 h), SED 8.7, P 0.027    |
| 1305      | Transglutaminase-like enzymes, putative cysteine proteases               | 68.7 (1 h), 134.3 (4 h), SED 9.7, P 0.003 |
| 1362      | Aspartyl aminopeptidase                                                  | 35 (1 h), 71.7 (4 h), SED 10.9, P 0.028  |
| 1364      | N-acetylglutamate synthase (N-acetylspermidine aminotransferase)         | 10.3 (1 h), 24.3 (4 h), SED 3.8, P 0.021  |
| 1410      | Methionine synthase I, cobalamin-binding domain                          | 143 (1 h), 225 (4 h), SED 39, NS        |
| 1446      | Asparaginase                                                             | 7 (1 h), 7.7 (4 h), SED 2.26, NS        |
| 1465      | Predicted alternative 3-dehydroquinase synthase                         | 4 (1 h), 6 (4 h), SED 1.29, NS          |
| 1505      | Serine proteases of the peptidase family 59A                            | 7 (1 h), 6 (4 h), SED 2.77, NS          |
| 1506      | Dipeptidyl aminopeptidases/acylaminoacyl-peptidases                      | 110 (1 h), 165 (4 h), SED 22.5, NS      |
| 1509      | Lysine 2,3-aminomutase                                                  | 28.3 (1 h), 49.3 (4 h), SED 7.5, P 0.048 |
| 1605      | Chorismate mutase                                                       | 12 (1 h), 25 (4 h), SED 7.3, NS         |
| 1703      | Putative periplasmic protein kinase ArgK and related GTPases of G3E family | 41 (1 h), 51.3 (4 h), SED 6.86, NS       |
| 1748      | Saccharopine dehydrogenase and related proteins                          | 63 (1 h), 124 (4 h), SED 22.7, P 0.054  |
| 1760      | L-serine deaminase                                                      | 40 (1 h), 79 (4 h), SED 15.58, NS       |
| 1770      | Protease II                                                             | 20 (1 h), 15 (4 h), SED 6.76, NS        |
| 1775      | Benzylo-CoA reductase/2-hydroxyglutaryl-CoA dehydratase subunit, Bcr/C/Bad/D/HgdB | 7.7 (1 h), 18.3 (4 h), SED 6.37, NS |
| 1897      | Homoserine trans-succinylase                                             | 32 (1 h), 91 (4 h), SED 17.1, P 0.026   |
| 1921      | Selenocysteine synthase [seryl-tRNASe selenium transferase]             | 4 (1 h), 10.7 (4 h), SED 3.23, NS       |
| 1932      | Phosphoserine aminotransferase                                           | 50.3 (1 h), 92.3 (4 h), SED 15.3, P 0.081 |
| 1982      | Arginine/lysine/ornithine decarboxylases                                | 37.7 (1 h), 63.7 (4 h), SED 18.43, NS   |
| 1984      | Allophanate hydrolase subunit 2                                          | 4 (1 h), 9.7 (4 h), SED 2.11, NS        |
| 2008      | Threonine aldolase                                                      | 3.7 (1 h), 9.1 (4 h), SED 1.9, P 0.052  |
| 2021      | Homoserine acetyltransferase                                             | 10.7 (1 h), 14 (4 h), SED 2.79, NS      |
| 2040      | Homocysteine/selenocysteine methylase (S-methylmethionine-dependent)     | 6.67 (1 h), 8.33 (4 h), SED 1.491, NS    |
| 2049      | Allophanate hydrolase subunit 1                                          | 11 (1 h), 22.7 (4 h), SED 2.9, P 0.016  |
| 2066      | Glutaminase                                                              | 103 (1 h), 216 (4 h), SED 33.9, P 0.029 |
| 2171      | Tetrahydrodipicolinate N-succinyltransferase                            | 6.3 (1 h), 10.3 (4 h), SED 3.35, NS     |
| 2195      | D- and tripeptidases                                                    | 6.3 (1 h), 18.7 (4 h), SED 2.6, P 0.009  |
| 2303      | Choline dehydrogenase and related flavoproteins                         | 11.7 (1 h), 11.3 (4 h), SED 3.4, NS     |
| 2309      | Leucyl aminopeptidase (aminopeptidase T)                                 | 24 (1 h), 50 (4 h), SED 11.28, NS       |
| 2317      | Zn-dependent carboxypeptidase                                            | 48 (1 h), 89.7 (4 h), SED 12.3, P 0.028 |
| 2355      | Zn-dependent dipeptidase, microsomal dipeptidase homolog                | 16 (1 h), 23.7 (4 h), SED 3.07, NS      |
| 2423      | Predicted ornithine cyclodeaminase, mu-crystallin homolog                | 11.7 (1 h), 22.3 (4 h), SED 6.57, NS    |
| 2502      | Asparagine synthetase A                                                 | 54.3 (1 h), 105.7 (4 h), SED 18.9, P 0.053 |
| 2515      | 1-Aminocyclopropane-1-carboxylate deaminase                             | 7 (1 h), 8.3 (4 h), SED 24, NS          |
| 2610      | H+/Glucosamine symporter and related permeases                           | 40.3 (1 h), 68.7 (4 h), SED 5.4, P 0.006 |
| 2755      | Lysophospholipase L1 and related esterases                               | 175 (1 h), 299 (4 h), SED 39.1, P 0.034 |

(Continued)
omitted from Tables 6–8. KEGG pathway analysis (Figure 4) corroborated that most functional pathways were present within PRG attached bacteria at both 1 and 4 h post rumen incubation with only a few being uniquely found in the attached bacteria at 1 or 4 h of incubation only (Table 9).

### Plant Chemical Changes

Analysis of FT-IR spectra of residual plant material taken over time, showed that the scores for the first 20 principal components (together accounting for 95% of variance) were significantly different between spectra from different time points ($P < 0.001$), with plots of the scores for PC1 vs. PC2 (Figure 5) and PC2 vs. PC3 (Figure 6) showing clear clustering between spectra from samples incubated for up to 2 h compared with those incubated for longer periods. It should be noted that the circles drawn to denote clustering have been constructed by eye for ease of interpretation, and not using any statistical methodology. PC 1 accounted for 34.7% of total variance, PC2 accounted for 15.7%, and PC3 10.2%. The spectra for all samples at various time points were similar (Supplementary Figure 3).

Analysis of the loadings for PC 1 showed positive contributions (i.e., a decrease in chemical content) from variables at 1393 and 1598 cm$^{-1}$, possibly being attributable to the amino amide I absorption of proteins (Schmitt and Flemming, 1998; Supplementary Figure 3). The loadings of PC2 showed similarities with PC1 but additional positive contributions were detected at 1029 cm$^{-1}$, a region that has also been associated with cellulose content (Kacurakova et al., 2000; Alonso-Simon et al., 2004; Supplementary Figure 3) and 1664 cm$^{-1}$, which is in the amide I absorption region of amino groups. PC2 was negatively correlated with absorbances at 1394 and 1591 cm$^{-1}$ (Supplementary Figure 3), PC 2 was positively correlated with absorbances at 2908 and 2850 cm$^{-1}$, which have been associated with the asymmetric and symmetric stretching on CH$_2$ moieties in fatty acids (Schmitt and Flemming, 1998; Supplementary Figure 3). The loadings for PC3 show positive contributions at 1359 cm$^{-1}$, corresponding to a CH2 stretch of cellulose (Kacurakova et al., 2000), and several peaks relating to hemicellulose components and pectin at 979, 995, 1045, and 1080 cm$^{-1}$ (Supplementary Figure 3). PC3 was also negatively correlated with peaks at 161 and 1529 cm$^{-1}$, corresponding to amide 1 and amide 11 in proteins (Schmitt and Flemming, 1998; Supplementary Figure 3).

### DISCUSSION

In this study, we demonstrate that colonization of PRG by rumen bacteria is biphasic with primary (up to 4 h) and secondary (post 4 h) events observed based on changes seen in attached bacterial diversity. We also demonstrate that these changes in diversity correlate with changes in functional capacity and changes in the metabolome of PRG itself. Thus, despite the resilience and redundancy observed within the rumen microbiome it is apparent, on a DNA level, that diversity and function are linked.
TABLE 7 | Carbohydrate metabolism clusters of orthologous genes (COG) categories showing significant read abundance differences within the primary (1 h) and secondary (4 h) bacteria attached to perennial ryegrass.

| COG number | Function | Average COG normalized read abundance | 1 h | 4 h | SED | P  |
|------------|----------|--------------------------------------|-----|-----|-----|----|
| 21         | Transketolase | 60 | 92.7 | 15.47 | NS  |
| 33         | Phosphoglucomutase | 8.33 | 9.33 | 1.599 | NS  |
| 36         | Pentose-5-phosphate-3-epimerase | 63 | 123 | 24.4 | NS  |
| 57         | Glyceraldehyde-3-phosphate dehydrogenase/erythrose-4-phosphate dehydrogenase | 43.7 | 71.7 | 6.52 | 0.013 |
| 58         | Glucan phosphorylase | 205 | 363 | 51.7 | 0.038 |
| 120        | Ribose 5-phosphate isomerase | 3.7 | 10.7 | 4.19 | NS  |
| 126        | 3-Phosphoglycerate kinase | 89.7 | 164.7 | 15 | 0.008 |
| 129        | Dihydroxyacetone dehydrogenase/phosphogluconate dehydrogenase | 69 | 125 | 22.4 | NS  |
| 148        | Enolase | 38.3 | 91.7 | 14.38 | 0.021 |
| 149        | Triosephosphate isomerase | 43 | 83.7 | 10.14 | 0.016 |
| 153        | Galactokinase | 45 | 96.7 | 10.41 | 0.008 |
| 158        | Fructose-1,6-bisphosphatase | 5.7 | 3.7 | 2.36 | NS  |
| 166        | Glucose-6-phosphate isomerase | 60.3 | 101.7 | 11.67 | 0.024 |
| 176        | Transaldolase | 18.3 | 36 | 6.61 | NS  |
| 191        | Fructose/Tagatose bisphosphate aldolase | 65 | 123 | 19.55 | 0.041 |
| 205        | 6-Phosphofructokinase | 108 | 195 | 29.8 | 0.044 |
| 235        | Ribulose-5-phosphate 4-epimerase and related epimerases and aldolases | 84 | 165 | 26 | 0.037 |
| 246        | Mannitol-1-phosphate/altronate dehydrogenases | 91 | 180 | 24.8 | 0.023 |
| 269        | 3-Hexulose-6-phosphate synthase and related proteins | 4 | 12.3 | 4.52 | NS  |
| 279        | Phosphoheptose isomerase | 11.7 | 23.7 | 6.94 | NS  |
| 296        | 1,4-Alpha-glucan branching enzyme | 150 | 286 | 50.7 | 0.054 |
| 297        | Glycogen synthase | 67 | 146 | 31.6 | 0.068 |
| 362        | 6-Phosphogluconate dehydrogenase | 22.7 | 37.3 | 5.68 | 0.061 |
| 363        | 6-Phosphogluconolactonase/Glucosamine-6-phosphate isomerase/deaminase | 73 | 141 | 24 | 0.047 |
| 364        | Glucose-6-phosphate 1-dehydrogenase | 9.3 | 7.7 | 3.2 | NS  |
| 366        | Glycosidases | 262 | 569 | 88.2 | 0.025 |
| 380        | Trehalose-6-phosphate synthase | 24.7 | 16.3 | 4.53 | NS  |
| 383        | Alpha-mannosidase | 26.3 | 47.7 | 7.02 | 0.038 |
| 395        | ABC-type sugar transport system, permease component | 141 | 337 | 48.5 | 0.016 |
| 406        | Fructose-2,6-bisphosphatase | 51.7 | 43.7 | 15.94 | NS  |
| 451        | Nucleoside-diphosphate-sugar epimerases | 429 | 764 | 100.9 | 0.029 |
| 469        | Pyruvate kinase | 82 | 136 | 30.4 | NS  |
| 524        | Sugar kinases, ribokinase family | 128 | 225 | 32.5 | 0.04 |
| 574        | Phosphoenolpyruvate synthase/pyruvate phosphate dikinase | 194 | 400 | 41.4 | 0.008 |
| 580        | Glycerol uptake facilitator and related permeases (Major Intrinsic Protein Family) | 38 | 70.3 | 14.23 | 0.085 |
| 588        | Phosphoglycerate mutase 1 | 16.3 | 30.7 | 7.99 | NS  |
| 647        | Predicted sugar phosphatases of the HAD superfamily | 8 | 15 | 2.77 | NS  |
| 662        | Mannose-6-phosphate isomerase | 21.3 | 36 | 8.09 | NS  |
| 696        | Phosphoglyceromutase | 57.3 | 122.7 | 15.07 | 0.012 |
| 698        | Ribose 5-phosphate isomerase RpoB | 82 | 145 | 29.8 | NS  |
| 702        | Predicted nucleoside-diphosphate-sugar epimerases | 23.3 | 17.7 | 9.39 | NS  |
| 726        | Predicted xylanase/chitin deacetylase | 46 | 82 | 22 | NS  |
| 738        | Fucose permease | 81.7 | 137.3 | 14.09 | 0.017 |
| 800        | 2-keto-3-deoxy-6-phosphogluconate aldolase | 30.7 | 48.3 | 7.8 | NS  |
| 1015       | Phosphopentomutase | 36.7 | 75.7 | 11.18 | 0.025 |
| 1080       | Phosphoenolpyruvate-protein kinase (PTS system EI component in bacteria) | 31.7 | 59.3 | 15.36 | NS  |
| 1082       | Sugar phosphate isomerases/epimerases | 24 | 55 | 12.06 | NS  |

(Continued)
| COG number | Function                                                                 | Average COG normalized read abundance |
|------------|--------------------------------------------------------------------------|---------------------------------------|
|            |                                                                          | 1 h        | 4 h        | SED       | P         |
| 1086       | Predicted nucleoside-diphosphate sugar epimerases                        | 239        | 425        | 62.7      | 0.041     |
| 1105       | Fructose-1-phosphate kinase and related fructose-6-phosphate kinase (PfkB) | 178        | 329        | 47.5      | 0.033     |
| 1109       | Phosphomannomutase                                                       | 12         | 39         | 7.46      | 0.022     |
| 1129       | ABC-type sugar transport system, ATPase component                        | 184        | 420        | 77.6      | 0.038     |
| 1172       | Ribose/xyllose/arabinose/galactoside ABC-type transport systems, permease components | 44         | 108        | 27.4      | NS        |
| 1175       | ABC-type sugar transport systems, permease components                     | 177        | 388        | 66.7      | 0.034     |
| 1263       | Phosphotransferase system IIC components, glucose/maltose/N-acetylglucosamine-specific | 186        | 390        | 103.1     | NS        |
| 1264       | Phosphotransferase system IIB components                                 | 175        | 360        | 96.9      | NS        |
| 1299       | Phosphotransferase system, fructose-specific IIC component               | 107        | 287        | 30.2      | 0.004     |
| 1312       | D-mannonate dehydratase                                                  | 39         | 62.7       | 8.51      | 0.05      |
| 1349       | Transcriptional regulators of sugar metabolism                            | 19         | 37.7       | 9.06      | NS        |
| 1363*      | Cellulase M and related proteins                                         | 39.7       | 68.3       | 10.09     | 0.047     |
| 1440       | Phosphotransferase system cellulose-specific component IIB                | 3          | 7          | 1.633     | NS        |
| 1445       | Phosphotransferase system fructose-specific component IIB                 | 9.3        | 38.3       | 7.67      | 0.019     |
| 1449       | Alpha-amylose/alpha-mannosidase                                          | 14.7       | 22.3       | 2.98      | NS        |
| 1455       | Phosphotransferase system cellulose-specific component IIC                | 26         | 59         | 20.4      | NS        |
| 1472       | Beta-glucosidase-related glycosidases                                    | 473        | 901        | 103.9     | 0.015     |
| 1482       | Phosphomannose isomerase                                                 | 42         | 90.7       | 1.6       | 0.008     |
| 1486       | Alpha-galactosidases/6-phospho-beta-glucosidases, family 4 of glycosyl hydrolases | 28         | 68.3       | 16.12     | NS        |
| 1501       | Alpha-glucosidases, family 31 of glycosyl hydrolases                      | 22         | 45         | 53.7      | 0.013     |
| 1523       | Type II secretory pathway, pullulanase PuA and related glycosidases      | 133        | 226        | 33.9      | 0.052     |
| 1548       | Predicted transcriptional regulator/sugar kinase                          | 3.33       | 6          | 1.563     | NS        |
| 1554       | Trehalose and maltose hydrolases (possible phosphorylases)                | 9          | 14.7       | 2.79      | NS        |
| 1593       | TRAP-type C4-dicarboxylate transport system, large permease component     | 50         | 116        | 21.8      | 0.039     |
| 1621       | Beta-fructosidases (levanase/invertase)                                  | 79         | 167        | 31.5      | 0.049     |
| 1626       | Neutral trehalase                                                        | 5          | 6.7        | 2.47      | NS        |
| 1638       | TRAP-type C4-dicarboxylate transport system, periplasmic component        | 28         | 57.3       | 11.88     | NS        |
| 1640       | 4-Alfa-glucanotransferase                                                | 123        | 199        | 20.7      | 0.022     |
| 1803       | Methylglyoxal synthase                                                   | 6.7        | 13         | 4.74      | NS        |
| 1820       | N-acetylglucosamine-6-phosphate deacetylase                              | 14.3       | 28.7       | 7.23      | NS        |
| 1830       | DhnA-type fructose-1,6-bisphosphate aldolase and related enzymes          | 4          | 11         | 1.16      | 0.004     |
| 1869       | ABC-type ribose transport system, auxiliary component                     | 2          | 3.33       | 1.563     | NS        |
| 1877       | Trehalose-6-phosphatase                                                  | 30         | 23         | 7.79      | NS        |
| 1925       | Phosphotransferase system, HPr-related proteins                          | 7          | 9.7        | 3.28      | NS        |
| 1974       | Beta-galactosidase                                                       | 76.3       | 113        | 19.69     | NS        |
| 1904       | Glucuronate isomerase                                                    | 45.7       | 104.7      | 12.39     | 0.009     |
| 1925       | Phosphotransferase system, HPr-related proteins                          | 7          | 9.7        | 3.28      | NS        |
| 1929       | Glycerate kinase                                                        | 32         | 54.3       | 8.61      | NS        |
| 1940       | Transcriptional regulator/sugar kinase                                   | 66         | 155        | 31.8      | 0.048     |
| 2074       | 2-Phosphoglycerate kinase                                                | 41.7       | 79.7       | 12.5      | 0.038     |
| 2115       | Xylose isomerase                                                        | 21.7       | 37         | 9.46      | NS        |
| 2133       | Glucose/sorbose dehydrogenases                                           | 4.7        | 12.3       | 7.91      | NS        |
| 2160       | L-arabinose isomerase                                                   | 76         | 132        | 28.9      | NS        |
| 2182       | Maltose-binding periplasmic proteins/domains                             | 4.67       | 8.33       | 1.886     | NS        |
| 2190       | Phosphotransferase system IIA components                                 | 77         | 187        | 45.7      | NS        |
| 2211       | Na+/Melibiose symporter and related transporters                         | 77         | 131        | 27.7      | NS        |
| 2213       | Phosphotransferase system, mannitol-specific IIBC component              | 29.7       | 55.3       | 17.8      | NS        |
| 2271       | Sugar phosphate permease                                                 | 9.3        | 6          | 2.11      | NS        |

(Continued)
| COG number | Function                                                                 | 1 h | 4 h | SED | P     |
|------------|--------------------------------------------------------------------------|-----|-----|-----|-------|
| 2273       | Galactose mutarotase and related enzymes                                 | 6.3 | 11  | 3.97| NS    |
| 2376       | Sugar phosphate permease                                                 | 59  | 97  | 26.2| NS    |
| 2379*      | Beta-glucanase/Beta-glucan synthetase                                    | 10.33| 16.33| 1.97| 0.038 |
| 2407       | L-fucose isomerase and related proteins                                  | 81  | 137 | 22.9| NS    |
| 2513       | PEP phosphonomutase and related enzymes                                  | 60.7| 97.7| 16.11| NS    |
| 2706       | 3-Carboxymuconate cyclase                                               | 1   | 27  | 24.5| NS    |
| 2723       | Beta-glucosidase/6-phospho-beta-glucosidase/beta-galactosidase           | 195 | 418 | 97.2| NS    |
| 2730       | Putative glycerate kinase                                               | 78  | 159.3| 15.18| 0.006 |
| 2731       | Beta-galactosidase, beta subunit                                         | 7   | 2.7 | 2.6 | NS    |
| 2814       | Arabinose efflux permease                                               | 5.3 | 11.7| 5.28| NS    |
| 2893       | Phosphotransferase system, mannose/fructose-specific component IIA      | 18.3| 35.7| 13.98| NS    |
| 2942       | N-acetyl-D-glucosamine 2-epimerase                                       | 36.7| 70  | 14.2| NS    |
| 3001       | Fructosamine-3-kinase                                                   | 19.3| 22.7| 3.4 | NS    |
| 3010       | Putative N-acetylmannosamine-6-phosphate epimerase                       | 6.7 | 11.7| 2.36| NS    |
| 3090*      | Endoglucanase                                                           | 9.7 | 20.3| 3.4 | 0.035 |
| 3250       | TRAP-type C4-dicarboxylate transport system, small permease component   | 524 | 875 | 90.4| 0.018 |
| 3265       | Gluconate kinase                                                        | 3.3 | 9   | 2.67| NS    |
| 3345       | Beta-galactosidase/beta-glucuronidase                                    | 109 | 207 | 33  | 0.041 |
| 3386       | Gluconolactonase                                                        | 8   | 8.33| 0.667| NS   |
| 3405       | Alpha-galactosidase                                                     | 24  | 44  | 5.63| 0.024 |
| 3408*      | Endoglucanase Y                                                         | 20.7| 37.3| 5.34| 0.036 |
| 3414       | Phosphotransferase system, galactitol-specific IIB component             | 2   | 4.33| 1.667| NS   |
| 3444       | Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIB | 25  | 55  | 22.8| NS    |
| 3459       | Glycogen debranching enzyme                                             | 145 | 289 | 37.3| 0.018 |
| 3534       | Cellobiose phosphorylase                                                | 147 | 273 | 33.2| 0.019 |
| 3537       | Alpha-L-arabinofuranosidase                                             | 48.3| 98  | 2.91| <0.001|
| 3588       | Fructose-1,6-bisphosphate aldolase                                       | 4.7 | 8.3 | 2.49| NS    |
| 3623       | Putative L-xylulose-5-phosphate 3-epimerase                             | 11  | 15  | 5.16| NS    |
| 3635       | Putative alpha-1,2-mannosidase                                          | 21  | 48  | 7.37| 0.022 |
| 3661       | Predicted phosphoglycerate mutase, AP superfamily                      | 58  | 115.7| 14.11| 0.015 |
| 3664*      | Beta-xylodsidase                                                        | 198 | 363 | 72.1| NS    |
| 3669       | Alpha-glucuronidase                                                     | 73.7| 147 | 18.7| 0.017 |
| 3690       | Alpha-L-fucosidase                                                     | 68  | 132 | 21.1| NS    |
| 3715       | Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIC | 36  | 63  | 20.6| 0.038 |
| 3716       | Phosphotransferase system, mannose/fructose/N-acetylgalactosamine-specific component IIB | 44  | 91  | 26.1| NS    |
| 3717*      | Beta-1,4-xylanase                                                       | 41.7| 69  | 8.07| 0.028 |
| 3730       | Phosphotransferase system sorbitol-specific component IIC               | 6.3 | 13  | 3.13| NS    |
| 3732       | Phosphotransferase system sorbitol-specific component IIBC              | 12.3| 27  | 12.78| NS    |
| 3775       | 5-keto 4-deoxyxuronate isomerase                                        | 14.67| 23  | 1.67| 0.007 |
| 3833       | Phosphotransferase system, galactitol-specific IIC component            | 24  | 58.7| 11.57| 0.04  |
| 3839       | ABC-type maltose transport systems, permease component                  | 127 | 281 | 32.5| 0.009 |
| 3866       | Pectate lyase                                                          | 24  | 30  | 5.29| NS    |
| 3869       | ABC-type sugar transport systems, ATPase components                     | 39.3| 82.7| 13.12| 0.03  |
| 3925       | N-terminal domain of the phosphotransferase system fructose-specific component IIB | 2   | 1   | 0.577| NS    |
| 3934       | Endo-beta-mannanase                                                    | 8.67| 10.67| 1.7 | NS    |
| 3957       | Arabinogalactan endo-1,4-beta-galactosidase                             | 33  | 67.7| 10.51| 0.03  |
In terms of temporal diversity of the bacteria attached to PRG, the phyla Firmicutes and Bacteroidetes dominated and changes in phyla level diversity were not evident over time. In our previous study investigating changes in diversity of bacteria attached to PRG (16S rRNA based) over time within the rumen, we also noted that Firmicutes and Bacteroidetes dominated, but Fibrobacteres sequence abundances were higher than noted in this study (4% compared with an average of 0.6% in this study; Huws et al., 2016). Piao et al. (2014) when investigating diversity (16S rDNA sequencing) of rumen bacteria attached to switchgrass showed similar results to those in our study. On a genus level we found that the most abundant classified genera were Butyrivibrio, Selenomonas, Prevotella, Eubacterium, Porphyromonadaceae, and Ruminococcus, with significant \((P > 0.05)\) increases in Bacillaceae, Lachnospiraceae, and Prevotellaceae were seen during secondary colonization events compared with abundances present during primary colonization. Previously we also noted that Lachnospiraceae, Veillonellaceae, Prevotellaceae, and Ruminococcaceae were dominant, but we also reported that 16S rRNA read abundances for Fibrobacteraceae and Coriobacteriaceae were reasonably similar between both studies but Fibrobacteraceae were lower in this study. We also found that read abundance for Eubacteriaceae and Clostridiaceae were higher in this study than our previous study (Huws et al., 2016). Again, the study conducted by Piao et al. (2014) on bacteria attached to switchgrass showed similar results to this study in terms of the most abundant families. In this study, we saw increases in Bacillaceae, Lachnospiraceae, Porphyromonadaceae, and Prevotellaceae during secondary colonization compared with abundances present during primary colonization. We noted increases in Lachnospiraceae only between primary and secondary colonization events in our previous study (Huws et al., 2016). On a genus level we found that the most abundant classified genera were Butyrivibrio, Selenomonas, Prevotella, Eubacterium, Porphyromonadaceae, and Ruminococcus, with significant \((P > 0.05)\) increases in Acidaminococcus, Bacillus, Blautia, Butyrivibrio, and Prevotella.
TABLE 8 | Lipid metabolism and transport clusters of orthologous genes (COG) categories showing significant read abundance differences within the primary (1 h) and secondary (4 h) bacteria attached to perennial ryegrass.

| COG number | Function | Average COG normalized read abundance |
|------------|----------|--------------------------------------|
|            |          | 1 h   | 4 h   | SED  | P     |
| 20         | Undecaprenyl pyrophosphate synthase | 34.7  | 71.7  | 13.6 | 0.053 |
| 183        | Acetyl-CoA acetyltransferase       | 45.7  | 89.3  | 17.95| NS    |
| 204        | 1-acyl-sn-glycerol-3-phosphate acyltransferase | 31    | 42.3  | 7.54 | NS    |
| 245        | 2C-methyl-D-erythritol 2,4-cyclodiphosphate synthase | 38    | 79.7  | 14   | 0.041 |
| 331        | (Acyl-carrier-protein) S-malonyltransferase | 37    | 69    | 11.8 | 0.053 |
| 332        | 3-Oxacyl-[acyl-carrier-protein] synthase III | 57.3  | 91.7  | 10.3 | 0.029 |
| 365        | Acyl-coenzyme A synthetases/AMP-(fatty) acid ligases | 106   | 211   | 28.1 | 0.02  |
| 416        | Fatty acid/phospholipid biosynthesis enzyme | 19.7  | 47.7  | 9.5  | 0.043 |
| 439        | Biotin carboxylase                 | 47    | 96.7  | 14.3 | 0.026 |
| 511        | Biotin carboxyl carrier protein    | 36    | 107.3 | 14.7 | 0.008 |
| 558        | Phosphatidylglycerophosphate synthase | 11.7  | 27.7  | 5.7  | 0.048 |
| 575        | CDP-diglyceride synthetase         | 67    | 136.3 | 16   | 0.012 |
| 615        | Cytidylyltransferase               | 26.7  | 45.7  | 7.76 | NS    |
| 623        | Enoyl-[acyl-carrier-protein] reductase (NADH) | 5.33  | 4.67  | 1.886| NS    |
| 657        | Esterase/lipase                    | 75    | 142   | 21.7 | 0.037 |
| 671        | Membrane-associated phospholipid phosphatase | 19.7  | 31    | 6.84 | NS    |
| 688        | Phosphatidylserine decarboxylase   | 13.3  | 24.7  | 8.08 | NS    |
| 764        | 3-Hydroxymyristoyl/3-hydroxydecanoyl-[acyl carrier protein] dehydratases | 22    | 39    | 11.63| NS    |
| 777        | Acetyl-CoA carboxylase beta subunit | 13    | 28.7  | 8.19 | NS    |
| 821        | Enzyme involved in the deoxyxylulose pathway of isoprenoid biosynthesis | 61    | 146   | 25.3 | 0.028 |
| 825        | Acetyl-CoA carboxylase alpha subunit | 16    | 22.3  | 9.63 | NS    |
| 1022       | Long-chain acyl-CoA synthetases (AMP-forming) | 92.3  | 133.3 | 3.7 | <0.001 |
| 1024       | Enoyl-CoA hydratase/carnithine racemase | 25.7  | 45.3  | 8.48 | NS    |
| 1154       | Deoxyxylulose-5-phosphate synthase | 109   | 219   | 40.1 | 0.051 |
| 1182       | Acyl carrier protein phosphodiesterase | 6     | 6.67  | 1.202| NS    |
| 1183       | Phosphatidylserine synthase        | 8.3   | 13.3  | 3.5  | NS    |
| 1211       | 4-Diphosphocytidyl-2-methyl-D-erthritol synthase | 77    | 175   | 20.8 | 0.009 |
| 1250       | 3-Hydroxyacyl-CoA dehydrogenase    | 21.7  | 47    | 6.5  | 0.018 |
| 1257       | Hydroxymethylglutaryl-CoA reductase | 4.3   | 13.7  | 2.5  | 0.02  |
| 1260       | Myo-inositol-1-phosphate synthase  | 27    | 50    | 5.2  | 0.011 |
| 1502       | Phosphatidylserine/phosphatidylglycerophosphate/cardiolipin synthases and related enzymes | 62.7  | 95.7  | 10.8 | 0.038 |
| 1562       | Phytoene/squalene synthetase       | 4     | 4.33  | 1.453| NS    |
| 1577       | Mevalonate kinase                 | 3     | 10.3  | 2.4  | 0.038 |
| 1607       | Acyl-CoA hydrolase                 | 4     | 4.7   | 2.03 | NS    |
| 1657       | Squalene cyclase                  | 3.67  | 6     | 1.453| NS    |
| 1788       | Acyl-CoA:acetate/3-ketoacid CoA transferase, alpha subunit | 3.33  | 2     | 1.667| NS    |
| 1835       | Predicted acyltransferases        | 9     | 17.3  | 4.01 | NS    |
| 1884       | Methylmalonyl-CoA mutase, N-terminal domain/subunit | 132   | 196   | 21.7 | 0.042 |
| 1924       | Activator of 2-hydroxyglutaryl-CoA dehydratase (HSP70-class ATPase domain) | 155   | 277   | 57.5 | NS    |
| 1946       | Acyl-CoA thioesterase             | 1.7   | 10.7  | 9.18 | NS    |
| 1960       | Acyl-CoA dehydrogenases           | 45    | 92    | 15.1 | 0.036 |
| 2030       | Acyl dehydratase                  | 7     | 12.7  | 2.33 | NS    |
| 2031       | Short chain fatty acids transporter | 2     | 7     | 0.6  | <0.001 |
| 2057       | Acyl-CoA:acetate/3-ketoacid CoA transferase, beta subunit | 3     | 11    | 3.11 | NS    |
| 2067       | Long-chain fatty acid transport protein | 9.3   | 14    | 2.73 | NS    |
| 2084       | 3-Hydroxyisobutyrate dehydrogenase and related beta-hydroxyacid dehydrogenases | 22    | 36.3  | 6.94 | NS    |

(Continued)
Mayorga et al. Perennial Ryegrass Attached Rumen Bacteria

TABLE 8 | Continued

| COG number | Function                                                                 | Average COG normalized read abundance |
|------------|--------------------------------------------------------------------------|---------------------------------------|
|            |                                                                          | 1 h    | 4 h    | SED   | P       |
| 2185       | Methylmalonyl-CoA mutase, C-terminal domain/subunit (cobalamin-binding) | 123.3  | 184.3  | 16.5  | 0.021   |
| 2267       | Lysophospholipase                                                        | 30     | 40     | 6.53  | NS      |
| 2272       | Carboxylesterase type B                                                 | 107    | 192    | 21.9  | 0.018   |
| 3000       | Sterol desaturase                                                        | 8.7    | 5      | 2.4   | NS      |
| 4799       | Acetyl-CoA carboxylase, carboxytransferase component (subunits alpha and beta) | 63     | 102    | 14.3  | 0.053   |
| 4981       | Enoyl reductase domain of yeast-type FAS1                                | 2.33   | 1.33   | 0.745 | NS      |

NS: Not Significant.

FIGURE 4 | Kyto encyclopedia of genes and genomes (KEGG) pathways exhibited by perennial ryegrass attached rumen bacteria following 1 and 4 h of rumen incubation. Blue lines show pathways present by plant attached bacteria following 1 h of rumen incubation. Red lines show pathways present by plant attached bacteria following 4 h of rumen incubation. Pink/Purple lines show pathways present by plant attached bacteria at both 1 and 4 h of rumen incubation. Boxes 1–7 have been denoted in order to formulate Table 9 listing pathways in blue and red in order to note unique pathways present at one incubation time only.

seen during secondary colonization compared with abundances present during primary colonization. In our previous study we also noted that Butyrivibrio, Selenomonas, Prevotella, Pseudobutyrivibrio, dominated the attached microbiota irrespective of time (Huws et al., 2016). We also found that Olsenella and Fibrobacter were reasonably dominant previously, which was not apparent in this study. The previous study also indicated that Pseudobutyrivibrio increased in abundance during the secondary phase of colonization. Again, the study conducted by Piao et al. (2014) showed similar results to those in this study in terms of the most abundant bacterial genera attached to switchgrass over time. The similarities between our in vitro study and these in sacco studies (Piao et al., 2014; Huws et al., 2016) demonstrate that in vitro rumen incubations are reasonably representative of attachment events that occur within the rumen itself. Also, the use of shotgun metagenomic sequencing in these studies, as compared with results from other studies using 16S rRNA (RNA and DNA) based sequencing illustrates that non-amplification based techniques are beneficial for taxonomical identification as well as allowing insight into the functionality of the rumen microbiota.

In terms of the temporal functional capacity of the attached bacteria, there was a clear difference between the function of the primary attached bacteria and that of the secondary attached
| Box no | Present (h) | EC number | Classification | Pathway |
|-------|------------|-----------|----------------|---------|
| 1     | 1          | 2.3.1.68  | Glutamine N-acyltransferase | Biosynthesis of secondary metabolites |
| 1     | 1          | 2.1.1.68  | Caffeate methyltransferase | Biosynthesis of secondary metabolites |
| 1     | 4          | 1.14.19.3 | Delta6-desaturase | Linoleic acid metabolism/biosynthesis of unsaturated fatty acids |
| 2     | 4          | 1.14.14.1 | Cytrochrome P450 | Arachidonic acid metabolism |
| 1     | 3.10.1.1   | N-sulfoglucoasamine sulfhydrolase | Glucosaminoglycan degradation/lysozyme |
| 2     | 1          | 2.4.1.155 | Alpha-1,3(6)-mannnosylglycoprotein | N-glycan biosynthesis |
| 2     | 1          | 3.1.6.12  | Arylsulfatase | Glucosaminoglycan degradation/lysosome |
| 2     | 1          | 3.1.1.23  | Acylglycerol lipase | Glycolipid metabolism/retrograde endocannabinoid signaling |
| 2     | 1          | 1.1.1.101 | Acylglycerone-phosphate reductase | Glycerocephospholipid metabolism/Ether lipid metabolism |
| 2     | 4          | 2.7.8.2   | Diaclyglycerol cholinephosphotransferase | Phosphonate and phosphinate/glycerophospholipid/ether lipid metabolism |
| 2     | 4          | 2.1.1.17  | Phosphodiyethanolamine N-methyltransferase | Glycerophospholipid metabolism/synthesis of secondary metabolites |
| 2     | 2.7.8.20   | Glycerocephosphotransferase | Glycerol lipid metabolism |
| 2     | 4          | 3.1.3.66  | Inositol polyphosphate-4-phosphatase | Inositol phosphate metabolism/inositol signaling system |
| 3     | 1          | 3.2.1.20/3.2.1.3 | Maltose glucoamylase | Galactose/starch and sucrose metabolism/carbohydrate digestion and absorption |
| 3     | 1          | 2.4.1.17  | Glucuronosyltransferase | Pentose and glucuronate interconversions/ascorbat metabolism/steroid synthesis etc. |
| 3     | 1          | 1.1.1.43  | Phosphoglucomonate 2-dehydrogenase | Pentose/glutathione phosphate pathway/microbial metabolism in diverse environments |
| 3     | 4          | 4.1.2.29  | 6-Phospho-5-dehydro-2 deoxy-D gulono aldolase | Inositol phosphate metabolism |
| 3     | 4          | 2.7.1.60  | N-acylmannosamine kinase | Amino sugar and nucleotide sugar metabolism |
| 3     | 4          | 2.7.1.13  | Dehydroglucuronokinase | Pentose phosphate pathway |
| 3     | 4          | 4.4.1.16  | Selenocysteine lyase | Selenocompound metabolism |
| 3     | 4          | 1.1.1.87  | Homoisoisocitrate dehydrogenase | Lysine biosynthesis/microbial metabolism in diverse environments/biosynthesis antibiotics |
| 4     | 1          | 1.14.13.180 | Oxygen oxidoreductase | Biosynthesis of secondary metabolites/Microbial metabolism in diverse environments |
| 4     | 1          | 2.3.1.5   | Acrylamide N-acetyltransferase | Nitrotoluene degradation/Drug metabolism |
| 4     | 1          | 3.5.3.4   | Allantoicase | Purine metabolism/microbial metabolism diverse environments |
| 4     | 1          | 1.14.18.1 | Tyrosinase | Tyrosine/riboflavin metabolism/Biosynthesis of secondary metabolites |
| 4     | 4          | 1.10.3.1  | Catechol oxidase | Tyrosine metabolism/Biosynthesis of secondary metabolites |
| 4     | 1          | 1.14.16.2 | Tyrosine 3-monooxygenase | Tyrosine metabolism |
| 4     | 4          | 1.4.3.4   | Monochlor oxide oxidase | Amino acid metabolism/Biosynthesis of secondary metabolites |
| 4     | 3          | 3.7.1.5   | Acylpyruvate hydrolyase | Tyrosine metabolism/Microbial metabolism in diverse environments |
| 4     | 3          | 3.7.1.3   | Kynureninase | Tryptophan metabolism |
| 4     | 4          | 4.1.1.43  | Phenylpyruvate decarboxylase | Phenylalanine and Tryptophan metabolism |
| 5     | 1          | 5.4.99.77 | Inositol synthase | Steroid biosynthesis/Biosynthesis of secondary metabolites |
| 5     | 1          | 1.1.1.62/1.1.1.239 | Beta-estradiol A-dehydrogenase | Steroid hormone biosynthesis |
| 5     | 1          | 1.14.13.50 | Pentachlorophenol monoxygenase | Chlorocyclohexane, chlorobenzene, fluorobenzoate degradation/Microbial metabolism in diverse environments |
| 5     | 1          | 1.1.1.46  | Glutathione-independent formaldehyde dehydrogenase | Chloroalkane and chloroalkene degradation/Methane metabolism |
| 5     | 1          | 1.13.11.2 | Catechol 2,3 dioxygenase | Chlorocyclohexane, chlorobenzene, benzoate, xylene, styrene degradation/Microbial metabolism in diverse environments |
| 5     | 1          | 1.13.11.39 | Biphenyl-2,3-diol 1,2 dioxygenase | Chlorocyclohexene, chlorobenzene, dioxin degradation/Degradation aromatic compounds |
| 5     | 1          | 1.2.1.32  | Aminomuconate-semialdehyde dehydrogenase | Tryptophan metabolism |

(Continued)
| Box no | Present (h) | EC number | Classification | Pathway |
|--------|------------|-----------|----------------|---------|
| 5      | 1          | 1.13.11.37 | Hydroxyphenol 1,2 dioxygenase | Chlorohexane, chlorobenzene, benzoate degradation/Microbial metabolism in diverse environments |
| 5      | 1          | 4.1.1.55   | 4,5-Dihydroxyphenylalate decarboxylase | Polycyclic aromatic hydrocarbon degradation/ Microbial metabolism in diverse environments |
| 5      | 4          | 1.3.1.3    | 3-Oxo-5-beta-sterol 4-dehydrogenase | Steroid hormone biosynthesis |
| 5      | 4          | 5.5.1.1    | Muconate cycloisomerase | Chlorocyclohexene, chlorobenzene, benzoate, fluorobenzoate, toluene degradation/degradation of aromatic compounds |
| 5      | 5          | 1.14.13.1  | Salicylate 1-monooxygenase | Dioxane, polycyclic aromatic hydrocarbon, naphthalene degradation/Microbial metabolism in diverse environments |
| 5      | 4          | 5.5.1.2    | 3-Carboxy-cis, cis-muconate cycloisomerase | Benzoate degradation/ Degradation of aromatic compounds |
| 5      | 4          | 3.1.1.24   | 3-Oxoadipate enol-lactonase | Benzoate degradation/ Degradation of aromatic compounds |
| 5      | 4          | 1.2.1.7    | Benzaldehyde dehydrogenase | Xylene, toluene, aminobenzoate degradation/Microbial metabolism in diverse environments |
| 5      | 5          | 3.5.99.4   | N-isopropylamidmedic acid isopropyl aminohydrolase | Atrazine degradation |
| 5      | 4          | 1.14.13.20 | 2,4-Dichlorophenol 6-monooxygenase | Chlorocyclohexene and chlorobenzene degradation/Microbial metabolism in diverse Environments |
| 6      | 1          | 1.13.11.2  | Catechol 2,3 dioxygenase | Degradation of aromatic compounds/Microbial metabolism in diverse environments |
| 6      | 1          | 4.1.1.47   | Tartarate-semialdehyde synthase | Glyoxylate and dicarboxylate metabolism |
| 6      | 1          | 1.2.1.31   | L-amino adipate-semialdehyde dehydrogenase | Lysine biosynthesis and degradation/ Biosynthesis of amino acids |
| 6      | 4          | 4.1.1.8    | Oxalyl-CoA decarboxylase | Glyoxylate and dicarboxylate metabolism |
| 6      | 4          | 2.6.1.44/2.6.1.40 | Alanine-glyoxylate transaminase | Amino acid metabolism and degradation |
| 6      | 4          | 5.1.99.1   | Methylmalonyl-CoA | Valine, leucine, isoleucine degradation/Propanoate metabolism/ Carbon metabolism |
| 6      | 4          | 1.3.1.32   | Maleylactetate reductase | Chlorocyclohexene, chlorobenzene, benzoate, fluorobenzoate, and toluene degradation/microbial metabolism in diverse environments |
| 7      | 1          | 2.6.1.11/2.6.1.17 | Acetylornithine/N-Succinylalamopimelate aminotransferase | Biosynthesis of amino acids/ 2-oxocarboxylic acid metabolism |
| 7      | 1          | 1.3.99.12  | Short/branched chain acyl-CoA dehydrogenase | Valine, leucine, and isoleucine degradation |
| 7      | 1          | 2.3.1.178  | L-2,4-diaminobutyric acid acetyl transferase | Glycine, serine, and threonine metabolism |
| 7      | 1          | 3.5.3.4    | Allantoicase | Purine metabolism/Microbial metabolism in diverse environments |
| 7      | 1          | 6.3.4.16   | Carbamoyl-phosphate synthase (ammonia) | Biosynthesis amino acids/Nitrogen and carbon metabolism |
| 7      | 1          | 6.3.2.5    | Phosphopantothenate cysteine ligase | Pantotholate and CoA biosynthesis |
| 7      | 4          | 4.2.1.33   | 3-Isopropylmalate dehydratase | Amino acid biosynthesis/ 2-oxocarboxylic acid metabolism |
| 7      | 4          | 3.5.1.54   | Allophanate hydrolase | Arginine biosynthesis/Atrazine degradation/Microbial metabolism in diverse environments |
| 7      | 4          | 1.4.3.4    | Monoamine oxidase | Amino acid metabolism/Isoquinolone alkaloid biosynthesis/Biosynthesis of secondary metabolites |

bacteria. The main functions seen were broadly within the COG categories metabolism, information, storage and processing, and cellular processes and signaling with gene abundances in all three of these categories being higher at 4 h of colonization compared with abundances at 1 h of colonization. Specifically, amino acid, carbohydrate, and lipid storage and transport were the main functionalities demonstrated by the attached bacteria (all residing within the function metabolism). Most of the genes within amino acid, carbohydrate and lipid storage and transport functional categories were increased in abundance during secondary colonization. KEGG pathway analysis showed that most pathways were present within PRG attached bacteria following both 1 and 4 h of rumen incubation, thus this coupled with the COG abundance data suggests that secondary colonization events is associated largely with increases in abundance of genes present during primary colonization. These increases correlate with increases in the genera Acidaminococcus, Bacillus, Butyrivibrio, and Prevotella suggesting that these are the bacteria responsible for the increase in amino acid, carbohydrate and lipid metabolism seen during secondary colonization. Acidaminococcus are asaccharolytic but have the capacity of producing ammonia (Eschenlauer et al., 2002). Butyrivibrio
FIGURE 5 | Score plot of principal components PC 1 vs. PC 2 for plant material from which the attached microbes had been removed. Score data sets are of 60 spectra from three analytical replicates and at least two spectral analyses. Circles indicate clusters.

FIGURE 6 | Score plot of principal components PC 2 vs. PC 3 for plant material from which the attached microbes had been removed. Score data sets are of 60 spectra from three analytical replicates and at least two spectral analyses. Circles indicate clusters.
spp. are also known for their proteolytic, biohydrogenating, and carbohydrate degradation within the rumen context (Hobson and Stewart, 1997; Krause et al., 2003). Rumen Prevotella spp. are often referred to as amylolytic and proteolytic, but they also have carbohydrate metabolic capacity (Gardener et al., 1995; Krause et al., 2003; Accetto and Avguštin, 2015; Kishi et al., 2015). Interestingly, the number of COG carbohydrate families classifying as cellulases was on average only 3.2% of the total normalized reads within those sequences classified as COG families involved in carbohydrate metabolism. Conversely in the study by Hess et al. (2011), 23% of the glycosyl hydrolases identified in the switchgrass attached bacteria post 24 h of rumen incubation were putative cellulases. The sequencing depth in the study by Hess et al. (2011) was 268 GB whereas we obtained on average 0.9 GB which may explain the differential in identifiable cellulases, coupled with the use of different forages substrates. Nonetheless, it is more likely a consequence of the fact that Hess et al. (2011) harvested bacteria from 24 h incubations when fermentation will be at a very advanced state compared to our study. Indeed, DM degradation in this study show that by 24 h 76% of the plant material was degraded as compared to 35% at 8 h of incubation. Irrespective, our data is suggestive that changing the cell wall characteristics of the plant material to focus on decreasing recalcitrance of structural carbohydrates may allow more efficient breakdown of the cell wall and increase speed of bioavailability of intra-plant nutrients to the microbes and the ruminant. Conversely, developing novel strategies to increase the cellulases, particularly endocellulase, capacity of the microbiota may result in more efficient breakdown of the cell wall and increases speed of bioavailability of intra-plant nutrients to the microbes and the ruminant.

Multivariate analysis of the FT-IR spectra corroborated the metagenetic data by showing increases in carbohydrates, amino acids, and lipid metabolism from primary to secondary colonization. The plant protein and lipid changes may have occurred within the plant itself irrespective of having an attached microbial community as we know that fresh forage is capable of degrading its own protein and lipids within the first 2 h of ruminal incubation (Kingston-Smith et al., 2003, 2008; Lee et al., 2004). A recent publication by Kingston-Smith et al. (2013) used FT-IR to investigate the metabolite fingerprint of the interactome generated during colonization of fresh PRG. In that work, richness of the spectra derived from a combination of metabolic activities of plant and bacterial chemistries (forage plus attached bacteria) meant that analysis of the resultant metabolite profiles did not demonstrate clear differences between 2 and 4 h although a slight change from 8 h onwards was noted. Hence, the results reported here further our understanding of forage degradation by illustrating the changes in plant chemistry that are specifically associated with sequential microbial colonization events when fresh forage is incubated under rumen-like conditions.

In conclusion, the data obtained in this study illustrate that temporal changes in the diversity of bacteria attached to PRG, between primary (up to 4 h) and secondary (post 4 h) colonization events, correlate with increases in amino acid, carbohydrate and lipid storage and transport functional capacity. This data suggests that these changes in gene abundance result in increased metabolism of plant amino acids, carbohydrates and lipids during secondary colonization events. The data suggests that the capacity of the rumen microbes to degrade the more recalcitrant components of the plant cell wall may be the rate limiting factor in increasing bioavailability of nutrients to the microbes and ultimately the ruminant during the first 4 h post-ingestion. Future strategies to increase ruminant nutrient use efficient should investigate the benefits of reducing the recalcitrant nature of the plant cell wall and/or increasing the cellulolytic capacity of the rumen microbiome within early colonization events in particular.

**AUTHOR CONTRIBUTIONS**

SH, EK, AK, MT, and CN conceived the project. OM completed the laboratory work under supervision of SH and EK. TW, MH and SH completed the sequencing and downstream analysis of the sequences. SH wrote the paper with input from all co-authors. GA helped OM with FT-IR analysis.

**FUNDING**

We acknowledge funding from COLCIENCIAS, CORPOICA (Colombia) and the Biological Sciences Research Council, UK via grant number BB/10013/1; BBS/E/W/10964A-01. SH is also funded 75% by the Coleg Cymraeg Cenedlaethol.

**ACKNOWLEDGMENTS**

We are also grateful to Mark Scott for his technical assistance in setting up the experiments.

**SUPPLEMENTARY MATERIAL**

The Supplementary Material for this article can be found online at: http://journal.frontiersin.org/article/10.3389/fmicb.2016.01854/full#supplementary-material

Supplementary Table 1 | Average sequence summary information obtained from attached primary bacterial biofilm communities over all time points.

Supplementary Figure 1 | Representative PCR-DGGE derived un-weight pair group method with arithmetic mean (UPGMA) dendograms showing temporal attached bacterial diversity in the presence of fresh perennial ryegrass for experimental replicate 1 (Replicate 2 and 3 showed very similar results). The numbers represent the different incubation times and scale relates to percent similarity.

Supplementary Figure 2 | Rarefaction curve showing metagenomic sequencing depth obtained for each sample.

Supplementary Figure 3 | FT-IR normalized spectra showing the change in signal intensity (absorbance) for decolonized plant material (plant material with the attached microbes removed) as function of incubation time. Spectral data are from 60 spectra from three analytical replicates and at least two spectral analyses.
Payne, R. W., Murray, D. A., Harding, S. A., Baird, D. B., and Soutar, D. M. (2007). *GenStat® for Windows™ 9th Edition, Introduction*. Hemel Hempstead, UK: VSN International.

Piao, H., Lachman, M., Malfatti, S., Sczyrba, A., Knierim, B., Auer, M., et al. (2014). Temporal dynamics of fibrolytic and methanogenic rumen microorganisms during in situ incubation of switchgrass determined by 16S rRNA gene profiling. *Front Microbiol.* 5:307. doi: 10.3389/fmicb

Russell, J. B., and Rychlik, J. L. (2001). Factors that alter rumen microbial ecology. *Science* 292, 1119–1122. doi: 10.1126/science.1058830

Schmitt, J., and Flemming, H.-C. (1998). FTIR-spectroscopy in microbial and material analysis. *Int. Biodeterio Biodegrad.* 41, 1–11. doi: 10.1016/S0964-8305(98)80002-4

Sheng, G.-P., Yu, H. Q., and Wang, C.-M. (2006). FTIR-spectral analysis of two photosynthetic H₂-producing strains and their extracellular polymeric substances. *Appl. Microbiol. Biotechnol.* 73, 204–210. doi: 10.1007/s00253-006-0442-2

Van Soest, P. J. (1967). Development of a comprehensive system of feed analyses and its application to forages. *J. Anim. Sci.* 26, 119–128. doi: 10.2527/jas1967.261119x

**Conflict of Interest Statement:** The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Copyright © 2016 Mayorga, Kingston-Smith, Kim, Allison, Wilkinson, Hegarty, Theodorou, Newbold and Huws. This is an open-access article distributed under the terms of the Creative Commons Attribution License (CC BY). The use, distribution or reproduction in other forums is permitted, provided the original author(s) or licensor are credited and that the original publication in this journal is cited, in accordance with accepted academic practice. No use, distribution or reproduction is permitted which does not comply with these terms.