Illumina sequencing analysis of the ruminal microbiota in high-yield and low-yield lactating dairy cows

Jinjin Tong*[1], Hua Zhang*[1], Delian Yang*, Benhai Xiong¶ and Linshu Jiang*[2]

*Beijing Key Laboratory for Dairy Cow Nutrition, Beijing University of Agriculture, Beijing, 102206, P. R. China

¶State Key Laboratory of Animal Nutrition, Institute of Animal Science, Chinese Academy of Agricultural Sciences, Beijing, 100193, P. R. China

Abstract

In this study, differences in the ruminal bacterial community between high-yield and low-yield lactating dairy cows fed the same diets were investigated. Sixteen lactating dairy cows with similar parity were divided into two groups based on their milk yield: high-yield (HY) and low-yield (LY) groups. On day 21, rumen content samples were collected, and the microbiota composition was determined using Illumina MiSeq sequencing of the 16S rRNA gene. During the study period, dry matter intake (DMI) and milk yield were measured daily, and milk composition was assessed 3 times per week. The results showed that the milk of the LY group tended to have higher fat (P=0.08), protein (P=0.01) and total solid (P=0.04) contents than that of the HY group, though the HY group had higher ruminal acetate (P=0.05), propionate (P=0.02) and volatile fatty acid (VFA) (P=0.02) concentrations. Principal coordinate analysis indicated significant differences in ruminal bacterial community composition and structure between the HY group and LY group. Overall, Bacteroidetes (HY group: 52.91±3.06%; LY group: 61.88±3.03%) was the predominant phylum, followed by Firmicutes (HY group: 41.10±2.74%; LY group: 32.11±2.97%). The abundances of *Ruminococcus 2*, Lachnospiraceae and *Eubacterium coprostanoligenes* were significantly higher in the HY group than in the LY group. In addition, 3 genera—*Anaerostipes*, *Bacteroidales* and *Anaeroplasma*—were identified as biomarker species with the greatest impacts on the ruminal community structure in the

---

[1] These 2 authors contributed equally to this article.

2 Corresponding author: kjxnb@vip.sina.com

LY group. These findings facilitate the understanding of bacterial synthesis within the rumen and reveal an important mechanism underlying differences in milk production in dairy cows.

Key words: bacterial community, 16S rRNA, rumen, dairy cows

Introduction

A symbiotic relationship exists with regard to the rumen microbiota of cattle. The rumen is a highly specialised organ of ruminant animals that promotes a community of mutualistic microbial species while simultaneously absorbing nutrients derived from digestion of plant fibre and cellular material [1]. In dairy cows, the rumen microbial community plays a critical role in the volatile fatty acid (VFA) production, B-vitamin synthesis, and microbial protein biosynthesis, which are critical for the animal's well-being and efficient milk production [2]. Moreover, the structure of the bacterial community has been correlated with the production traits [3], production variables [4-7], and milk production and composition [8] of dairy cows. It has been reported that rumen microbial dynamics involve both core and variable microbiotal components [9-11]. In addition, similar to the microbial community in the gut of non-ruminants, the structure and function of the microbial community in the cow rumen are shaped by dynamic physical, chemical, and predatory environments. In turn, the microbial community regulates nutrient cycling to the host [12]. However, a more in-depth comparison is warranted to improve our understanding of differences in rumen bacterial community composition between high-yield and low-yield dairy cows.

Recent efforts to study the rumen microbiome have focused on identifying and quantifying ruminal microbial communities [8, 13]. As a powerful molecular approach for taxonomic analyses, the application of 16S rRNA gene sequencing technology has provided novel insight into the microbiome ecology of gastrointestinal tracts [14, 15]. Indeed, this technique has been widely used to study microbial diversity and the metabolic capabilities of microbiomes in different ecological niches [16], fermented food [17, 18], waste-water treatment facilities [19], and human and animal gastrointestinal tracts [20-22]. The objective of the present study was to examine differences in ruminal bacterial community compositions between high-yield

58 and low-yield lactating cows.

59 **Materials and Methods**

60 **Animals and Experimental Design**

61 The experimental protocol was approved by the Institutional Animal Care and Use
62 Committee at the Beijing University of Agriculture, in compliance with regulations
63 for the administration of affairs concerning experimental animals (The State Science
64 and Technology Commission of P. R. China, 1988). According to the principle of
65 parity and lactation days, 16 Holstein lactating dairy cows of similar parity were used
66 and assigned to a high-yield group (average production 31.90±1.76 kg/d, mean±SD)
67 or a low-yield group (average production 19.30±1.76 kg/d), with 8 each. The test
68 period was 21 d, with a pre-feeding period of 14 d and a treatment period of 7 d.
69 These lactating dairy cows were the fed same diet, the composition of which is shown
70 in Table 1.

71 **Table 1 Ingredients and nutrient composition (% of DM) of the basal diet**

Item	Content
Ingredient, % of DM	
Alfalfa hay	6.90
Corn silage	46.32
Oat grass	2.40
Ground corn	9.88
Soybean meal	5.10
Steam-flaked corn	4.40
DDGS[1]	4.40
Corn bran	3.70
Extruded soybean	3.00
Barley	2.66
Wheat barn	2.66
Sodium cyclamate	2.40
Oat	1.50
Canola meal	1.07
Cottonseed meal	1.07
Magalac[2]	0.90
$NaHCO_3$	0.59
Limestone	0.48
NaCl	0.27

Premix[3]	0.30
Nutrient composition[4]	
CP	17.4
NDF	31.1
ADF	16.6
Ether extract	5.00
Ca	0.78
P	0.44
NE_L, Mcal/kg	1.76

[1]DDGS=dried distillers grain with solubles.

[2]Church and Dwight Co. Inc., Princeton, NJ.

[3]Formulated to provide (per kg of DM) 4,560 mg of Cu, 3,000 mg of Fe, 12,100 mg of Zn, 4,590 mg of Mn, 60 mg of Co, 200 mg of Se, 270 mg of I, 10,000 IU of vitamin E, 450,000 IU of vitamin D, 2,000,000 IU of vitamin A, and 3,000 mg of nicotinic acid.

[4]Chemical composition is based on chemical analysis of the total mixed ration (TMR), as described.

Rumen fluid sampling and parameter measurement

Rumen fluid samples were collected from the oral cavity at 3-4 h after the morning feeding on day 7. The rumen contents were strained through 4 layers of cheesecloth with a mesh size of 250 μm. Ruminal pH was immediately measured using a portable pH meter (Testo 205, Testo AG, Germany). The filtered rumen fluid samples were centrifuged at 10,000 × g for 15 min at 4°C, aliquoted into 5-mL cryopreservation tubes, frozen in liquid nitrogen tank and stored at -80°C for analysis of the ruminal bacterial community. Another 10 mL of clear supernatant samples was mixed with 2 mL of 250 g/L of metaphosphoric acid and stored at −20°C for VFA determination, as described by Pan et al. [23].

DNA extraction and polymerase chain reaction (PCR) amplification

Microbial DNA was extracted from rumen fluid samples using E.Z.N.A.® Bacterial DNA Kit (Omega Bio-Tek, Norcross, U.S.) according to the manufacturer's protocols. The yield and purity of the extracted DNA were assessed with a NanoDrop 1000 instrument (NanoDrop, Wilmington, DE).

16S rRNA analysis

The V3-V4 regions of the 16S ribosomal RNA gene were amplified by PCR (95°C for 3 min, followed by 27 cycles at 95°C for 30 s, 55°C for 30 s, and 72°C for 45 s and a final extension at 72°C for 10 min) using the primers 338F 5'-barcode-ACTCCTACGGGAGGCAGCAG)-3' and 806R 5'-GGACTACHVGGGTWTCTAAT-3', where the barcode is an eight-base sequence unique to each sample. The PCR reactions were performed in triplicate in a 20-μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM deoxyribonucleotide triphosphates (dNTPs), 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, 0.2 μL of bovine serum albumin (BSA) and 10 ng of the template DNA. Amplicons were excised from 2% agarose gels and purified using AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, U.S.) according to the manufacturer's instructions and then quantified using QuantiFluor™ -ST (Promega, U.S.). Purified amplicons were pooled in equimolar ratios and pair-end sequenced (2 × 300) on the Illumina MiSeq platform according to standard protocols. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number: SRP136923).

The raw fastQ files were quality filtered by Trimmomatic and merged by FLASH under the following criteria. First, the reads were truncated at any site receiving an average quality score of <20 over a 50-bp sliding window. Second, sequences with overlapping segments longer than 10 bp were merged according to their overlapping region, with a mismatch of no more than 2 bp. Finally, sequences of each sample were separated according to barcodes (exactly matching) and primers (allowing 2 nucleotide mismatching), and reads containing ambiguous bases were removed.

Operational taxonomic units (OTUs) were clustered with a 97% similarity cut-off using UPARSE (version 7.1 http://drive5.com/uparse/) with a novel 'greedy' algorithm that simultaneously performs chimaera filtering and OTU clustering. The taxonomy of each 16S rRNA gene sequence was analysed according to the RDP Classifier algorithm (http://rdp.cme.msu.edu/) against the Silva (SSU123) 16S rRNA database using a confidence threshold of 70%.

125 **Statistical analysis**

126 Data were analysed with a mixed-model procedure (SAS Institute, Inc., Cary,
127 NC), and Duncan's multiple comparison tests were employed to assess differences
128 between means. Differences were considered statistically significant when $P<0.05$
129 and considered a trend when $P<0.10$.

130 **Results**

131 **Dry matter intake, milk yield, and milk composition**

132 Dry matter intake (DMI) was significantly greater in the high-yield (HY) group
133 than in the low-yield (LY) group ($P=0.03$). Milk production (19.3±1.76 and 31.9±.76
134 kg/d, respectively; $P<0.01$), 4% fat-corrected milk (FCM) (18.95±1.73 and
135 29.20±1.73 kg/d, respectively. $P<0.01$) and energy-corrected milk (ECM)
136 (21.07±1.93 and 32.09±1.93 kg/d, respectively; $P<0.01$) were significantly lower in
137 the LY group than in the HY group (Table 2). The milk fat content tended to be
138 higher ($P=0.08$) (Table 2), and the milk protein content was significantly higher in the
139 LY group than in the HY group ($P<0.01$). No difference was observed in milk lactose
140 content between the LY and HY groups ($P=0.21$; Table 2). Fat, protein and lactose
141 yields were significantly greater in the HY group than in the LY group (Table 2).
142 However, somatic cell count (SCC) was not different between the HY and LY groups
143 ($P=0.13$; Table 2).

144 **Table 2. Milk and ECM from high-yielding and low-yielding dairy cows during the entire**
145 **sampling period [1]**

Items	LY	HY	SEM	*P*-value
Milk production (kg/d)	19.3	31.9	1.76	<0.0001
4%FCM production (kg/d)[2]	18.95	29.20	1.73	<0.0001
ECM production (kg/d)[3]	21.07	32.09	1.93	<0.0001
Milk composition				
Fat %	4.02	3.48	0.28	0.0788
Fat yield (kg/d)	0.75	1.10	0.07	0.0004
Protein %	3.50	3.07	0.11	0.0023

Protein yield (kg/d)	0.66	0.98	0.06	0.0002
Lactose %	4.87	5.05	0.14	0.2127
Lactose yield (kg/d)	0.92	1.61	0.10	<0.0001
Total solid content %	13.15	12.30	0.38	0.0417
SCC (log_{10})	2.46	3.10	2.78	0.1339

[1]Data are presented as least squares means.

SCC=somatic cell count.

Ruminal pH and VFA concentrations

Rumen pH was not different between the HY group and the LY group (Table 3). NH_3-N (mg/dL) was greater in the HY group than in the LY group (P<0.01), and there was a significant increase in acetate, propionate and butyrate levels in the HY group relative to the LY group (P<0.05).

In addition, total VFA levels were significantly greater in the HY group than in the LY group (P=0.02). A lower trend was observed for the acetate:propionate ratio in the HY group compared to the LY group (P=0.06).

Table 3. Effects of differences between high-yielding and low-yielding dairy cows on metabolites in the rumen

Items	LY	HY	SEM	*P*-value
pH	6.73	6.71	0.02	0.69
NH_3-N, mg/dL	7.99	13.28**	1.03	0.01
Molar proportion				
Acetate	62.19	68.97*	1.78	0.05
Propionate	21.94	26.35**	0.97	0.02
Isobutyrate	0.83	0.84	0.03	0.93
Butyrate	12.01	14.23**	0.45	0.01
Isovalerate	1.22	1.48	0.07	0.08
Valerate	1.57	1.76	0.06	0.14
Acetate:propionate ratio	2.85	2.64	0.06	0.06
Total VFA, m*M*	99.76	113.63	3.14	0.02

*P<0. 05; **P<0. 01: Values within a sampling day followed by superscripted asterisks differ.

SEM=standard error of the mean.

Diversity and richness of microbial communities

In total, 2,382,338 merged sequences were acquired for the 16 samples from the dairy

cows, and 1,191,169 high-quality sequences, with an average read length of 440 bp, were classified as bacterial. On average, at least 54,144 sequences were obtained per sample, and greater than 99% depth coverage was achieved. The rarefaction curve generated tended to plateau, showing that the number of OTUs did not rise with an increasing volume of data. This finding showed that the data volume of sequencing was reasonable. The results of this study show that the sequencing data were reasonable and could reflect changes in most bacterial flora (Figure 1).

No significant differences were observed in alpha diversity index results between the HY and LY groups ($P>0.05$) (Table 4). However, the coverage of the HY group was significantly higher than that of the LY group ($P<0.01$), indicating greater community diversity for the HY group.

Table 4. Alpha diversity index of rumen bacteria

Item	LY	HY	SEM	*P*-value
Sobs	1196.13	1214.88	17.21	0.60
Shannon	5.4104	5.4852	0.04	0.42
Simpson	0.01	0.01	0.00	0.74
ACE	1375.65	1360.65	18.75	0.70
Chao	1378.64	1376.82	20.10	0.97
Coverage	0.9921	0.9938**	0.00	0.001

*$P<0.05$; **$P<0.01$: Values within a sampling day followed by superscript asterisks differ.

SEM=standard error of the mean;

Principal coordinate analysis (PCoA) results showed that the HY group was distinct from the LY group (Figure 2). Principal coordinates 1 and 2 accounted for 24.94% and 12.25%, respectively, of the total variation.

Twenty-one bacterial phyla were identified across all samples. Bacteroidetes, Firmicutes and Proteobacteria were the three dominant phyla, representing 57.59%, 35.86%, and 1.53%, respectively, of the total sequences (Figure 3). Thus, at the phylum level, Bacteroidetes and Firmicutes were particularly dominant. The HY group exhibited a greater abundance of Firmicutes and lower abundance of

Bacteroidetes than did the LY group (P<0.01), whereas Proteobacteria was less abundant (P<0.05).

At the genus level, taxa with a relative abundance of ≥1% in at least one sample were further analysed, and the relevant genera are presented in Figure 4 and Figure 5. Twenty-one genera were identified, 6 of which exhibited significantly different abundances between the groups. Specifically, 4 genera were more abundant in the HY group at P<0.01, including *Ruminococcaceae-NK4A214-group*, *Ruminococcus 2*, *Lachnospiraceae-BS11-gut-group*, and *[Eubacterium]-coprostanoligenes-group*, and 2 were more abundant in the HY group at P<0.05: *Succiniclasticum* and *Christensenellaceae-R-7-group*.

Correlations between bacterial communities and ruminal variables

As shown in Figure 6, the relative abundances of the genera *Bacteroides* and *Ruminococcus 2* were positively correlated with ruminal propionate and NH_3-N concentrations (r>0.4, P<0.05) but negatively correlated with the ruminal ratio (acetate:propionate ratio) (r<-0.4, P<0.05). In addition, *norank_o__Mollicutes_RF9* was positively correlated with ruminal acetate and VFA concentrations (r>0.4, P<0.05). *Candidatus-Saccharimonas* was positively correlated with the ruminal propionate concentration (r>0.4, P<0.05) but negatively correlated with the ruminal ratio (r<-0.4, P<0.05). Moreover, the ratio was negatively correlated with *Schwartzia* (r<-0.6, P<0.05).

Discussion

High-throughput sequencing has been widely used in studies of microbial flora in ruminants as a means of quickly and efficiently determining the microbial community structure of the rumen [24]. Differences in rumen microbiota between high- and low-yield dairy cows can therefore be characterised using high-throughput sequencing methods. The present study also linked rumen fluid VFAs with milk components and rumen microbes.

Changes in rumen fermentation parameters and milk composition

Higher values for rumen fermentation parameters and milk composition were found in

the HY group compared to the LY group. This finding suggests that due to host–microbiota interactions, different cows may harbour different microbial species compositions, which are closely related to distinct differences in rumen fermentation parameters and milk composition. Furthermore, recent findings suggest an important relationship between VFAs and milk components [2, 25, 26]. Specifically, of the three principle VFAs, acetic and butyric acids are substrates for oxidation and are precursors of lipids [27, 28]. Propionic acid is the only glucogenic VFA, accounting for 65-80% of the net glucose supply in lactating dairy cows [29, 30]. In a previous study, milk yield was most highly related to rumen concentrations of butyrate and propionate [31]. In the present study, propionate and butyrate concentrations as well as fat, protein and lactose yields were significantly greater in the HY group than in the LY group, which is consistent with previous research [32]. The NH_3-N concentration in rumen fluid can reflect the balance of protein degradation and synthesis under varying feed conditions. NH_3-N is an intermediate product of feed protein, non-protein nitrogen degradation and microbial protein synthesis, and it is mainly affected by feed protein degradation, rumen wall absorption, microorganism utilisation and rumen chyme outflow rate [33-35]. Yang et al. [36] reported that the concentration of NH_3-N should be higher than 5 mg/dL; otherwise, it will influence the "uncoupling" effect of ruminal fermentation and reduce the efficiency of microbial protein synthesis. According to our results, the NH_3-N concentration was within the normal range, though that in the HY group was significantly higher than that in the LY group. These results indicate that rumen microbes promote protein degradation, providing a better understanding of difference in milk proteins between the two groups.

Differences in rumen microbial composition between HY and LY groups

No differences were observed in bacterial community richness and diversity between the groups. Three phyla predominated in both groups, which was consistent with previous studies reporting that the principal phyla of microbes in the rumen are Bacteroidetes, Firmicutes, and Proteobacteria. The proportions of these three phyla account for approximately 94% of the total [35, 37, 38]. Interestingly, our results showed that the abundance of Firmicutes in the HY group was higher than that in the

LY group, though the abundances of the two other dominant phyla were lower in the former than in the latter.

As previously described by Pan et al. [23], cows fed a high proportion of grain have a higher abundance of Firmicutes and a lower abundance of Proteobacteria than control cows, and other studies have shown that feeding a high amount of grain can promote milk production [39, 40]. Thus, the present study provides a better understanding of why cows fed the same diet can have different milk production. The present findings further demonstrate that Firmicutes plays an important role in milk production.

In agreement with other research results [11, 41], *Prevotella* was the most abundant genus in all samples. Although *Prevotella* was more abundant in the LY group than in the HY group, the difference was not significant. In contrast, *Ruminococcaceae-NK4A214-group*, *Ruminococcus 2*, *Lachnospiraceae-BS11-gut-group* and *[Eubacterium]-coprostanoligenes-group* were significantly different between the two groups, with higher abundances in the HY group than in the LY group. Jiang et al. [42] reported that the increase in the relative abundance of *Ruminococcus* partly explains why adding live yeast to the diet increases the in vivo digestibility of DM and NDF and the performance of cows. This result illustrates that high-performance cows have higher abundances of *Ruminococcus* in the rumen fluid, which is consistent with the present research results. Members of the family Lachnospiraceae are gram-positive obligate anaerobes that are mostly non-spore-forming bacteria [43, 44]. Huws et al. [45] showed that Ruminococcaceae and Lachnospiraceae play predominant roles in biohydrogenation pathways within the rumen. As the primary succinate-utilising bacterial taxon, *Succiniclasticum* accounted for 7.45% of the total bacterial community in the HY group, with significantly greater abundance than in the LY group. A higher level of *Succiniclasticum* has been associated with greater production of succinate from starch degradation [46]. Moreover, the abundances of Christensenellaceae and Ruminococcaceae NK4A214 in the HY group were significantly higher than in the LY group, though little information about these two genera has been reported in the literature. The reasons for the altered status of genera

in cows with different milk production are unclear.

Conclusion

In summary, high-yield dairy cows have better ruminal fermentation patterns than do low-yield cows, which was partially attributed to the greater abundances of *Bacteroides*, *Ruminococcus 2*, *Ruminococcaceae NK4A214*, Lachnospiraceae, *Succiniclasticum*, *Eubacterium* and *Christensenella* in the former. Furthermore, rumen fermentation in high-yield cows exhibited higher VFA levels than that found in low-yield cows. Rumen microbiotal composition between high-yield and low-yield dairy cows differs, and microbial species diversity and distribution contribute to production-related phenotypes. Overall, our findings enhance our understanding of rumen bacteria in cows with different milk yields and provide new strategies for improving dairy cow production performance.

Acknowledgements

The study was supported by the National Nature Science Foundation of China (Grant No. 31772629 and No. 31702302), Beijing Municipal Education Commission Project (SQKM201710020011), Open Project Program of Beijing Key Laboratory of Dairy Cow Nutrition, Beijing University of Agriculture and the National Key Research and Development Plan (2016YFD0700205, 2016YFD0700201, 2017YFD0701604). The authors thank the members of the Beijing Key Laboratory for Dairy Cow Nutrition, Beijing University of Agriculture (Beijing, China) for their assistance in the sampling and analysis of the samples.

Figure 1. The rarefaction curve of sequencing based on OTUs[1].

[1]OTUs, operational taxonomic units.

Figure 2. Principal coordinate analysis (PCoA) of bacterial community structures of the ruminal microbiota in the high-yielding group (green circles) and the low-yielding group (red triangles). PCoA plots were constructed using the unweighted UniFrac method.

Figure 3. Percent composition of predominant phyla in the rumen.

Figure 4. Percent composition of genera in the rumen.

Figure 5. Percent composition and significance of genera in the rumen.

Figure 6. Correlation analyses between the relative abundances of bacteria genera and ruminal fermentation parameters. Only genera with abundances significantly associated with ruminal VFA, propionate and acetate concentrations are presented. Green represents a negative correlation between the abundance of the species and the ratio ($r<-0.4$), and red represents a positive correlation ($r>0.4$, $0.01<P<=0.05$ *; $0.001<P\leq0.01$ **; $P\leq0.001$ ***; Values with a significant correlation followed by superscripted asterisks differ).

References

1. Delong EF, Stephen L, Stackebrandt E, Rosenberg E. Prokaryotic biology and symbiotic associations: Springer; 2013.
2. Krehbiel CR. INVITED REVIEW: Applied nutrition of ruminants: Fermentation and digestive physiology 1. Professional Animal Scientist. 2014;30(2):129-39.
3. Schären M, Frahm J, Kersten S, Meyer U, Hummel J, Breves G, et al. Interrelations between the rumen microbiota and production, behavioral, rumen-fermentation, metabolic, and immunological attributes of dairy cows. Journal of Dairy Science. 2018.
4. Guan LL, Nkrumah JD, Basarab JA, Moore SS. Linkage of microbial ecology to phenotype: correlation of rumen microbial ecology to cattle's feed efficiency. Fems Microbiology Letters. 2008;288(1):85-91.
5. Li F, Guan LL. Metatranscriptomic Profiling Reveals Linkages between the Active Rumen Microbiome and Feed Efficiency in Beef Cattle. Applied & Environmental Microbiology. 2017;83(9):AEM.00061-17.
6. Myer PR, Smith TPL, Wells JE, Kuehn LA, Freetly HC. Rumen Microbiome from Steers Differing in Feed Efficiency. Plos One. 2015;10(6):e0129174.
7. Shabat SK, Sasson G, Doronfaigenboim A, Durman T, Yaacoby S, Berg Miller ME, et al. Specific microbiome-dependent mechanisms underlie the energy harvest efficiency of ruminants. Isme Journal. 2016.
8. Jami E, White BA, Mizrahi I. Potential role of the bovine rumen microbiome in modulating milk composition and feed efficiency. Plos One. 2014;9(1):e85423.
9. Creevey CJ, Kelly WJ, Henderson G, Leahy SC. Determining the culturability of the rumen bacterial microbiome. Microbial Biotechnology. 2014;7(5):467-79.
10. Henderson G, Cox F, Ganesh S, Jonker A, Young W, Collaborators GRC, et al. Rumen microbial community composition varies with diet and host, but a core microbiome is found across a wide geographical range. Scientific Reports. 2016;6:14567.
11. Wu S, Baldwin RL, Li W, Li C, Connor EE, Li RW. The Bacterial Community Composition of the Bovine Rumen Detected Using Pyrosequencing of 16S rRNA Genes. Metagenomics. 2012;1(1):b1-11.
12. Church DC. The ruminant animal. Digestive physiology and nutrition. 1988.
13. Mcsweeney C, Mackie R. Commission on Genetic Resources for Food and Agriculture. Micro-organisms and ruminant digestion: State of knowledge, trends and future prospects. Background Study

344 Paper. 2012.
345 14. Dowd SE, Callaway TR, Wolcott RD, Sun Y, Mckeehan T, Hagevoort RG, et al. Evaluation of
346 the bacterial diversity in the feces of cattle using 16S rDNA bacterial tag-encoded FLX amplicon
347 pyrosequencing (bTEFAP). Bmc Microbiology. 2008;8(1):125.
348 15. Edwards JE, Mcewan NR, Travis AJ, Wallace RJ. 16S rDNA library-based analysis of ruminal
349 bacterial diversity. Antonie Van Leeuwenhoek. 2004;86(3):263-81.
350 16. Bucbe M, Reich M, Murat C, Morin E, Nilsson RH, Uroz S, et al. 454 Pyrosequencing analyses
351 of forest soils reveal an unexpectedly high fungal diversity. New Phytologist. 2009;184(2):449-56.
352 17. Hamberger A, Horn MA, Dumont MG, Murrell JC, Drake HL. Anaerobic consumers of
353 monosaccharides in a moderately acidic fen. Applied & Environmental Microbiology.
354 2008;74(10):3112.
355 18. Humblot C, Guyot JP. Pyrosequencing of Tagged 16S rRNA Gene Amplicons for Rapid
356 Deciphering of the Microbiomes of Fermented Foods Such as Pearl Millet Slurries. Applied &
357 Environmental Microbiology. 2009;75(13):4354-61.
358 19. Sanapareddy N, Hamp TJ, Gonzalez LC, Hilger HA, Fodor AA, Clinton SM. Molecular Diversity
359 of a North Carolina Wastewater Treatment Plant as Revealed by Pyrosequencing. Applied &
360 Environmental Microbiology. 2009;75(6):1688.
361 20. Mao SY, Huo WJ, Zhu WY. Microbiome-metabolome analysis reveals unhealthy alterations in
362 the composition and metabolism of ruminal microbiota with increasing dietary grain in a goat model.
363 Environmental Microbiology. 2016;18(2):525.
364 21. Gonzalo MF, Denman SE, Yang C, Jane C, Makoto M, Mcsweeney CS. Methane Inhibition
365 Alters the Microbial Community, Hydrogen Flow, and Fermentation Response in the Rumen of Cattle.
366 Frontiers in Microbiology. 2016;7(1087).
367 22. Danielsson R, Dicksved J, Sun L, Gonda H, Müller B, Schnürer A, et al. Methane Production in
368 Dairy Cows Correlates with Rumen Methanogenic and Bacterial Community Structure. Frontiers in
369 Microbiology. 2017;8:226.
370 23. Pan X, Xue F, Nan X, Tang Z, Wang K, Beckers Y, et al. Illumina Sequencing Approach to
371 Characterize Thiamine Metabolism Related Bacteria and the Impacts of Thiamine Supplementation on
372 Ruminal Microbiota in Dairy Cows Fed High-Grain Diets. Frontiers in Microbiology. 2017;8:1818.
373 24. Highlander SK. High throughput sequencing methods for microbiome profiling: application to
374 food animal systems. Animal Health Research Reviews. 2012;13(1):40-53.
375 25. Armentano LE. Ruminant hepatic metabolism of volatile fatty acids, lactate and pyruvate. Journal
376 of Nutrition. 1992;122(3 Suppl):838-42.
377 26. Tang C, Zhang K, Zhan T, Zhao Q, Zhang J. Metabolic Characterization of Dairy Cows Treated
378 with Gossypol by Blood Biochemistry and Body Fluid Untargeted Metabolome Analyses. Journal of
379 Agricultural & Food Chemistry. 2017.
380 27. Bauman DE, Griinari JM. NUTRITIONAL REGULATION OF MILK FAT SYNTHESIS.
381 Annual Review of Nutrition. 2003;23(1):203-27.
382 28. Dijkstra J, Boer H, Van BJ, Bruining M, Tamminga S. Absorption of volatile fatty acids from the
383 rumen of lactating dairy cows as influenced by volatile fatty acid concentration, pH and rumen liquid
384 volume. Br J Nutr. 1993;69(2):385-96.
385 29. Reynolds CK, Aikman PC, Lupoli B, Humphries DJ, Beever DE. Splanchnic metabolism of dairy
386 cows during the transition from late gestation through early lactation. Journal of Dairy Science.
387 2003;86(4):1201-17.

30. Zarrin M, Grossen-Rösti L, Bruckmaier RM, Gross JJ. Elevation of blood β-hydroxybutyrate concentration affects glucose metabolism in dairy cows before and after parturition. Journal of Dairy Science. 2017;100(3):2323-33.

31. Seymour WM, Campbell DR, Johnson ZB. Relationships between rumen volatile fatty acid concentrations and milk production in dairy cows: a literature study. Animal Feed Science & Technology. 2005;119(1):155-69.

32. Sofyan A, Mitsumori M, Ohmori H, Uyeno Y, Hasunuma T, Akiyama K, et al. Differences in rumen fermentation characteristics between low‐yield and high‐yield dairy cows in early lactation. Animal Science Journal. 2017;88(7).

33. Colmenero JJO, Broderick GA. Effect of Dietary Crude Protein Concentration on Milk Production and Nitrogen Utilization in Lactating Dairy Cows 1. Journal of Dairy Science. 2006;89(5):1704.

34. Mutsvangwa T, Davies KL, Mckinnon JJ, Christensen DA. Effects of dietary crude protein and rumen-degradable protein concentrations on urea recycling, nitrogen balance, omasal nutrient flow, and milk production in dairy cows. Journal of Dairy Science. 2016;99(8):6298-310.

35. Jin D, Zhao S, Wang P, Zheng N, Bu D, Yves B, et al. Insights into Abundant Rumen Ureolytic Bacterial Community Using Rumen Simulation System. Frontiers in Microbiology. 2016;7(887).

36. Yang WZ, Beauchemin KA, Rode LM. Effects of Grain Processing, Forage to Concentrate Ratio, and Forage Particle Size on Rumen pH and Digestion by Dairy Cows 1. Journal of Dairy Science. 2001;84(10):2203-16.

37. Khafipour E, Krause DO, Plaizier JC. A grain-based subacute ruminal acidosis challenge causes translocation of lipopolysaccharide and triggers inflammation. Journal of Dairy Science. 2009;92(3):1060-70.

38. Khafipour E, Li S, Plaizier JC, Krause DO. Rumen microbiome composition determined using two nutritional models of subacute ruminal acidosis. Appl Environ Microbiol. 2009;75(22):7115-24.

39. Rugoho I, Cheng L, Aizimu W, Bryant RH, Edwards GR. Effects of post‐grazing herbage height and concentrate feeding on milk production and major milk fatty acids of dairy cows in mid‐lactation. Grass & Forage Science. 2017;72(2):211-9.

40. Ueda K, Mitani T, Kondo S. Effect of timing and type of supplementary grain on herbage intake, nitrogen utilization and milk production in dairy cows grazed on perennial ryegrass pasture from evening to morning. Animal Science Journal. 2017;88(1):107-18.

41. Stevenson DM, Weimer PJ. Dominance of Prevotella and low abundance of classical ruminal bacterial species in the bovine rumen revealed by relative quantification real-time PCR. Applied Microbiology & Biotechnology. 2007;75(1):165.

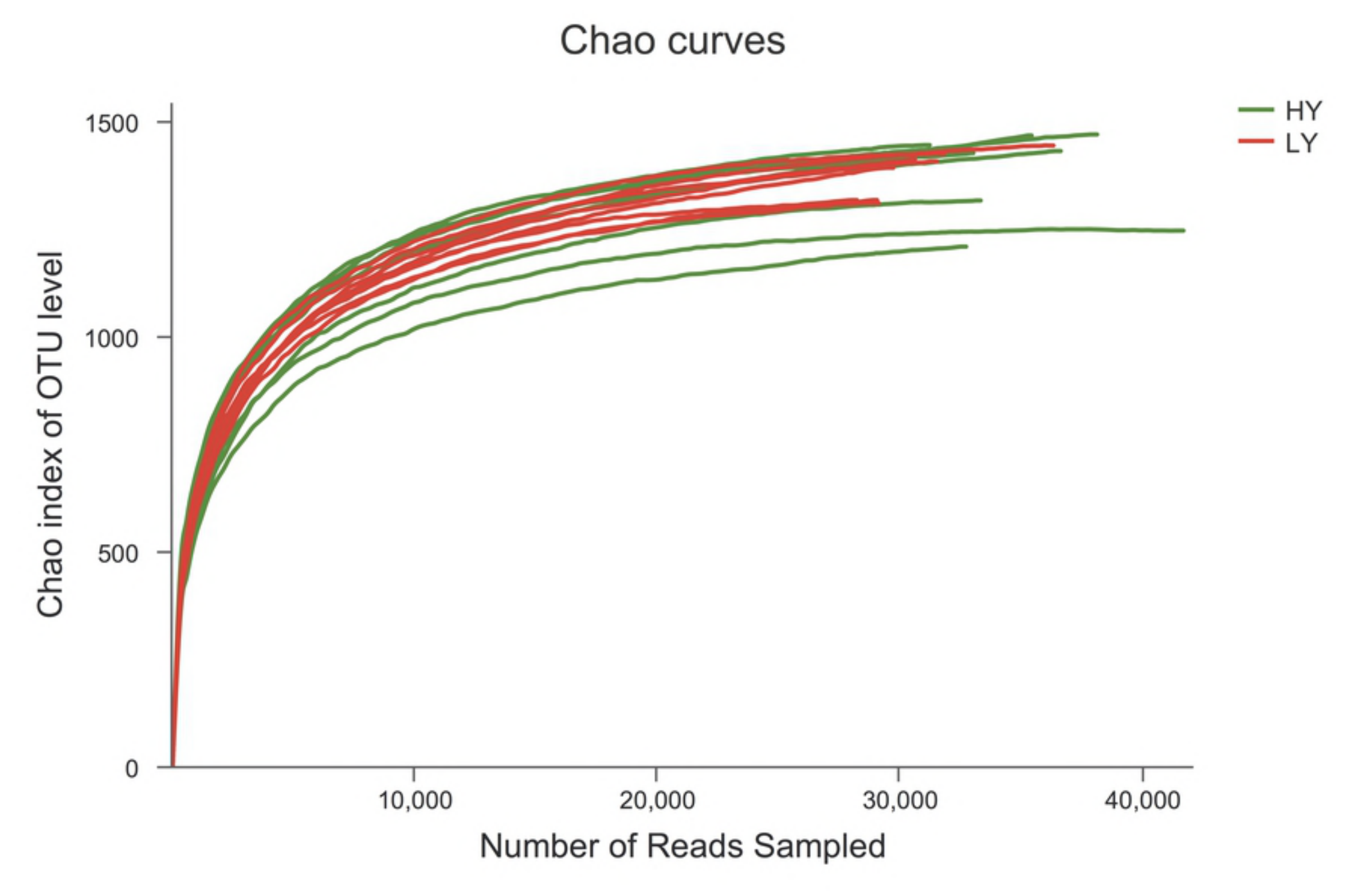
42. Jiang Y, Ogunade IM, Qi S, Hackmann TJ, Staples C, Adesogan AT. Effects of the dose and viability of Saccharomyces cerevisiae . 2. Diversity of ruminal microbes as analyzed by Illumina MiSeq sequencing and quantitative PCR. Journal of Dairy Science. 2016;100(1):325.

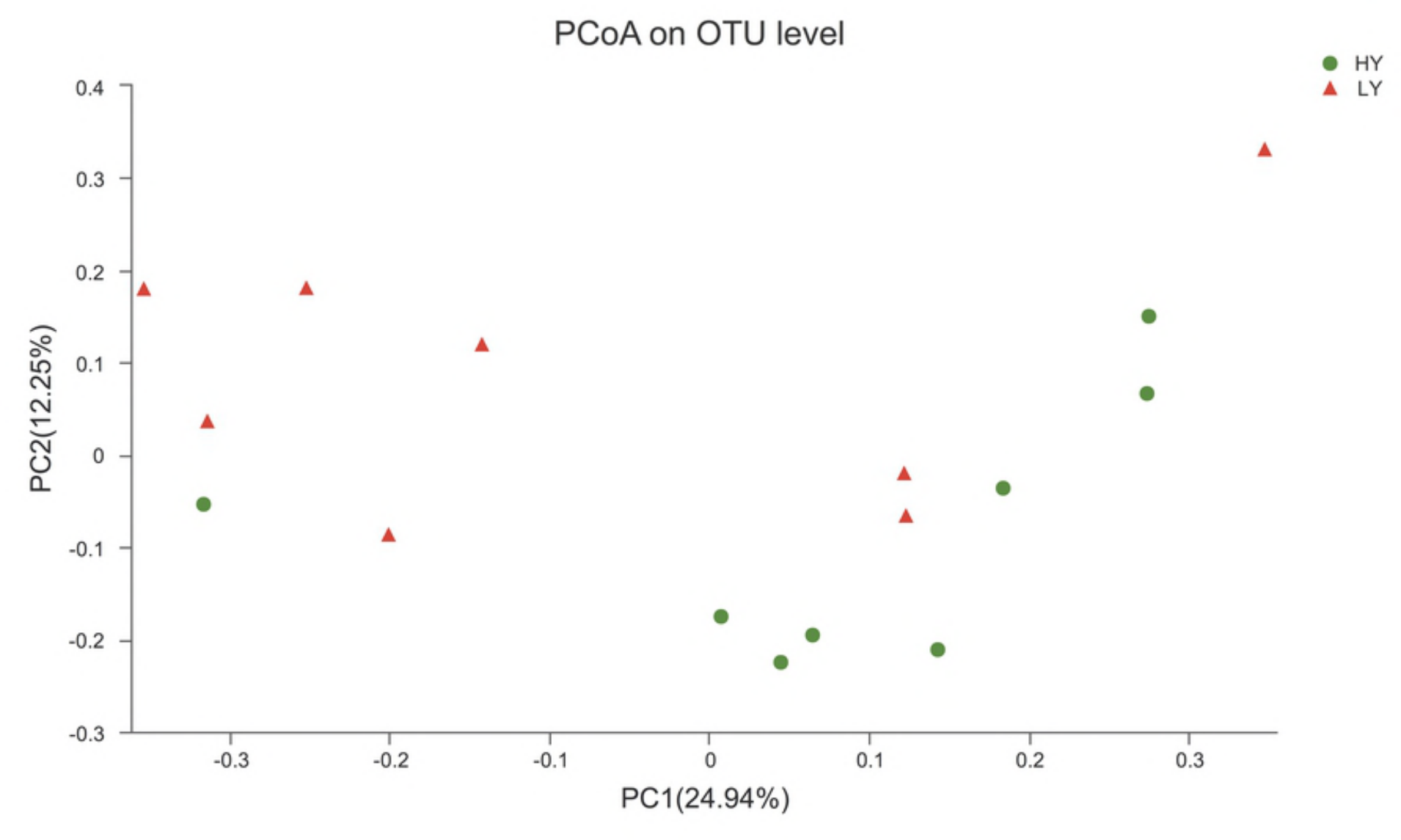
43. Cotta M, Forster R. The Family Lachnospiraceae, Including the Genera Butyrivibrio , Lachnospira and Roseburia: Springer US; 2006. : 727–35. p.

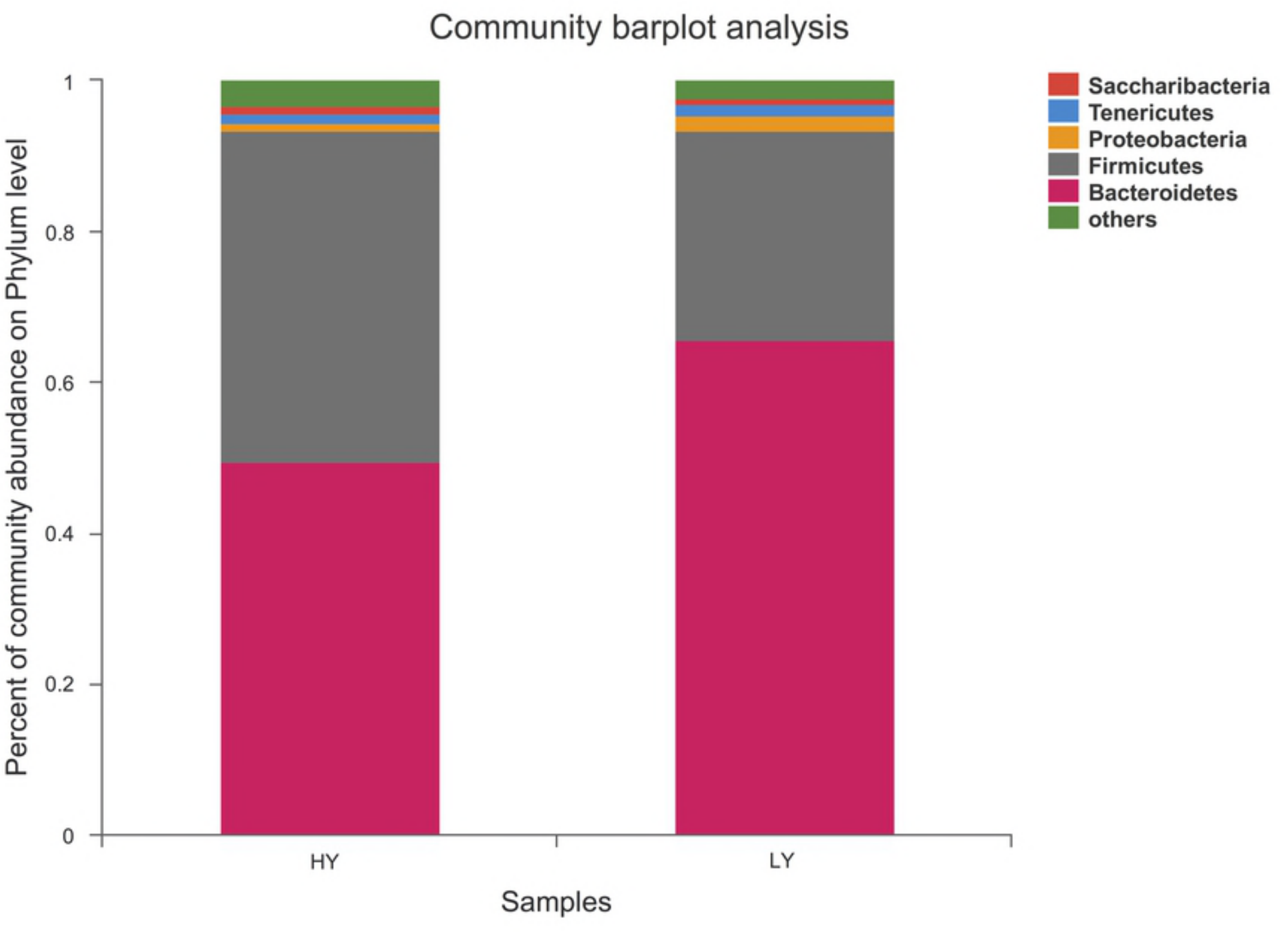
44. Hedberg ME, Moore ER, Svenssonstadler L, Hörstedt P, Baranov V, Hernell O, et al. Lachnoanaerobaculum gen. nov., a new genus in the Lachnospiraceae: characterization of Lachnoanaerobaculum umeaense gen. nov., sp. nov., isolated from the human small intestine, and Lachnoanaerobaculum orale sp. nov., isolated from saliva, and reclassifica. International Journal of Systematic & Evolutionary Microbiology. 2012;62(Pt 11):2685.

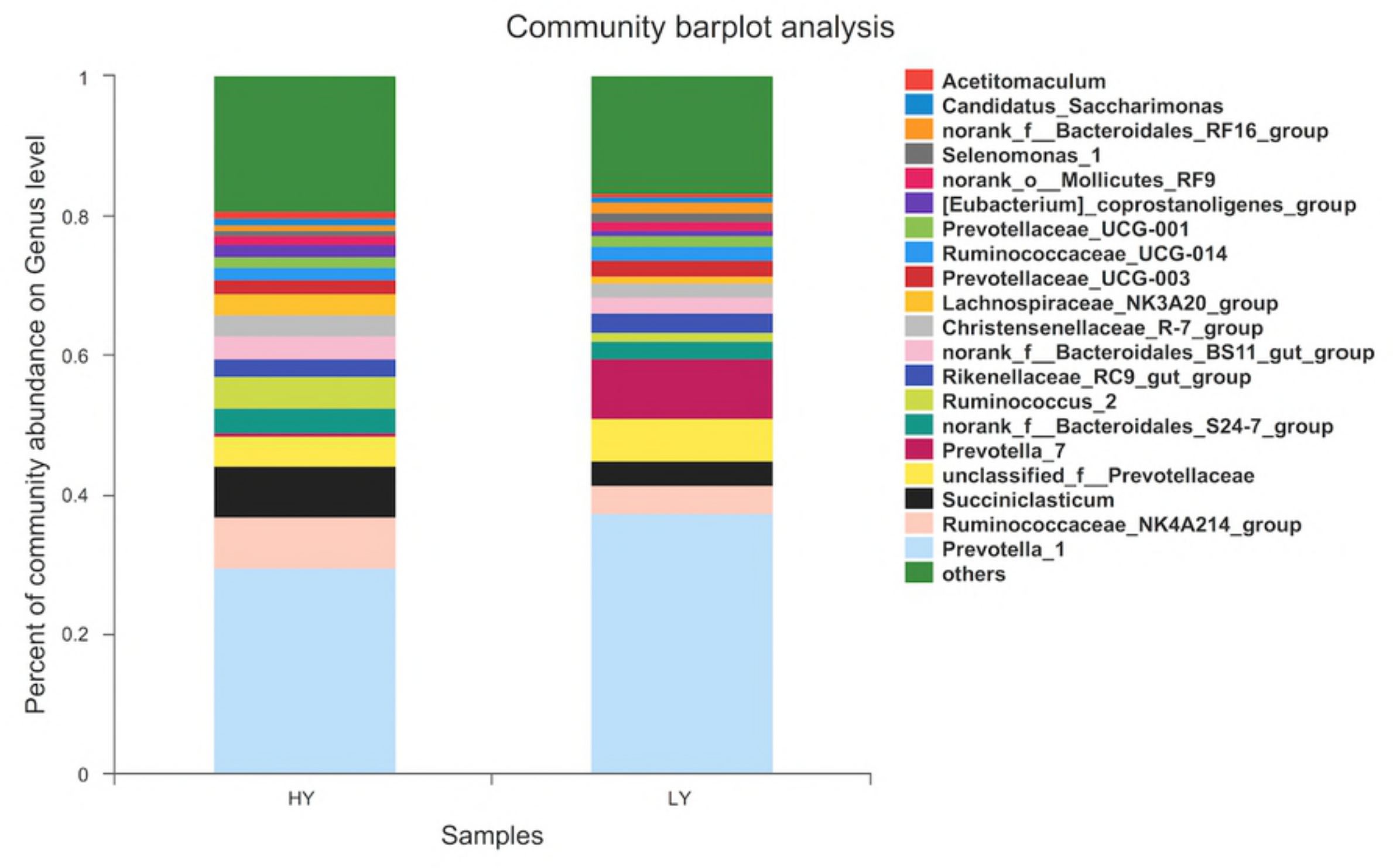
45. Huws SA, Kim EJ, Lee MRF, Scott MB, Tweed JKS, Pinloche E, et al. As yet uncultured bacteria phylogenetically classified as Prevotella, Lachnospiraceae incertae sedis and unclassified Bacteroidales, Clostridiales and Ruminococcaceae may play a predominant role in ruminal biohydrogenation. Environmental Microbiology. 2011;13(6):1500-12.

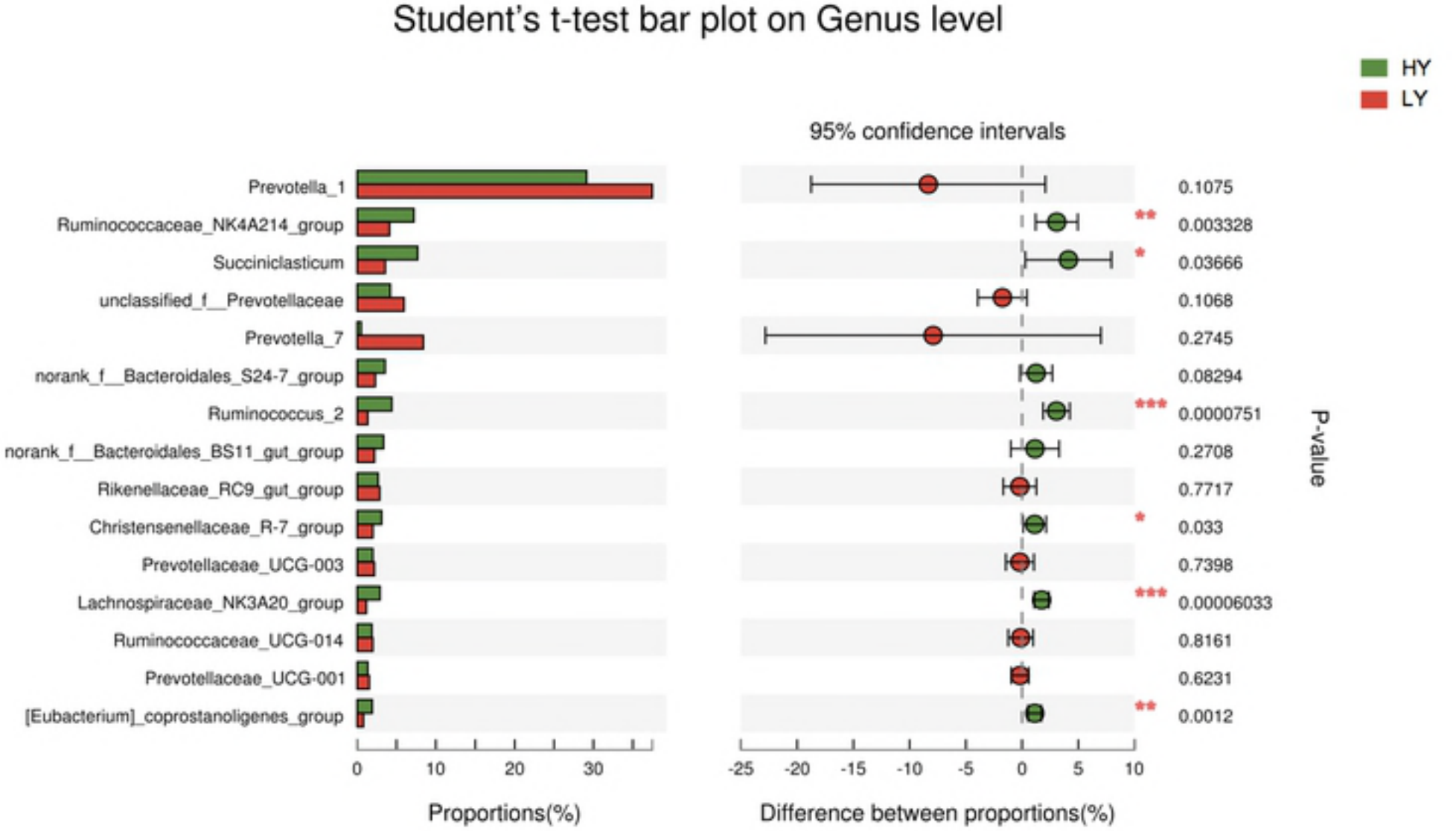
46. Hook SE, Steele MA, Northwood KS, Dijkstra J, France J, Wright AD, et al. Impact of subacute ruminal acidosis (SARA) adaptation and recovery on the density and diversity of bacteria in the rumen of dairy cows. Fems Microbiology Ecology. 2011;78(2):275–84.


Chao curves
HY
LY
1500
1000
500
0
Chao index of OTU level
10,000
20,000
30,000
40,000
Number of Reads Sampled


PCoA on OTU level
HY
LY
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
PC2(12.25%)
-0.3
-0.2
-0.1
0
0.1
0.2
0.3
PC1(24.94%)


Community barplot analysis
Percent of community abundance on Phylum level
1
0.8
0.6
0.4
0.2
0
HY
LY
Samples
Saccharibacteria
Tenericutes
Proteobacteria
Firmicutes
Bacteroidetes
others


Community barplot analysis
Percent of community abundance on Genus level
1
0.8
0.6
0.4
0.2
0
HY
LY
Samples
Acetitomaculum
Candidatus_Saccharimonas
norank_f__Bacteroidales_RF16_group
Selenomonas_1
norank_o__Mollicutes_RF9
[Eubacterium]_coprostanoligenes_group
Prevotellaceae_UCG-001
Ruminococcaceae_UCG-014
Prevotellaceae_UCG-003
Lachnospiraceae_NK3A20_group
Christensenellaceae_R-7_group
norank_f__Bacteroidales_BS11_gut_group
Rikenellaceae_RC9_gut_group
Ruminococcus_2
norank_f__Bacteroidales_S24-7_group
Prevotella_7
unclassified_f__Prevotellaceae
Succiniclasticum
Ruminococcaceae_NK4A214_group
Prevotella_1
others


Student's t-test bar plot on Genus level
HY
LY
95% confidence intervals
Prevotella_1
Ruminococcaceae_NK4A214_group
Succiniclasticum
unclassified_f__Prevotellaceae
Prevotella_7
norank_f__Bacteroidales_S24-7_group
Ruminococcus_2
norank_f__Bacteroidales_BS11_gut_group
Rikenellaceae_RC9_gut_group
Christensenellaceae_R-7_group
Prevotellaceae_UCG-003
Lachnospiraceae_NK3A20_group
Ruminococcaceae_UCG-014
Prevotellaceae_UCG-001
[Eubacterium]_coprostanoligenes_group
0.1075
** 0.003328
* 0.03666
0.1068
0.2745
0.08294
*** 0.0000751
0.2708
0.7717
* 0.033
0.7398
*** 0.00006033
0.8161
0.6231
** 0.0012
P-value
0 10 20 30
Proportions(%)
-25 -20 -15 -10 -5 0 5 10
Difference between proportions(%)