Running Title: Prepartum and Postpartum Rumen Microbiome

Prepartum and postpartum rumen microbiomes correlate with production traits in dairy cows

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Abstract

Microbes present in the rumen of dairy cows are essential for degradation of cellulosic and non-structural carbohydrates of plant origin. The prepartum and postpartum diets of high-producing dairy cows are substantially different, but in what ways the rumen microbiome changes in response and how those changes may influence production traits are not well elucidated. Here, we sequenced the 16S and 18S rRNA genes using the MiSeq platform to characterize the prepartum and postpartum rumen fluid microbiomes in 115 high-producing dairy cows, including both primiparous and multiparous animals. Discriminant analysis identified differences between the microbiomes of prepartum and postpartum samples and between primiparous and multiparous cows. 18S rRNA sequencing revealed an overwhelming dominance of the protozoan class Litostomatea, with over 90% of the eukaryotic microbial population belonging to that group. Additionally, fungi were relatively more prevalent and Litostomatea relatively less prevalent in prepartum samples compared with postpartum ones. The core rumen microbiome (common to all samples) consisted of 64 bacterial taxa, of which members of the genus *Prevotella* were the most prevalent. The Chao1 diversity index was greater for prepartum multiparous cows than for postpartum multiparous cows. Multivariable models identified bacterial taxa associated with increased or reduced milk production, and general linear models revealed that a metagenomic-based prediction of productivity is highly associated with production of actual milk and milk components. In conclusion, the structure of the rumen fluid microbiome shifts between the prepartum and postpartum periods and its profile can accurately predict production traits.

Keywords: microbiome/milk production/prepartum/postpartum/rumen.

Introduction

High-throughput sequencing technologies have opened new frontiers in microbial analysis by allowing cost-effective characterization of complex microbial communities in biological samples, and have significantly improved our knowledge of bovine rumen microbial diversity. Over 27,000 carbohydrate-active genes, 50 proteins with enzymatic activity against cellulosic substrates, and 15 uncultured microbial genomes were revealed in a study of rumen samples using high-throughput sequencing (Hess et al, 2011). Diet can be a significant factor shaping the microbial diversity of the rumen content of dairy cows (de Menezes et al, 2011) and beef cows (Petri et al, 2013). Variation in the rumen microbiome of dairy cattle has also been linked to levels of methane emission (Ross et al, 2013a), and metagenomic profiling of the rumen microbiome can actually be used to predict phenotypes related to enteric methane gas production (Ross et al, 2013b).

Jami & Mizrahi (2012) suggested the presence of a core rumen microbiome, but also reported significant variability in bacterial genera abundances among animals. Using 454 pyrosequencing of ruminal metagenomic DNA they described the bacterial communities across five different age groups (from 1-day-old calves to 2-year-old cows) (Jami et al, 2013). The same group of researchers recently showed the potential role of the bovine rumen microbiome in modulating milk composition (Jami et al, 2014). They were able to identify connections between milk fat yield and the Firmicutes to Bacteroides ratio. Interesting correlations were also present at the genus level. However, only 15 primiparous animals, one diet, and one sample per animal were used in that study, suggesting that additional work evaluating variation across diets and animals might improve the characterization of potential relationships between the rumen microbiome and production traits.

The transition period (usually defined as the three weeks before and the three weeks after calving) is undeniably the most challenging period for a high-producing Holstein dairy

cow. During these six weeks, the cow undergoes physiological stress as she prepares for and then recovers from parturition, dramatically altering her metabolism so as to supply the mammary gland with nutrients necessary for milk synthesis, while often consuming insufficient dry matter that leads to negative energy balance and immunosuppression. Adaptation of the rumen microbiota to dietary changes during this period is of paramount importance and is best elucidated with the use of metagenomic tools. Koren et al, (2012) showed dramatic changes in pregnant women's gut microbiota and suggested the existence of important host-microbial interactions that impact host metabolism during pregnancy. Similar findings await description in dairy cattle. In this study, we characterize the rumen microbiomes of prepartum and postpartum high-producing Holstein cows and investigate their associations with productivity.

Materials and methods

Animal handling, data and sample collection

The experimental procedures used in this study were reviewed and approved by the Institutional Animal Care and Use Committee of Cornell University (Protocol number: 2013-0082). The study was conducted at a single commercial dairy farm milking 2,800 Holstein cows near Ithaca, NY, USA. One week before the expected calving date and one week after parturition, rumen fluid samples were collected from primiparous (n = 48) and multiparous (n = 67) cows. We opted to sample the rumen using a non-invasive procedure with the aid of a scientifically evaluated and commercially available oro-ruminal sampling device (Flora Rumen Scoop, profs-product, Guelph, Canada)(Geishauser et al, 2012). After sample collection, an aliquot (50 mL) was stored in a sterile conical tube and kept on ice until transported to the laboratory in Ithaca, NY, where samples were preserved in a -80°C freezer.

Data regarding daily milk yield were recorded using the DeLaval ALPRO™ milk point controller 780 (Kansas City, MI, USA) and later data were retrieved from DairyComp 305 (Tulare, CA, USA) database. Daily milk production for each cow was averaged to a weekly basis, and milk fat and protein percentages were recorded on a monthly basis. Quartiles for average milk production, and milk fat and protein percentages for the first 150 days postpartum were determined for all cows and later used as ordinal categorical data in the statistical models.

Prepartum cows were fed a diet with high fiber (NDF = 38.2%, ADF = 43.3%) and low energy density (1.39 Mcal/kg), whereas postpartum cows were fed a diet of low fiber (NDF = 24.1%, ADF = 30.1%) and high energy density (1.69 Mcal/kg) derived from higher starch and fat supplementation (Supplementary Table S1).

DNA extraction

Rumen fluid samples were thawed and homogenized by vortexing for 3 min. A 1-ml aliquot of each rumen fluid sample was centrifuged for 10 min at room temperature at 13,200 rpm (16,100 rcf) in an Eppendorf 5415R centrifuge. The supernatant was discarded and the remaining pellet was resuspended in 400 ml of nuclease-free water. Isolation of genomic DNA was performed by using a QIAamp DNA minikit (Qiagen) according to the manufacturer's instructions, except that 400 mg of lysozyme was added to the bacterial suspension and incubated for 12 h at 56°C to maximize bacterial DNA extraction. DNA concentration and purity were evaluated by optical density using a NanoDrop ND-1000 spectrophotometer (NanoDrop Technologies, Rockland, DE, USA) at wavelengths of 230, 260 and 280 nm.

DNA amplification, purification, and quantification of the 16S rRNA and 18S rRNA genes

The 16S rRNA and 18S rRNA genes were amplified by PCR from individual metagenomic DNA samples using barcoded primers. For amplification of the V4 hypervariable region of the bacterial/archaeal 16S rRNA gene, primers 515F and 806R were used accordingly to a previously described method (Caporaso et al, 2012) optimized for the Illumina Miseq platform. Likewise, for amplification of the V9 hypervariable region of the 18S rRNA gene (Amaral-Zettler et al, 2009), primers 1391F and 1510R were used following (Caporaso et al, 2012) optimized for the Illumina Miseq platform. The earth microbiome project (http://www.earthmicrobiome.org/; (Gilbert et al, 2010) was used to select 140 different 12-bp error-correcting Golay barcodes for the 16S rRNA gene and another 140 different 12-bp error-correcting Golay barcodes for 18S rRNA gene, as described by (Caporaso et al, 2012). The 5'-barcoded amplicons were generated in triplicate using 12–300 ng DNA template (isolated from rumen samples), 1× GoTaq Green Master Mix (Promega, Madison, WI), 1 mM $MgCl_2$, and 10 μM of each primer. The PCR conditions for the 16S rRNA gene consisted of an initial denaturing step of 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 50°C for 1 min, and 72°C for 90 s, and the final elongation step of 72°C for 10 min. The PCR conditions for the 18S rRNA gene consisted of an initial denaturing step of 94°C for 3 min, followed by 35 cycles of 94°C for 45 s, 57°C for 1 min, and 72°C for 90 s, and the final elongation step of 72°C for 10 min. Replicate amplicons were pooled and purified with a QIAquick PCR Purification Kit (Qiagen, Valencia, CA, USA), and visualized by electrophoresis through 1.2% (wt/vol) agarose gels stained with 0.5 mg/ml ethidium bromide before sequencing. Blank controls, in which no DNA was added to the reaction, were performed. In all cases these blank controls failed to produce visible PCR products; these samples were not analyzed further. Purified amplicon DNA was quantified using the Quant-iT™ PicoGreen® dsDNA Assay Kit (Life Technologies Corporation, Carlsbad, CA, USA).

137

138 *Sequences library analysis and statistical analysis*

139 Amplicon aliquots were standardized to the same concentration and then pooled into
140 one of three different runs (140 samples per run) according to individual barcode primers of
141 the 16S rRNA gene. The same procedure was conducted for the 18S rRNA amplicons. Final
142 equimolar libraries were sequenced using the Miseq reagent kit V2-300 cycles on the MiSeq
143 platform (Illumina, Inc., San Diego, CA, USA). The 16S rRNA and 18S rRNA gene
144 sequences obtained from the MiSeq platform were processed through the open source
145 software pipeline Quantitative Insights Into Microbial Ecology (QIIME) version 1.7.0-dev
146 (Caporaso et al, 2010). Sequences were filtered for quality using established guidelines
147 (Bokulich et al, 2013). Sequences were binned into Operational Taxonomic Units (OTUs)
148 based on 97% identity using UCLUST (Edgar, 2010) against the Greengenes reference
149 database (McDonald et al, 2012) May 2013 release. Low-abundance clusters were filtered
150 and chimeric sequences were removed using USEARCH (Edgar, 2010). Representative
151 sequences for each OTU were compared against the Greengenes database for taxonomy
152 assignment and only full-length, high-quality reads (*-r*=0) were used for analysis.

153 The OTU results obtained from the analysis above were used to determine the core
154 microbiome for the prepartum and postpartum periods. The core microbiome was defined as
155 all taxa found to be ubiquitous across all samples. A multivariable model was built using JPM
156 Pro 11 (SAS Institute Inc., NC) to evaluate correlations between bacterial taxa in the core
157 microbiome at the prepartum and postpartum periods. Using the obtained OTU information,
158 we evaluated each sample's richness using the Chao1 index, which is a nonparametric
159 estimator of the minimum richness (number of OTUs) and is based on the number of rare
160 OTUs (singletons and doublets) within a sample. The Chao1 index means (± SD) were then

compared using a general linear model with JMP Pro 11 with time relative to calving, parity and milk quartiles as independent variables.

The prevalences of different bacterial taxa in each sample were used as covariates in stepwise discriminant analysis models built in JMP Pro 11. Variables were removed in a stepwise manner until only variables with a P value < 0.001 were retained in the final model. *Time relative to calving* and *parity* were used as categorical variables. In this way, differences in microbiome structure during the transition period of primiparous and multiparous cows were illustrated. A series of analyzes was performed to investigate how prepartum and postpartum microbial diversity relates to production traits. A screening analysis using JMP Pro 11 was performed to determine which bacterial taxa were associated with increased or decreased average milk production, and with average milk fat and protein percentages for the first 150 days in milk stratified by period relative to calving and by parity. Linear correlation matrixes (Pearson correlation coefficient) were generated to illustrate the level of correlation of the bacterial taxa selected by the screening model and the weekly milk averages. Metagenomic-based production predictions were estimated using multivariable generalized linear mixed models using JMP Pro 11; bacterial taxa that were found to be significantly associated with milk production (P value < 0.001) based on the variable screening model were offered to the model as independent variables and the variable of interest was the repeated weekly measurements of milk production. To control for repeated measures, the variable "animal identification" was included in the models as a random variable. Similar models were built for monthly average of milk fat percent and milk protein percent for the first 5 months following parturition.

Results

Sequencing results, core microbiome description, and genera prevalence

Quality-filtered reads for 16S sequences were demultiplexed, yielding 24,863,354 sequences in total with a median sequence length of 301 bases per read, and an average coverage of 108,102 sequences per sample. Similarly, quality-filtered reads for 18S sequences were demultiplexed, yielding 22,592,149 sequences in total with a median sequence length of 129 bases per read, and an average coverage of 98,226 sequences per sample.

The rumen fluid core microbiome was composed of 64 bacterial taxa. The core microbiome represented 89.6% and 91.2% of all bacterial genera present in the rumen in the prepartum and postpartum periods, respectively. The mean prevalence of each bacterial taxon present in the core microbiome is illustrated in Supplementary Table S2, and the prevalences of core microbiome bacterial genera for all cows are illustrated in supplementary Figure S1 (prepartum) and supplementary Figure S2 (postpartum). Taxa that could not be assigned to a genus but were present in all samples are still displayed based on the highest taxonomic level that could be assigned to them. Data analysis identified 2,132 different bacterial species; however, these represented only 46% of the sequences identified for all samples and therefore they were not included in further models to determine the core microbiome and associations with production traits. Twelve bacterial species that had an average prevalence of 1% and were consistently the most prevalent among prepartum and postpartum multiparous and primiparous cows are depicted in supplementary Figure S3.

The core microbiome in the prepartum period was predominantly composed of *Prevotella* (19.5% ± 0.82), Ruminococcaceae 2 (7.3% ± 0.21), Bacteroidales (7.2% ± 0.21), Lachnospiraceae *2* (5.4% ± 0.16), *Ruminococcus* (4.8% ± 0.18), Clostridia 2 (4.1%± 0.17), Clostridiales 2 (3.5% ± 0.12), Christensenellaceae (3.3% ± 0.16), Bacteroidales 2 (3.2% ± 0.08) and *Succiniclasticum* (3.1% ± 0.12). In comparison, the core microbiome in the postpartum period consisted of predominantly *Prevotella* (21.3% ± 1.20), Ruminococcaceae

2 (8.0% ± 0.34), *Ruminococcus* (7.3% ± 0.38), Bacteroidales (5.7% ± 0.24), Lachnospiraceae 2 (5.7% ± 0.16), Clostridia 2 (3.8%± 0.15), family S24-7 (3.8% ± 0.02), *Succiniclasticum* (3.4% ± 0.17), Clostridiales 2 (2.9% ± 0.11), and Bacteroidales 2 (2.7% ± 0.24). The prevalence of each bacterial phylum for each sample evaluated is depicted in Figure 1. Twenty-eight phyla were identified in at least 20 samples across the prepartum and postpartum samples, and 13 phyla composed the core microbiome. The two major phyla present in rumen samples were Firmicutes and Bacteroidetes. The mean prevalences of Firmicutes for the prepartum-primiparous, prepartum-multiparous, postpartum-primiparous, and postpartum-multiparous samples were 45.1%, 42.5%, 49.65% and 42.8%, respectively. The mean prevalences of Bacteroidetes for the prepartum-primiparous, prepartum-multiparous, postpartum-primiparous, and postpartum-multiparous samples were 36.9%, 38.4%, 33.6% and 40.7%, respectively. Other major phyla with prevalences over 1% include Verrucomicrobia, Euryarchaeota, Tenericutes and Proteobacteria. The mean prevalences of eukaryotic organisms based on 18S sequencing are presented in Figure 2. The protozoan class Litostomatea was the dominant eukaryotic taxon, its members accounting for more than 90% of the eukaryotes present in the rumen samples. An unclassified-metazoan OTU was the second most prevalent eukaryotic taxon, followed by a series of fungi (Saccharomyceta, Unclassified-Fungi, Agaricomycotina, Neocallimastigales) and a few other protozoa (Unclassified-Ciliophora, Unclassified-Intramacronucleata and Unclassified-Alveolata). Litostomatea and Unclassified-Alveolata prevalence decreased from the prepartum to postpartum period, whereas Unclassified-Metazoa, *Saccharomyceta,* Unclassified-Fungi, Agaricomycotina, Neocallimastigales, Mitosporic fungi, Pucciniomycotina, Unclassified-Basidiomycota and Unclassified-Parabasalia increased in prevalence over the same transition. In general, the fungal types identified showed variation similar to that of the Unclassified-Metazoa, having an increased prevalence from the prepartum to the postpartum period.

236

237 *Discriminant analysis results*

238 Differences in rumen microbial diversity between the prepartum and postpartum
239 periods are mainly illustrated by Canonical 1 (Figure 3), whereas differences between
240 primiparous and multiparous cows are mainly illustrated by Canonicals 2 and 3 (Figure 3).
241 The canonical scores for each bacterial taxon used to discriminate rumen microbiomes
242 according to period relative to calving and primiparous cows from multiparous cows are
243 presented in Figure 4.

244

245 *Richness indexes and association of the Firmicutes-Bacteroidetes ratio with production traits*

246 Chao1 index means for pre- and postpartum samples for multiparous and primiparous
247 cows stratified by milk production quartiles are illustrated in Figure 5. The Chao1 index
248 dropped significantly between the prepartum and postpartum periods in multiparous cows for
249 both the lower milk production quartile (1) and the higher milk production quartile (4).

250 The Firmicutes-Bacteroidetes ratio for cows within milk quartile 2 was significantly
251 higher in primiparous-postpartum cows versus multiparous-prepartum cows (Figure 6).
252 Likewise, the Firmicutes-Bacteroidetes ratio for cows within milk quartile 4 was significantly
253 higher for primiparous-postpartum cows compared to multiparous-prepartum, multiparous-
254 postpartum and primiparous-prepartum cows (Figure 6). The Firmicutes-Bacteroidetes ratio
255 was not correlated with milk fat percentage (Pearson R = -0.03, *P* value = 0.38) or milk
256 protein percentage (Pearson R = -0.83, *P* value = 0.40).

257

258 *Metagenomic-based production traits*

259 Bacterial taxa associated with either increased or reduced average milk production for
260 the first 150 days postpartum were obtained from screening analyses performed according to

the period relative to calving and parity. Those bacterial taxa were used in a multivariable model to evaluate correlations between the prevalence of these bacterial taxa and weekly average milk yield for the first 12 weeks postpartum. Primiparous-prepartum microbiome correlation patterns varying from -0.60 (negative correlation with milk production) to 0.50 (positive correlation with milk production) are illustrated in Figure 7 A. The bacterial taxon Micrococcaceae was consistently the most positively correlated with weekly milk production throughout the first 12 weeks postpartum, whereas *Ureibacillus* was the most negatively correlated throughout the same period (Figure 7 A). A similar pattern was observed for the primiparous-postpartum microbiome, the correlation varying from -0.60 to 0.60, with Deltaproteobacteria being the most negatively correlated with weekly average milk production and Erysipelotrichaceae the most positively correlated (Figure 7 B). Likewise, bacterial taxa in samples from multiparous-prepartum cows showed correlations with weekly milk production throughout the first 12 weeks postpartum that ranged from -0.60 to 0.40, with *Faecalibacterium* and *Virgibacillus* being the most negatively and most positively associated, respectively (Figure 8 A). Lastly, the multiparous-postpartum microbiome correlations also ranged from -0.60 to 0.40, with Prevotellaceae 2 being the most positively correlated with weekly milk production throughout the first 12 weeks postpartum, and R4-41B the most negatively correlated (Figure 8B).

Additionally, a multivariable regression model was built that used bacterial taxa significantly associated with average milk production in the first 150 days postpartum to predict weekly average of milk production compared to actual milk production. The microbiome-predicted milk production according to period relative to calving and parity was significantly correlated with actual weekly averages of milk production as illustrated in Figures 9A to 9D. Similar models were built for milk fat percent and milk protein percent and added to our supplemental data (Supplementary Figures S4 and S5).

A final multivariable model was built to evaluate correlations between the prepartum and postpartum core microbiomes for the most prevalent bacteria, and revealed strong correlations between the predominant core bacterial genera before and after parturition (Figure 10).

Discussion

We showed here that differences exist between the prepartum and postpartum rumen microbiomes in primiparous and multiparous Holstein cows and that these differences can be used to predict, with relatively high accuracy, certain production traits. Rumen microbes have an essential role in the deconstruction of plant lignocellulosic material (Hess et al, 2011) by enabling cows to harness the solar energy stored in plant fibers via their conversion into milk and meat, both important sources of high-quality protein and energy for human consumption. The transition from a prepartum high-fiber, low-energy diet to a postpartum low-fiber, high-energy diet represents the most common feeding scenario on dairy farms with high-producing dairy cows, and understanding its effects on the rumen microbiome and potential relationships with production is of great interest.

Use of the MiSeq Illumina sequencing platform generated a great number of sequences per read (108,102), exceeding the 80,000 sequences per sample estimated to be required for full coverage of all OTUs in rumen samples across different diets (Jami & Mizrahi, 2012). Indeed, 88.3% of all samples evaluated in the present study were above the threshold of 80,000 sequences per read, representing increased coverage and depth than those of previous studies that used 454 Roche pyrosequencing (16,000 to 36,000 sequences per sample; Jami & Mizrahi, 2012; Li et al, 2012; Petri et al, 2013).

Prepartum and postpartum rumen samples were readily distinguished by discriminant analysis based on bacterial profiles (Figure 3). These results are comparable to recent

findings describing rapid alterations of the gut microbiome in humans (David et al, 2014) and cattle (Petri et al, 2013) in a diet-dependent manner. Many well-known cellulolytic, amylolytic and acidophilic bacteria (*Fibrobacter, Ruminobacter, Selenomonas, Butyrivibrio, Succinivibrio*) were significant in discriminating the prepartum from the postpartum microbiome. Other significant bacteria distinguishing these two microbiomes were uncultured-unidentified rumen bacterial clones *YRC22* and *RFP12* and, previously unreported in rumen, bacteria such as *Solibacillus* and *Sporanaerobacter,* all with completely unknown and unexplored functions in rumen physiology. Bacteria from the family Christensenellaceae have previously been reported in human feces; these are strictly anaerobic, non-motile, non-spore-forming, gram-negative species which produce acetic acid and a small amount of butyric acid as fermentation end-products (Morotomi et al, 2012). Considering the high significance that Christensenellaceae had in our discriminant analysis model, it is likely that these bacteria play an important role in rumen dynamics, and their further investigation is warranted.

Discriminant analysis models also revealed that rumen samples derived from primiparous cows were readily distinguished from multiparous cows based on their microbiomes (Figure 3). A clear age effect on the rumen microbiome was described by Jami et al, (2013), in which diversity and within-group similarity increased with age. Similar results of increased microbial diversity and convergence toward a mature bacterial composition with age were also reported in a study of the gut microbiome of human populations from different geographical locations across different age groups (Yatsunenko et al, 2012). Heifers at one week before the expected calving date are considered as adult animals. However, they are fed a high-fiber, low-energy diet that differs dramatically from the low-fiber, high-energy diet fed to multiparous cows during the previous lactation period. The group of bacteria that largely distinguishes primiparous from multiparous cows is the

amylolytic/acidophilic bacteria (*Butyrivibrio*, *Succinivibrio*, *Selenomonas* and *Ruminobacter*). Nonetheless, some unusual bacterial types also featured in this discrimination, such as the candidate phylum SR1, which includes bacteria found in marine and terrestrial high-temperature environments (Davis et al, 2009), in mammalian digestive tracts (Ley et al, 2008), or in the oral cavity of humans (Dewhirst et al, 2010). Until now, these bacteria were not known to be present in the rumen of dairy cows.

The notion of diet influencing microbial diversity in cattle is a long-standing one, (Hungate et al, 1964) supported more recently by the use of molecular techniques to investigate rumen dynamics, function and the effects of diet (Mackie et al, 2003; Malmuthuge et al, 2012; Petri et al, 2013). As discussed above, use of the MiSeq Illumina platform can propel studies of rumen microbiology even further. Sequencing the 18S rRNA gene allowed us to identify rumen fungal and protozoan species that have also been shown to play important roles in rumen physiology (Bauchop, 1979; William & and Coleman, 1997). We showed here that over 90% of the sequences belonged to the protozoan class Litostomatea*,* ciliated protists that until recently were divided into two groups, the Haptoria and the Trichostomatia (Gao et al, 2008). The Trichostomatia subclass contains one of most studied ruminal protozoan taxa, the *Entodinium spp*., which are able to engulf starch and attach to amylolytic bacteria (Dennis et al, 1983); these protozoans have greater relative abundance in cows fed a low-fiber diet compared to cows fed a high-fiber diet (Carberry et al, 2012). These results are in line with our findings of increased relative abundance of Litostomatea in the postpartum period, corresponding to a low-fiber, high-energy diet. Also in line with our findings was the consistently increased abundance of fungal types in the prepartum period compared to the postpartum period. Generally, fungi present in the rumen can penetrate both the cuticle and the cell wall of lignified material, thus playing an essential role in fiber degradation (Hobson & Stewart, 1997).

The concept of a 'core' microbiome developed for the human gut implies that there is a population of microbes that remains stable independent of host genetics and diet; however, deviation from this core population might indicate occurrence of metabolic unbalance and disease (Ley et al, 2006; Turnbaugh et al, 2009; Turnbaugh et al, 2006). The same concept has been recently applied to the bovine rumen (Jami & Mizrahi, 2012; Li et al, 2012; Petri et al, 2013). Jami & Mizrahi (2012) identified 32 genera across 16 cows fed an *ad libitum* diet for many months. Li et al (2012) identified 45 genera that were common to 4 calves being fed milk-replacer. However, a study by Petri et al (2013) found that only the genus *Prevotella* was ubiquitous in 8 heifers fed either a forage diet, a forage-concentrate diet, a concentrate diet, or an acidosis-inducing diet. In our much larger sample population and across two different diets, the core rumen microbiome in the present study is defined by 64 bacterial taxa, suggesting that the description of the core rumen microbiome is perhaps influenced by sequencing coverage and depth. Evidence in support of this possibility comes from the work of Petri et al (2013), who reported prevalences of 32.3% and 43.2% for the two major phyla Bacteroidetes and Firmicutes, respectively, percentages comparable to ours despite differences in animal category, diets and methodology between the two studies. Many of the core rumen microbiome bacterial types identified in the present study belong to these two phyla and could potentially be present in other samples studied by Petri et al (2013). Use of the MiSeq platform allows greater throughput per run and smaller errors rates compared to 454 pyrosequencing, which ultimately leads to greater depth and breadth of coverage and potential identification of higher numbers of microbial genera (Loman et al, 2012; Frey et al, 2014). Petri et al (2013) reported an average of 3,260 to 6,832 sequences per sample depending upon diet/treatment and mentioned that a plateau was not reached for any of the dietary treatments, indicating that additional sequencing would be necessary to fully describe rumen bacterial communities under those conditions.

Recently, Jami et al (2014) reported that milk yield and composition were highly correlated with abundance of various bacterial members of the rumen microbiota. A strong correlation between the Firmicutes to Bacteroidetes ratio and milk-fat yield was shown. Considering the essential role of rumen bacteria in the breakdown of plant polysaccharides (Flint et al, 2008) and that volatile fatty acids produced by this breakdown are a major source of energy and have a direct effect on milk production (Brulc et al, 2009; Hurtaud et al, 1993), it is plausible that rumen microbiome profiles in the prepartum and early postpartum periods help determine production traits. Indeed, our screening analysis revealed that several bacterial taxa in prepartum and postpartum samples were associated with increased or reduced average milk production, milk fat percent, and milk protein percent for the first 150 days postpartum. We built many models using bacterial taxa significantly associated with production traits in an attempt to evaluate correlations between the rumen microbiome and weekly milk production or monthly milk fat and protein percentages. Bacteria significantly correlated with milk production were used to generate microbiome predictions for milk production, milk fat percent and milk protein percent. Although we were unable to replicate the strong correlation between the Firmicutes to Bacteroidetes ratio and milk fat percentage reported by Jami et al (2014), we did identify bacterial groups (stratified by parity and period relative to calving) that are highly correlated with production traits. In general, moderate to high correlations (R square = 0.42 to 0.82) of microbiome predictions for production traits were identified by our models. Some of the bacteria with the highest positive correlation with milk production are well-known rumen bacteria such as *Butyrivibrio* and Prevotellaceae 2, and their role on rumen function is already well described. *Butyrivibrio* undertake biohydrogenation of fatty acids (Polan et al, 1964), which generates conjugated linoleic acid as an intermediate (Kepler et al, 1966). Prevotellaceae 2 is the most prevalent bacterial family in the rumen of adult cattle, and some of the species within this family such as *Prevotella bryantii* when used as

411 probiotics decreased lactate production and increased milk fat percentages during the weeks
412 following inoculation (Chiquette et al, 2008). Conversely, other bacteria with high positive
413 correlations with milk production such as *Micrococcus,* Enterobacteriaceae*,*
414 Erysipelotrichaceae*, Virgibacillus,* Anaeroplasmatales 2, Thermoplasmata, and
415 Rhodobacteriaceae are very poorly characterized or unreported in rumen. Among all
416 production traits, milk production had the highest correlations with bacterial types and could
417 be more accurately predicted by microbiome profiles.
418

419 **Conclusions**

420 As expected, moving from a high-fiber, low-energy diet to a low-fiber, high-energy
421 diet led to a shift of the rumen microbiome. Differences between the prepartum and
422 postpartum rumen microbiomes included different prevalences of classic cellulolytic and
423 amylolytic bacteria coupled with variations in several other bacterial taxa previously
424 uncultured, unreported or with unknown function in the rumen. Moreover, the prepartum
425 microbiome was characterized by increased prevalence of fungi, which then shifted at the
426 immediate postpartum period to a pattern of increased prevalence of protozoa associated with
427 starch digestion. Milk production was predicted with relatively high accuracy by the rumen
428 microbiome; nonetheless, it remains to be determined how microbiome profiles are
429 associated with or indeed shape production traits. Future research will need to investigate the
430 validity of the microbiome predictions of this study across different environments in an
431 integrative manner that incorporates host genetics and metatranscriptomic information of the
432 rumen microbiome.

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436 **Conflict of interest statement**

437 The authors declare no conflict of interest.

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439 Supplementary information is available at ISME website.

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Supplementary information is available at ISME website.

Titles and legend to figures

Figure 1: Aggregate microbiome composition at the phylum level for 16S rRNA sequences according to period relative to calving (prepartum and postpartum) and parity (multiparous and primiparous) for each cow evaluated in the study. The y axis represents the relative abundance of OTUs for all samples evaluated within the specific period relative to calving and parity.

Figure 2 Graph bars illustrating the microbial taxa prevalence for 18S rRNA gene sequences. The mean microbial prevalence according to period relative to calving (prepartum and postpartum) and parity (multiparous and primiparous) is represented by x axis values. Error bars represent standard errors.

Figure 3 Discriminant analysis of rumen microbiome samples. Different OTU prevalences in each sample were used as covariates and *time relative to calving* and *parity* were used as categorical variables. Differences in the ruminal microbial profiles of primiparous (prepartum = red dots, postpartum = green dots) and multiparous (prepartum = blue dots, and postpartum = orange dots) are illustrated by Canonical 1, 2 and 3.

Figure 4 Canonical scores 1, 2 and 3 for bacterial taxa that were found to be significant for the discriminant analysis displayed in Figure 3.

Figure 5 Bar graphs illustrating the mean Chao1 index for different periods relative to calving and milk quartiles for primiparous cows (a) and multiparous cows (b). Error bars represent standard errors. *$P < 0.01$.

578 **Figure 6** Bar graph illustrating the Firmicutes-Bacteroidetes ratio for periods relative to

579 calving, parity and milk quartiles. Error bars represent standard errors. *$P < 0.01$.

580

581 **Figure 7** Heatmaps illustrating correlations between bacterial taxa significantly associated
582 with milk production and weekly average of milk production. The color and intensity of each
583 square represent the value of the correlation between bacteria generally significantly
584 associated with milk production and weekly average of milk production. Panel **a** represents
585 correlations for the primiparous-prepartum cow microbiomes. Panel **b** represents correlations
586 for the primiparous-postpartum cow microbiomes. The letters in front of the bacterial names
587 identify the lowest level of classification (k=kingdom, p=phylum, c=class, o=order, f=family,
588 and g=genus).

589

590 **Figure 8** Heatmaps illustrating correlations between bacterial taxa significantly associated
591 with milk production and weekly average of milk production. The color and intensity of each
592 square represent the value of the correlation between bacteria generally significantly
593 associated with milk production and weekly average of milk production. Panel **a** represents
594 correlations for the multiparous-prepartum cow microbiomes. Panel **b** represents correlations
595 for the primiparous-postpartum cows microbiomes. The letters in front of the bacterial names
596 identify the lowest level of classification (k=kingdom, p=phylum, c=class, o=order, f=family,
597 and g=genus).

598

599 **Figure 9** Linear regression illustration of microbiome predicted milk production and actual
600 milk production. The x axis represents the microbiome-predicted milk production according
601 to bacterial taxa that significantly affected milk production for weekly values, and the y axis
602 represents the actual average of weekly milk production. The legend shows the quartiles of
603 milk production.

604

Figure 10 Heatmap illustrating correlations between the most prevalent core microbiome components prepartum and postpartum. The color and intensity of each square represent the value of the correlation between bacteria observed at the prepartum and postpartum periods. The color legend represents the values of the correlations. The letters in front of the bacterial names identify the lowest level of classification (k=kingdom, p=phylum, c=class, o=order, f=family, and g=genus).

Supplementary information is available at ISME website.

Figure 1.

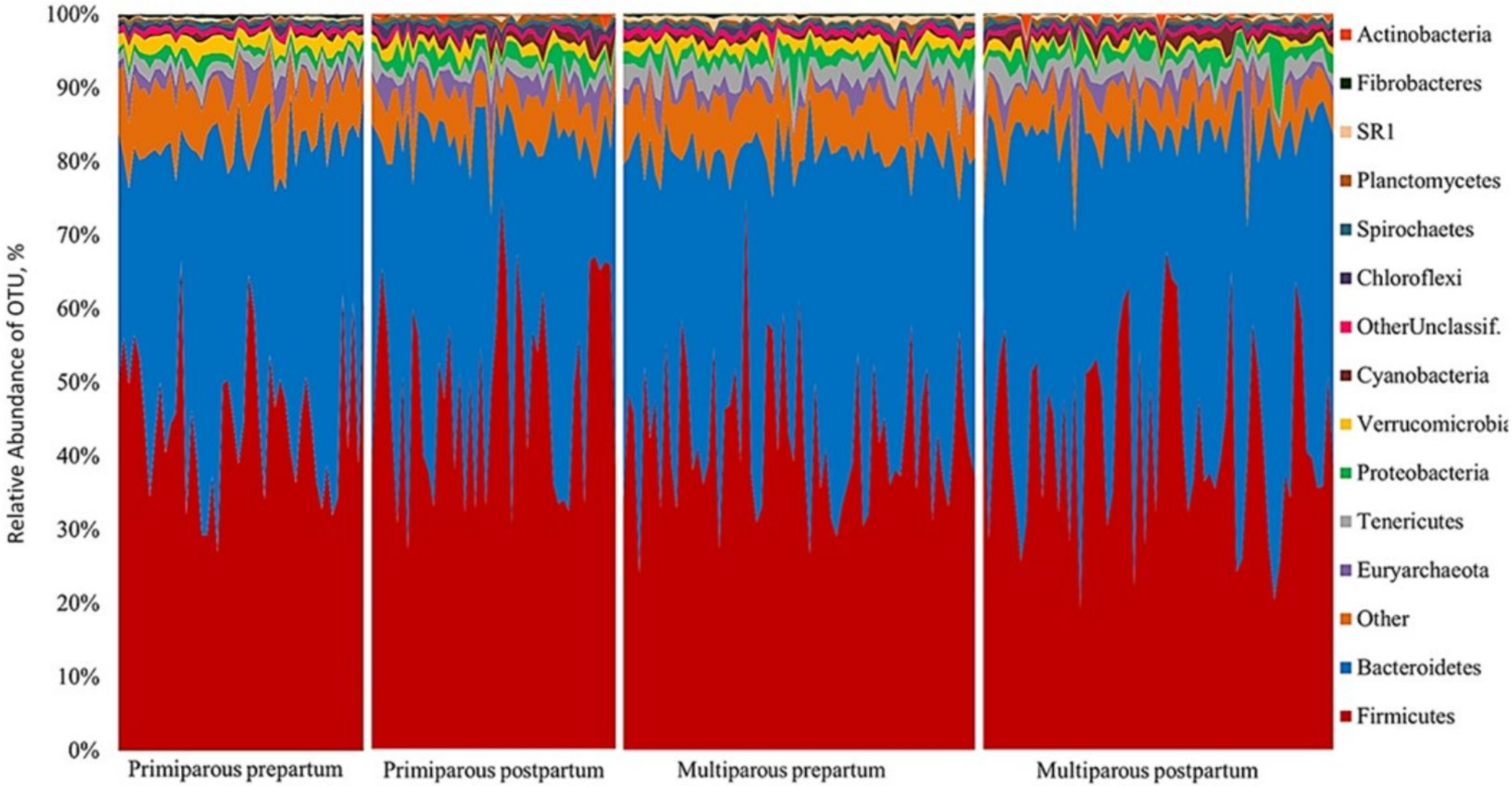

100%
90%
80%
70%
60%
50%
40%
30%
20%
10%
0%
Relative Abundance of OTU, %
Primiparous prepartum
Primiparous postpartum
Multiparous prepartum
Multiparous postpartum
Actinobacteria
Fibrobacteres
SR1
Planctomycetes
Spirochaetes
Chloroflexi
OtherUnclassif.
Cyanobacteria
Verrucomicrobia
Proteobacteria
Tenericutes
Euryarchaeota
Other
Bacteroidetes
Firmicutes

Figure 2.

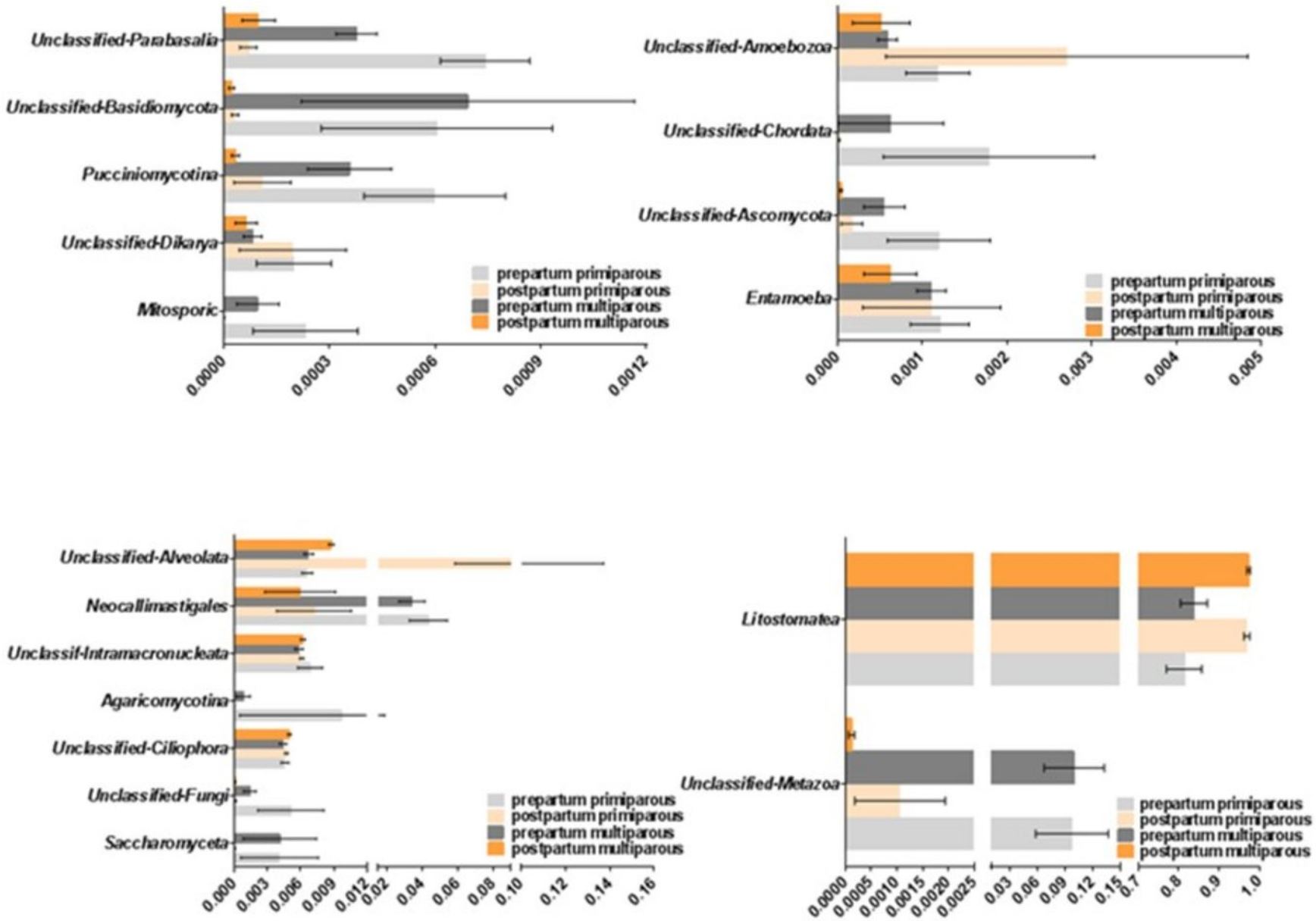

Unclassified-Parabasalia
Unclassified-Basidiomycota
Pucciniomycotina
Unclassified-Dikarya
Mitosporic
prepartum primiparous
postpartum primiparous
prepartum multiparous
postpartum multiparous
0.0000
0.0003
0.0006
0.0009
0.0012
Unclassified-Amoebozoa
Unclassified-Chordata
Unclassified-Ascomycota
Entamoeba
0.000
0.001
0.002
0.003
0.004
0.005
Unclassified-Alveolata
Neocallimastigales
Unclassif-Intramacronucleata
Agaricomycotina
Unclassified-Ciliophora
Unclassified-Fungi
Saccharomyceta
0.000
0.003
0.006
0.009
0.012
0.02
0.04
0.06
0.08
0.10
0.12
0.14
0.16
Litostomatea
Unclassified-Metazoa
0.0000
0.0005
0.0010
0.0015
0.0020
0.0025
0.03
0.06
0.09
0.12
0.15
0.7
0.8
0.9
1.0

Figure 3.

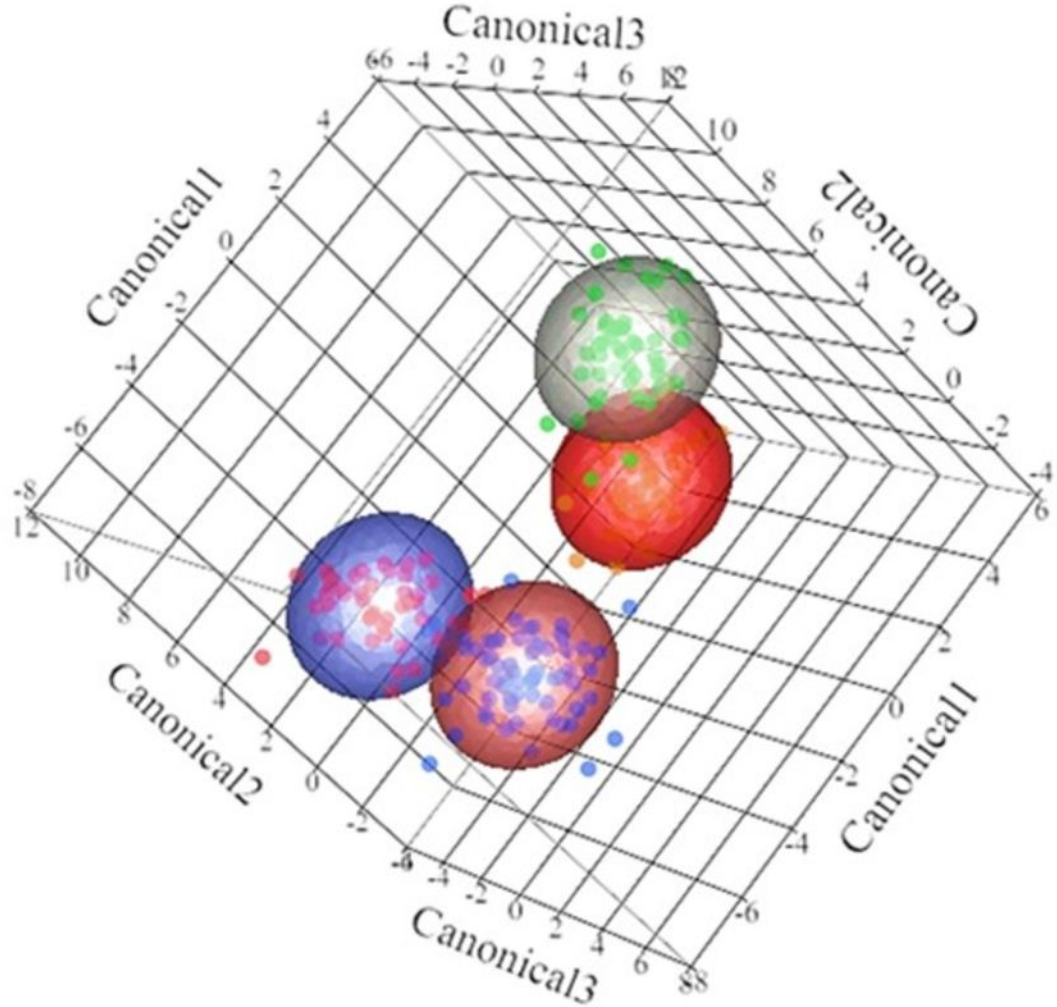

Canonical3
Canonical1
Canonical2
Canonical2
Canonical1
Canonical3

Figure 4.

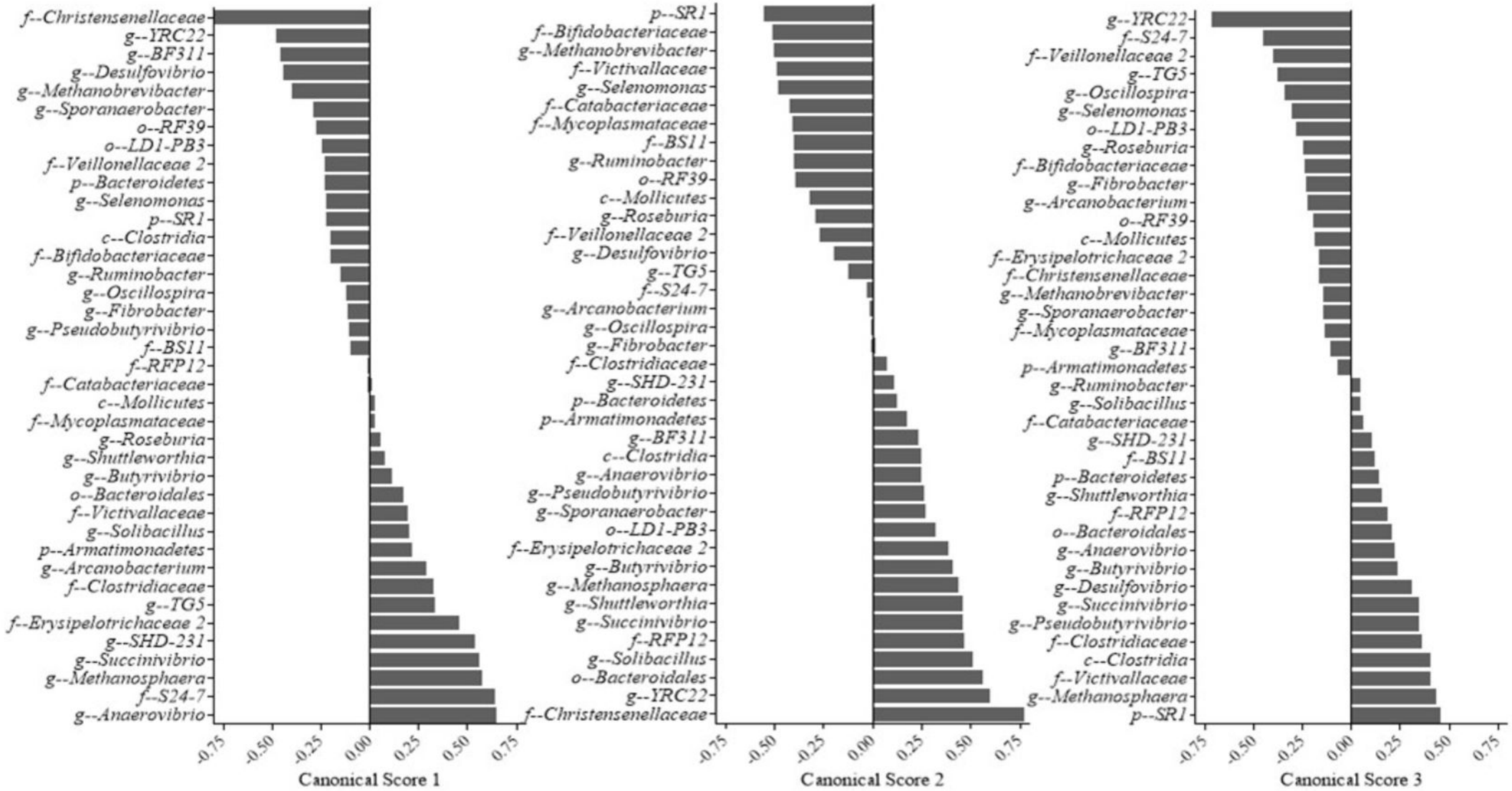

f--Christensenellaceae
g--YRC22
g--BF311
g--Desulfovibrio
g--Methanobrevibacter
g--Sporanaerobacter
o--RF39
o--LD1-PB3
f--Veillonellaceae 2
p--Bacteroidetes
g--Selenomonas
p--SR1
c--Clostridia
f--Bifidobacteriaceae
g--Ruminobacter
g--Oscillospira
g--Fibrobacter
g--Pseudobutyrivibrio
f--BS11
f--RFP12
f--Catabacteriaceae
c--Mollicutes
f--Mycoplasmataceae
g--Roseburia
g--Shuttleworthia
g--Butyrivibrio
o--Bacteroidales
f--Victivallaceae
g--Solibacillus
p--Armatimonadetes
g--Arcanobacterium
f--Clostridiaceae
g--TG5
f--Erysipelotrichaceae 2
g--SHD-231
g--Succinivibrio
g--Methanosphaera
f--S24-7
g--Anaerovibrio
-0.75
-0.50
-0.25
0.00
0.25
0.50
0.75
Canonical Score 1
p--SR1
f--Bifidobacteriaceae
g--Methanobrevibacter
f--Victivallaceae
g--Selenomonas
f--Catabacteriaceae
f--Mycoplasmataceae
f--BS11
g--Ruminobacter
o--RF39
c--Mollicutes
g--Roseburia
f--Veillonellaceae 2
g--Desulfovibrio
g--TG5
f--S24-7
g--Arcanobacterium
g--Oscillospira
g--Fibrobacter
f--Clostridiaceae
g--SHD-231
p--Bacteroidetes
p--Armatimonadetes
g--BF311
c--Clostridia
g--Anaerovibrio
g--Pseudobutyrivibrio
g--Sporanaerobacter
o--LD1-PB3
f--Erysipelotrichaceae 2
g--Butyrivibrio
g--Methanosphaera
g--Shuttleworthia
g--Succinivibrio
f--RFP12
g--Solibacillus
o--Bacteroidales
g--YRC22
f--Christensenellaceae
-0.75
-0.50
-0.25
0.00
0.25
0.50
0.75
Canonical Score 2
g--YRC22
f--S24-7
f--Veillonellaceae 2
g--TG5
g--Oscillospira
g--Selenomonas
o--LD1-PB3
g--Roseburia
f--Bifidobacteriaceae
g--Fibrobacter
g--Arcanobacterium
o--RF39
c--Mollicutes
f--Erysipelotrichaceae 2
f--Christensenellaceae
g--Methanobrevibacter
g--Sporanaerobacter
f--Mycoplasmataceae
g--BF311
p--Armatimonadetes
g--Ruminobacter
g--Solibacillus
f--Catabacteriaceae
g--SHD-231
f--BS11
p--Bacteroidetes
g--Shuttleworthia
f--RFP12
o--Bacteroidales
g--Anaerovibrio
g--Butyrivibrio
g--Desulfovibrio
g--Succinivibrio
g--Pseudobutyrivibrio
f--Clostridiaceae
c--Clostridia
f--Victivallaceae
g--Methanosphaera
p--SR1
-0.75
-0.50
-0.25
0.00
0.25
0.50
0.75
Canonical Score 3

Figure 5.

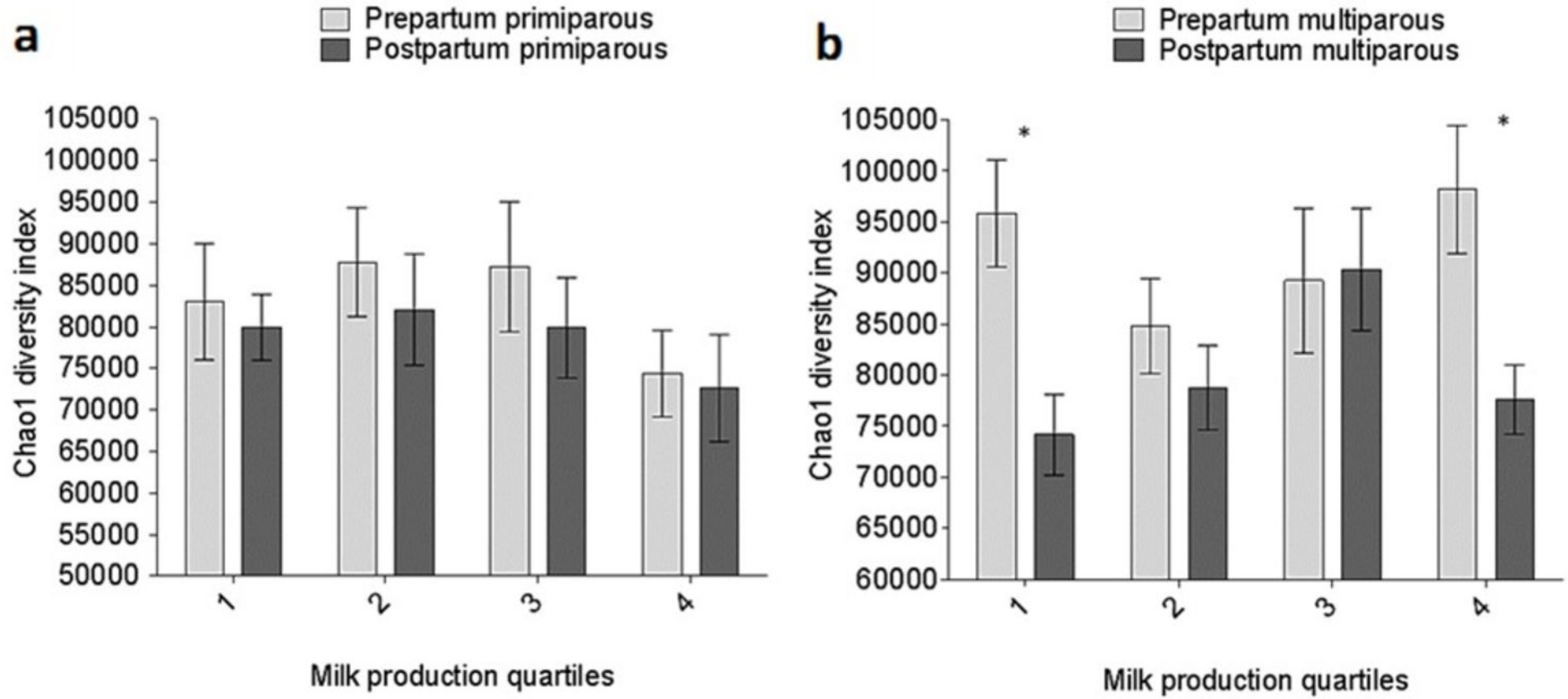

a
Prepartum primiparous
Postpartum primiparous
Chao1 diversity index
105000
100000
95000
90000
85000
80000
75000
70000
65000
60000
55000
50000
1
2
3
4
Milk production quartiles
b
Prepartum multiparous
Postpartum multiparous
Chao1 diversity index
105000
100000
95000
90000
85000
80000
75000
70000
65000
60000
*
*
1
2
3
4
Milk production quartiles

Figure 6.

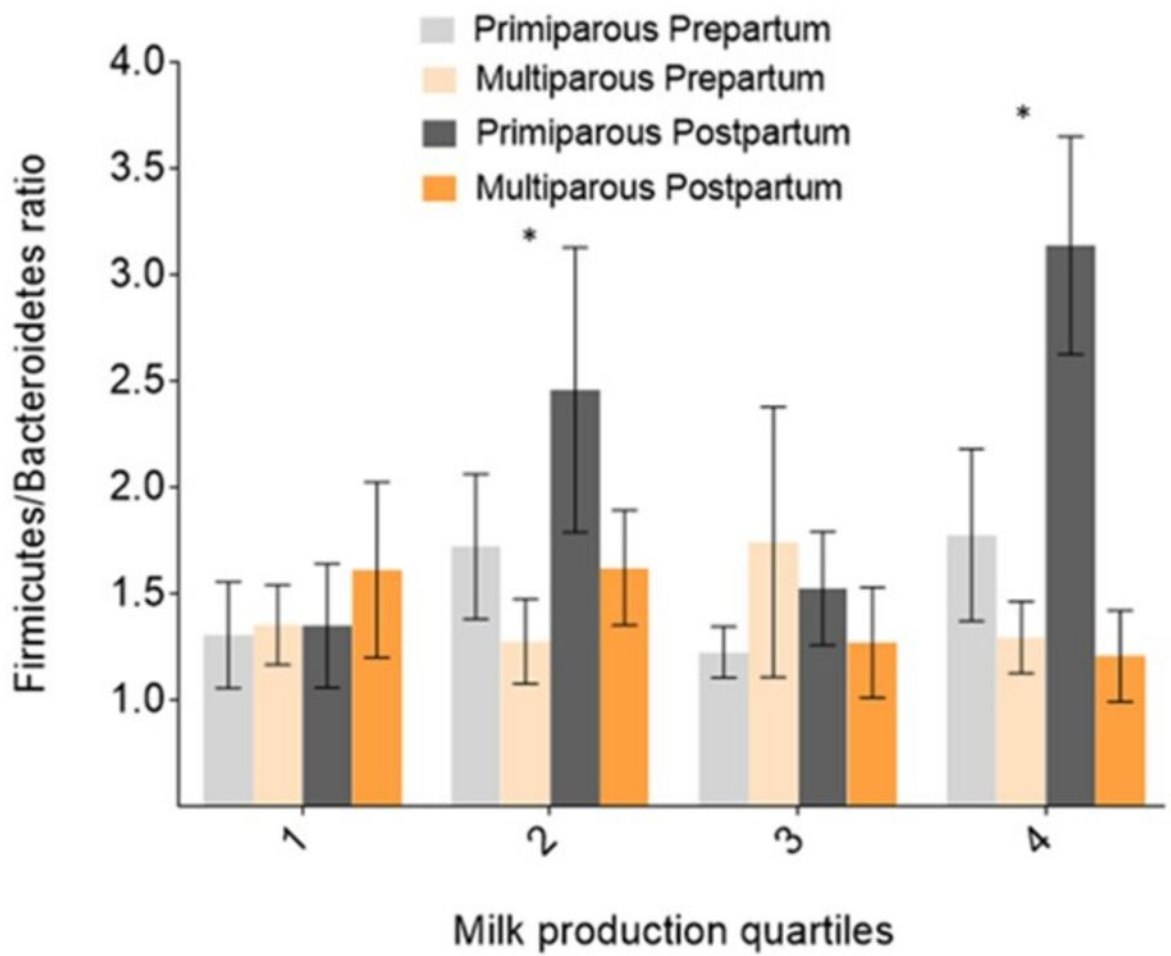

Primiparous Prepartum
Multiparous Prepartum
Primiparous Postpartum
Multiparous Postpartum
Firmicutes/Bacteroidetes ratio
4.0
3.5
3.0
2.5
2.0
1.5
1.0
*
*
1
2
3
4
Milk production quartiles

Figure 7.

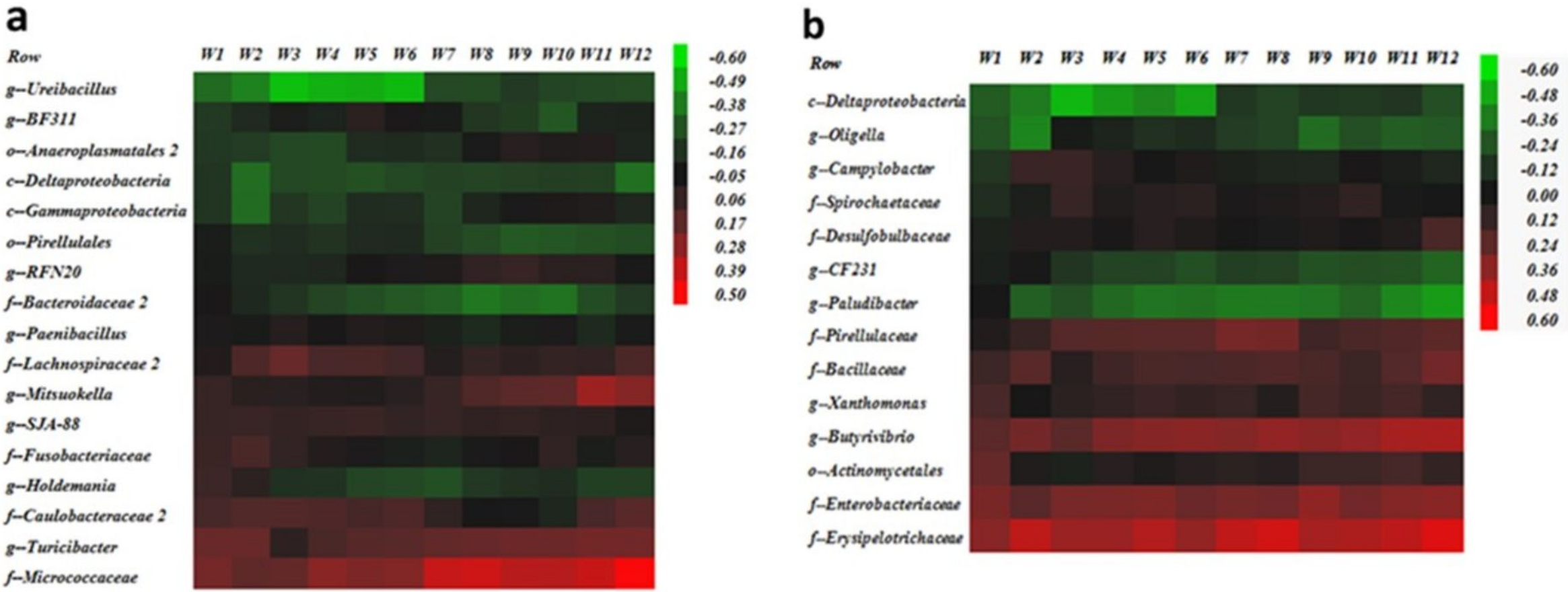

a
Row
W1 W2 W3 W4 W5 W6 W7 W8 W9 W10 W11 W12
g--Ureibacillus
g--BF311
o--Anaeroplasmatales 2
c--Deltaproteobacteria
c--Gammaproteobacteria
o--Pirellulales
g--RFN20
f--Bacteroidaceae 2
g--Paenibacillus
f--Lachnospiraceae 2
g--Mitsuokella
g--SJA-88
f--Fusobacteriaceae
g--Holdemania
f--Caulobacteraceae 2
g--Turicibacter
f--Micrococcaceae
-0.60
-0.49
-0.38
-0.27
-0.16
-0.05
0.06
0.17
0.28
0.39
0.50
b
Row
W1 W2 W3 W4 W5 W6 W7 W8 W9 W10 W11 W12
c--Deltaproteobacteria
g--Oligella
g--Campylobacter
f--Spirochaetaceae
f--Desulfobulbaceae
g--CF231
g--Paludibacter
f--Pirellulaceae
f--Bacillaceae
g--Xanthomonas
g--Butyrivibrio
o--Actinomycetales
f--Enterobacteriaceae
f--Erysipelotrichaceae
-0.60
-0.48
-0.36
-0.24
-0.12
0.00
0.12
0.24
0.36
0.48
0.60

Figure 8.

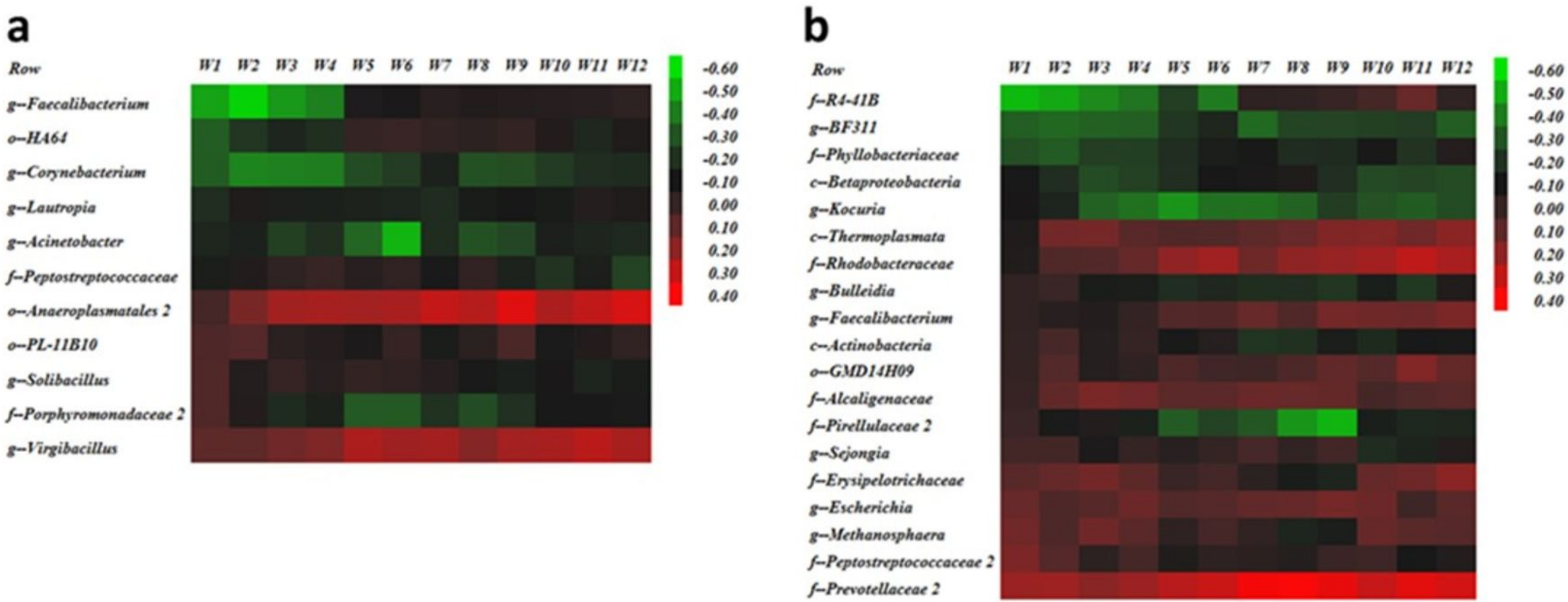

a
Row
W1 W2 W3 W4 W5 W6 W7 W8 W9 W10 W11 W12
g--Faecalibacterium
o--HA64
g--Corynebacterium
g--Lautropia
g--Acinetobacter
f--Peptostreptococcaceae
o--Anaeroplasmatales 2
o--PL-11B10
g--Solibacillus
f--Porphyromonadaceae 2
g--Virgibacillus
-0.60 -0.50 -0.40 -0.30 -0.20 -0.10 0.00 0.10 0.20 0.30 0.40
b
Row
W1 W2 W3 W4 W5 W6 W7 W8 W9 W10 W11 W12
f--R4-41B
g--BF311
f--Phyllobacteriaceae
c--Betaproteobacteria
g--Kocuria
c--Thermoplasmata
f--Rhodobacteraceae
g--Bulleidia
g--Faecalibacterium
c--Actinobacteria
o--GMD14H09
f--Alcaligenaceae
f--Pirellulaceae 2
g--Sejongia
f--Erysipelotrichaceae
g--Escherichia
g--Methanosphaera
f--Peptostreptococcaceae 2
f--Prevotellaceae 2
-0.60 -0.50 -0.40 -0.30 -0.20 -0.10 0.00 0.10 0.20 0.30 0.40

Figure 9.

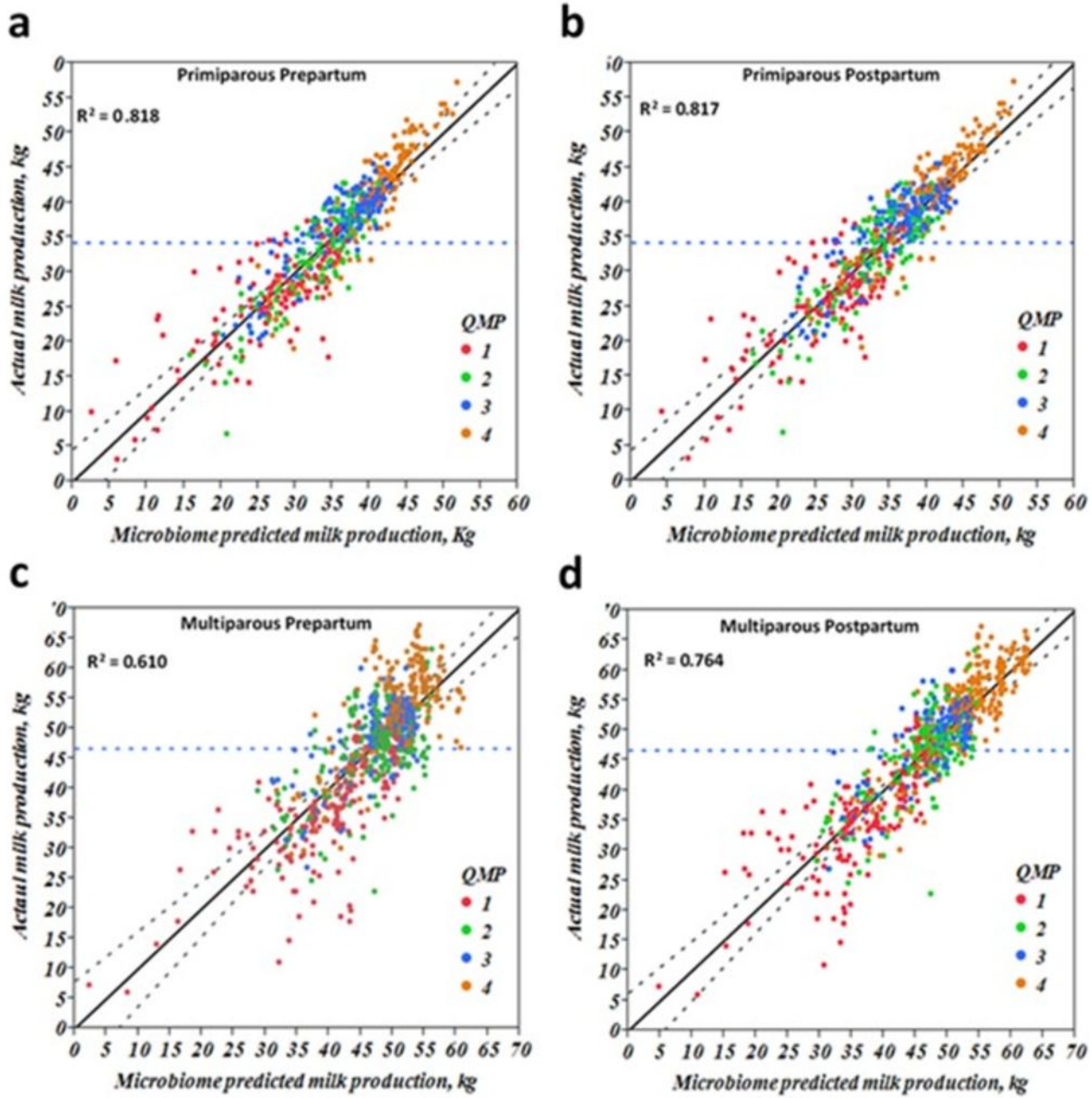
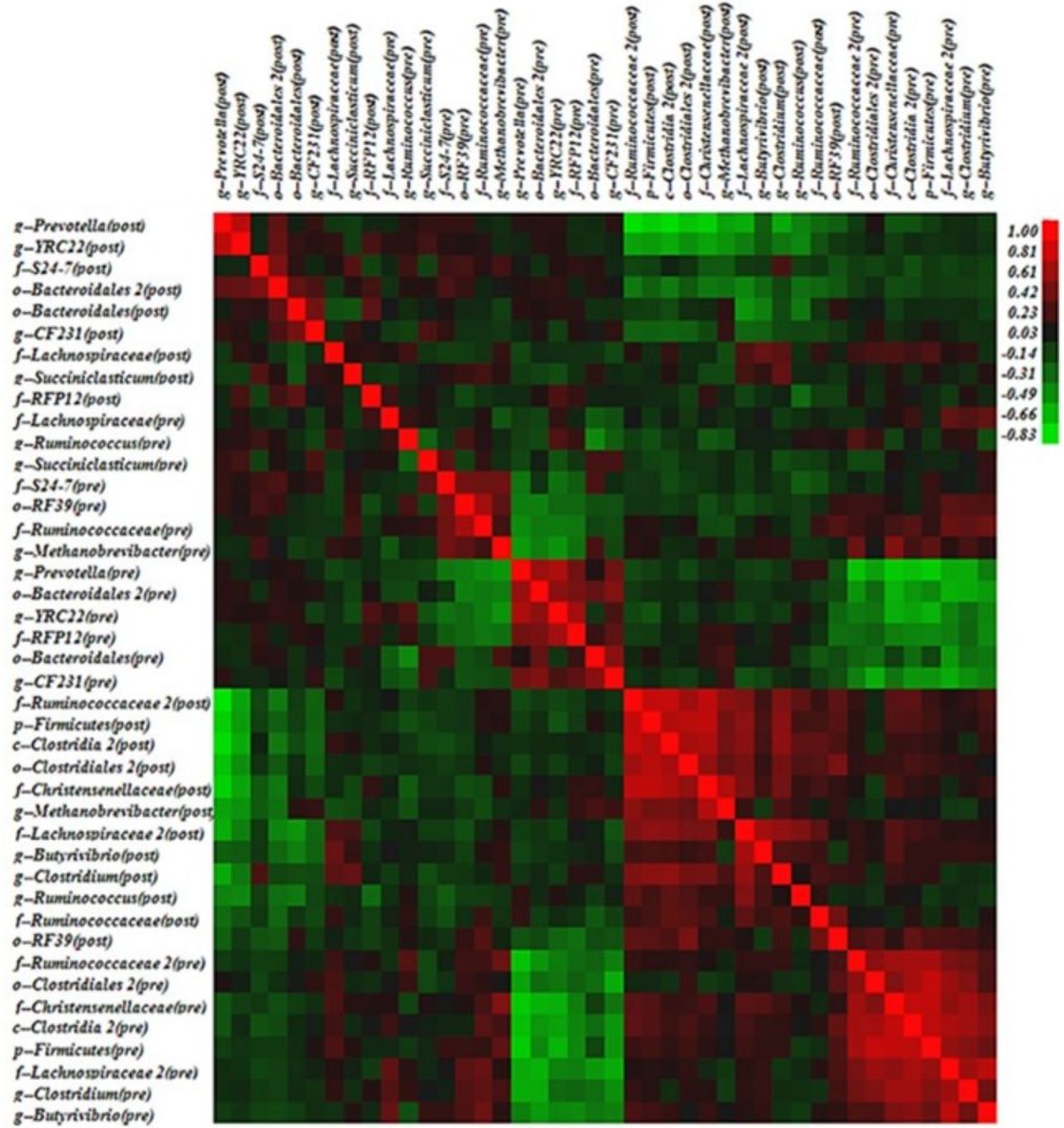

a
Primiparous Prepartum
R² = 0.818
Actual milk production, kg
Microbiome predicted milk production, Kg
QMP
1
2
3
4
b
Primiparous Postpartum
R² = 0.817
Actual milk production, kg
Microbiome predicted milk production, kg
QMP
1
2
3
4
c
Multiparous Prepartum
R² = 0.610
Actual milk production, kg
Microbiome predicted milk production, kg
QMP
1
2
3
4
d
Multiparous Postpartum
R² = 0.764
Actual milk production, kg
Microbiome predicted milk production, kg
QMP
1
2
3
4

Figure 10.


g--Prevotella(post)
g--YRC22(post)
f--S24-7(post)
o--Bacteroidales 2(post)
o--Bacteroidales(post)
g--CF231(post)
f--Lachnospiraceae(post)
g--Succiniclasticum(post)
f--RFP12(post)
f--Lachnospiraceae(pre)
g--Ruminococcus(pre)
g--Succiniclasticum(pre)
f--S24-7(pre)
o--RF39(pre)
f--Ruminococcaceae(pre)
g--Methanobrevibacter(pre)
g--Prevotella(pre)
o--Bacteroidales 2(pre)
g--YRC22(pre)
f--RFP12(pre)
o--Bacteroidales(pre)
g--CF231(pre)
f--Ruminococcaceae 2(post)
p--Firmicutes(post)
c--Clostridia 2(post)
o--Clostridiales 2(post)
f--Christensenellaceae(post)
g--Methanobrevibacter(post)
f--Lachnospiraceae 2(post)
g--Butyrivibrio(post)
g--Clostridium(post)
g--Ruminococcus(post)
f--Ruminococcaceae(post)
o--RF39(post)
f--Ruminococcaceae 2(pre)
o--Clostridiales 2(pre)
f--Christensenellaceae(pre)
c--Clostridia 2(pre)
p--Firmicutes(pre)
f--Lachnospiraceae 2(pre)
g--Clostridium(pre)
g--Butyrivibrio(pre)
1.00
0.81
0.61
0.42
0.23
0.03
-0.14
-0.31
-0.49
-0.66
-0.83