

Study on the Diversity of Epiphytic Bacteria on Corn and Alfalfa using Illumina MiSeq/NovaSeq High-throughput Sequencing System

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Research Article

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Abstract

Purpose

To investigate the diversity of the epiphytic bacteria on corn and alfalfa collected in Hengshui city and Xingtai city, hebei province, China.

Methods

Illumina MiSeq/NovaSeq High-throughput sequencing system was used to conduct Paired-end sequencing of community DNA fragments from surface of corn and alfalfa in Hengshui and Xingtai. QIIME2 and R language were employed to sort and calculate the number of sequences and taxonomic units for each sample. Thereafter, the abundance, distribution, alpha diversity index of species, beta diversity and the differences of abundance among the samples were analyzed.

Result

At phylum level, the advantage bacterium group are Proteobacteria (70%), Firmicutes (13%), Actinobacteria (9%) and Bacteroidetes (7%). The dominant genera are *Pseudomonas* (8%), *Acinetobacter* (4%), *Chryseobacterium* (3%), *Hymenobacter* (1%). *Enterobacteriaceae* (24%) are the most predominant bacteria on both corn and alfalfa samples. Alpha diversity analysis and beta diversity analysis showed that the diversity of epiphytic microbial community was significantly affected by plant species, but not by region. The diversity and richness of epiphytic bacterial community of alfalfa were significantly higher than that of corn, yet corn had more LAB than alfalfa samples.

Conclusion

This study contributes to expanding our understanding of the diversity of epiphytic microorganisms in corn and alfalfa silage and provide a basis for the selection of raw materials.

1. Introduction

Silage is the process of converting the fermentation substrate (soluble sugar) in raw materials into acidic materials such as lactic acid through the proliferation of lactic acid bacteria, creating an acidic environment and inhibiting the proliferation of harmful microorganisms, thereby preserving the nutritional content of raw materials (Zhang et al. 2011). As a silage storage technology, silage has the effects of reducing forage nutrient loss, facilitating animal digestion and absorption, increasing the value of forage utilization, expanding the source of forage, and adjusting the forage supply period (Shang et al. 2019). After silage, the nutrients will not be reduced. It also has an aromatic and sour taste, which stimulates the appetite of livestock and increases the feed intake.

Studies show that Lactic acid bacteria (LAB) play a key role during the silage fermentation processing, such as *Lactobacillus*, *Lactococcus*, *Leuconstoc*, *Streptococcus*, *Pediococcus* and *Enterococcus*

(Gharechahi et al. 2017). The number of LAB on crops determines the success of silage process. According to the different metabolites of lactic acid bacteria, they can be divided into homomorphous lactic acid bacteria and heteromorphous lactic acid bacteria. Homogeneous lactic acid bacteria can produce more lactic acid, which can improve the fermentation quality of silage. The heteromorphous lactic acid bacteria can produce lactic acid and volatile fatty acids to inhibit the growth of aerobic bacteria and improve the aerobic stability of silage.

The epiphytic microflora greatly affect the fermentation quality of silage. And the fermentation quality of silage can be affected by different crops or even the same crop grown under different environments conditions (Ali et al. 2020; Huang et al. 2019). Choosing the right forage ingredients can help improve the quality of silage. Corn and alfalfa are often used to make silage. Knowing epiphytic microflora on forage can provide scientific basis for effectively regulating the fermentation process of silage. At present, there are few reports on the microflora dwelling on corn and alfalfa. The populations of eubacteria on the corn samples collected immediately after inoculation were mainly composed of genera belonging to Proteobacteria (56.4±1.5%) orders Pseudomonadales, Xanthomonadales, and Enterobacteriales, and Bacteroidetes (37.4±1.7%) orders Sphingobacteriales and Flavobacteriales (Drouin et al, 2019). Lactobacillales were substantial contributors of the Firmicutes, with the *Leuconostocaceae* representing between 60% and 100% of the fresh forage sample composition (Drouin et al. 2019). Enterobacteriaceae are predominant on corn and alfalfa (Lin et al. 1992). Yeasts and molds are also major epiphytic microorganisms on both crops (Lin et al. 1992; Zhang et al. 2011). However, the number of LAB on the raw material for ensiling is far less than that of aerobic bacteria, *E. coli*, yeast, mold and other harmful microorganisms (Zhang et al. 2011). The number of LAB on the surface of corn is more than other raw material. However, there are few reports on the epiphytic microorganisms of silage raw materials. This study discussed the species and diversity of epiphytic bacteria on alfalfa and whole corn.

2. Materials And Methods

2.1 Collection of Samples

When alfalfa was in the budding stage to the early flowering stage, and when the corn silage material was in the late milking stage to the early waxing stage, alfalfa and corn were collected in Xingtai and Hengshui, Hebei Province. And the moisture content of them was about 50 ~ 60 %. The specific sampling results are shown in Table 1.

Table 1
Origin and grouping of microbial samples.

Places	Plants	Breed	Serial number	Groups		
Xingtai	corn	Jinchu 100	D1	D1-1	D1-2	D1-3
		Duobao No. 3	D2	D2-1	D2-2	D2-3
		Shengrui 565	D3	D3-1	D3-2	D3-3
		Yawangqingchu No. 8	D4	D4-1	D4-2	D4-3
	alfalfa	SR4030	E1	E1-1	E1-2	E1-3
		Saidi 5	E2	E2-1	E2-2	E2-3
		Zhongmu No. 1	E3	E3-1	E3-2	E3-3
Hengshui	corn	Duobao No. 3	F1	F1-1	F1-2	F1-3
		Shengrui 565	F2	F2-1	F2-2	F2-3
		Jinchu 100	F3	F3-1	F3-2	F3-3
		Yawangqingchu No. 8	F5	F5-1	F5-2	F5-3
	alfalfa	SR4030	G1	G1-1	G1-2	G1-3
		Zhongmu No. 1	G2	G2-1	G2-2	G2-3
		Saidi 5	G5	G5-1	G5-2	G5-3

2.2 Sample DNA Extraction and PCR Amplification, Quantification, Pooling and Sequencing

The DNA was extracted and quantified with Nanodrop. DNA extraction quality was detected using electrophoresis on a 1.2% agarose gel. And the variable region of rRNA gene (single or consecutive multiple) or specific gene fragment could be amplified by PCR. Subsequently, the PCR products were purified using the Vazyme VAHTSTM DNA Clean Beads and quantified by fluorescence. Sequencing libraries were prepared using Illumina's TruSeq Nano DNA LT Library Prep Kit. PCR products with appropriate concentration and the correct size of the target band were detected by 2% agarose gel electrophoresis. High-throughput sequencing was conducted using Illumina Miseq/NovaSeq.

2.3 Bioinformatics and statistical analysis

Microbiome bioinformatics were mainly performed with QIIME2 (2019.4) (Bolyen et al. 2018). Sequences were denoised using the DADA2 plugin (Callahan et al. 2016) while the OTU clustering procedure following the Vsearch (v2.13.4) (Rognes et al. 2016). The abundance, distribution, alpha diversity index of species, beta diversity and the differences of abundance among the samples were analyzed by QIIME2 and R language.

3. Results

3.1 Species Composition Analysis

Through the statistics of the ASV/OTU table after pumping, the specific composition table of microbial communities in each sample at each classification level can be obtained. Then we use the R script to plot the data in the table into a histogram (Fig. 1) to visually show the number of taxa at each classification level for different samples. In order to show the composition of all taxa at the same time, we draw a microbial classification hierarchy tree using ggtree in the R language.

The advantage bacterium group are Proteobacteria (70%), Firmicutes (13%), Actinobacteria (9%) and Bacteroidetes (7%) in all microbiome samples coming from corn and alfalfa in Hengshui and Xingtai (Fig. 2). The four dominant bacteria have more branches, which indicates that the genotypes of the dominant bacteria in the samples are diversified in evolutionary relations. Lactobacillales were found mainly in the samples of corn (Fig. 2). Enterobacteriaceae (24%) belonging to the Proteobacteria phylum are the most predominant bacteria on both corn and alfalfa samples. More than 1% of the reads from 4 genera belonging to Proteobacteria and Bacteroidetes including *Pseudomonas* (8%), *Acinetobacter* (4%), *Chryseobacterium* (3%) and *Hymenobacter* (1%) (Fig. 3).

3.2 Microbial diversity analysis of maize and alfalfa samples

3.2.1 Alpha diversity analysis

Alpha diversity represents the diversity of species within habitats. Chao1 (Chao. 1984) and Observed species indices measure community richness. Shannon and Simpson (Simpson. 1949) indices measure community diversity. Faith's PD (Faith., 1992) index represents diversity based on evolution. Pielou's evenness (Pielou. 1966) index represents the evenness. Good's coverage (Good. 1958) index represents coverage. And the specific results were plotted into a boxplot using the R script to visually show the difference of alpha diversity between different groups. It can be seen that the microbial community richness, diversity, evenness and evolutionary diversity of microbiome samples of alfalfa (group E and G) is higher than that of maize (group D and F) on average. However, the coverage of species in a community of microbiome samples of alfalfa is lower than that of maize (Fig. 4). The microbiome samples of alfalfa and maize in Xingtai have extreme significant differences in community richness and evolutionary diversity. And the microbiome samples of alfalfa and maize in Hengshui have highly significant differences in community richness, diversity and evenness. Thus, the diversity of epiphytic microorganism community is significantly affected by plant species. All alpha diversity indices of alfalfa in different areas have no significant difference. And alpha diversity indices of maize except the evolutionary diversity have no significant difference in Xingtai and Hengshui. We can conclude that the region has no significant effect on the diversity of epiphytic microbial community. The epiphytic microbial diversity of Shengrui 565 is lower than other breeds in the two places.

3.2.2 Beta diversity analysis

The microbial communities in alfalfa and maize samples were compared using NMDS based on the weighted UniFrac distance (Lozupone and Knight. 2005). Each point in the diagram represents a sample, and different colored dots indicate different samples (Fig. 5). Samples are clustered according to their similarity, and the closer the distance between two points is, the more similar the two samples are. Alfalfa group samples were aggregated in the NMDS analysis diagram, while the samples of the maize group were dispersed. Samples of corn (group D and F) are similar and the samples of alfalfa (group E and G) are similar. The results showed that epiphytic bacteria were more affected by species than by region.

3.3 Species difference analysis and biomarker

The number of ASV/OUT in group D, E, F and G are 6683, 8305, 6920, 8080 respectively (Fig. 6). And there are 545 ASV/OUTs in common.

We use the abundance data of the top 50 genera in average abundance to make a heat map. In the genus-level species composition heat map for species clustering, red patches indicate that the genera are more abundant in this sample than other samples, and blue patches indicate that the genera are less abundant in this sample than other samples. Lactic acid bacteria have an important effect on the silage fermentation, such as *Leuconostoc* and *Lactobacillus* in the top 50 genera in average abundance. *Leuconostoc* mainly exist in group D2, F1, F2 and F3. *Lactobacillus* mainly exist in group D1, D3, D4, F1, F2, F3, E1, E2 and E3 (Fig. 7). Group F1 and F2 have more *Clostridium sensu stricto 1* that are harmful to fermentation (Fig. 7).

Through the algorithm analysis of Random Forests (Breiman. 2001), we obtained the distribution of important species in each group (Fig. 8). The abscissa is the importance of species to the classifier model, the ordinate is the taxon name at the level of genus from top to bottom, and the importance of species in influencing grouping decreases successively. These highly important species can be considered markers of differences in these groups, and they are *Pedobacter*, *Nocardioides*, *Chryseobacterium*, *Burkholderia - Caballeronia - Paraburkholderia*, *Paracoccus*, *Pseudomonas*, *Acinetobacter*, *Allorhizobium - Neorhizobium - Pararhizobium - Rhizobium*, *Larkinella*, *Mucilaginibacter*, *Sphingomonas*, *Brevundimonas*, *Siphonobacter*, *Methylobacterium*, *Spirosoma*, *Hymenobacter*, *Bacillus*, *Actinomycespora*, *Taibaiella*, *Sphingobacterium*. *Bacillus* mainly exist in SR4030 and Saidi 5 in Xingtai.

4. Discussion

The results show that the diversity of epiphytic microorganism community is not affected by region, yet it is significantly affected by plant species. It may also be because the environment of the two places is similar and it cannot affect the epiphytic microorganisms of the plants. The species and number of epiphytic microorganisms on different silage raw materials are quite variable. Epiphytic microorganisms of forage are affected by forage species, stage of maturity, weather, mowing, field-wilting, the chopping process humidity, solar radiation, plant surface structure and plant nutrient distribution (Lin et al. 1992; Bai. 2011).

The diversity and richness of epiphytic bacterial community of alfalfa were significantly higher than that of corn, yet corn had more LAB than alfalfa samples. Many studies have shown that corn have more LAB than other crops. For example, the number of LAB on the surface of corn was twice that of sorghum and alfalfa, and 20 times that of ryegrass (Cai et al. 1999). And the total number of LAB on corn is 7 times that of grass and 15 times that of clover (Kasmaei et al. 2017). The contents of lactic acid and acetic acid in silage corn, elephant grass and sugarcane top were significantly increased by adding the epiphytic microorganisms of corn straw, and the aerobic stability of elephant grass silage was positively affected (Huang et al. 2020). Mogodiniyai found that the increase of lactic acid and acetic acid content in corn straw silage by epiphytic microorganisms may be related to the abundance of *Lactococcus* and *Leuconostoc* (Mogodiniyai et al. 2017). The crops have more LAB are more suitable for silage. Thus corn is possible better for silage than alfalfa.

Duobao No.3 and Shengrui 565 in Hengshui have more *Clostridium sensu stricto 1* that are harmful to fermentation. *Bacillus* mainly exist in SR4030 and Saidi 5 in Xingtai. There are some microorganism undesirable for the fermentation process and the silage quality, such as anaerobic bacilli of the genus *Clostridium*, aerobic bacteria of the genus *Bacillus*, coliform bacilli. (Fabiszewska et al. 2019). Tao Lian et al. used Miseq high-throughput sequencing technology to analyze the change of microflora structure in corn stalk before and after natural silage. The results showed that the number of bacteria belonging to *Firmicutes*, *Bacilli*, *Lactobasubcillales*, *Lactobacillaceae*, *Pediococcus* and *Lactobacillus* increased, while *Proteobacteria* and *Enterobacteriaceae* decreased (Tao et al. 2016). It was found that the aerobic stability was increased by 66~312 h after the quantity of *Clostridium* in silage and the content of ammonia-butyrate nitrogen were decreased in the aerobic stability test of silage corn (Jatkauskas et al. 2013).

Epiphytic bacteria of crops run through the whole fermentation process, affecting the quality of silage. These bacterial communities also have a succession process, indicating that the microorganism attached to the forage itself has a great impact on the quality of silage. However, the study of silage microbial community and its mechanism of succession is still unclear, and more information is needed to reveal this complex fermentation process (Xu et al. 2017).

Conclusion

In summary, the advantage bacterium group are Proteobacteria (70%), Firmicutes (13%), Actinobacteria (9%) and Bacteroidetes (7%) on corn and alfalfa in Xingtai and Hengshui. At the genus level, *Pseudomonas* (8%), *Acinetobacter* (4%), *Chryseobacterium* (3%), *Hymenobacter* (1%) were the main bacteria genera. This study showed that the diversity of epiphytic microbial community was significantly affected by species, but not by region. The composition richness and diversity of microbe of alfalfa are higher than that of maize in both Xingtai and Hengshui, yet corn have more LAB than alfalfa samples.

Declarations

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Author contributions

H.T. (Professor) planned and designed this study. M.W. (graduate student) analyzed data and wrote this article.

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Availability of data and materials

Data is available under request.

Ethics approval and consent to participate

Not applicable

Consent for publication

All coauthors have consent and approve the paper for publication.

Competing interests

The authors declare they have no conflict of interests.

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Figures

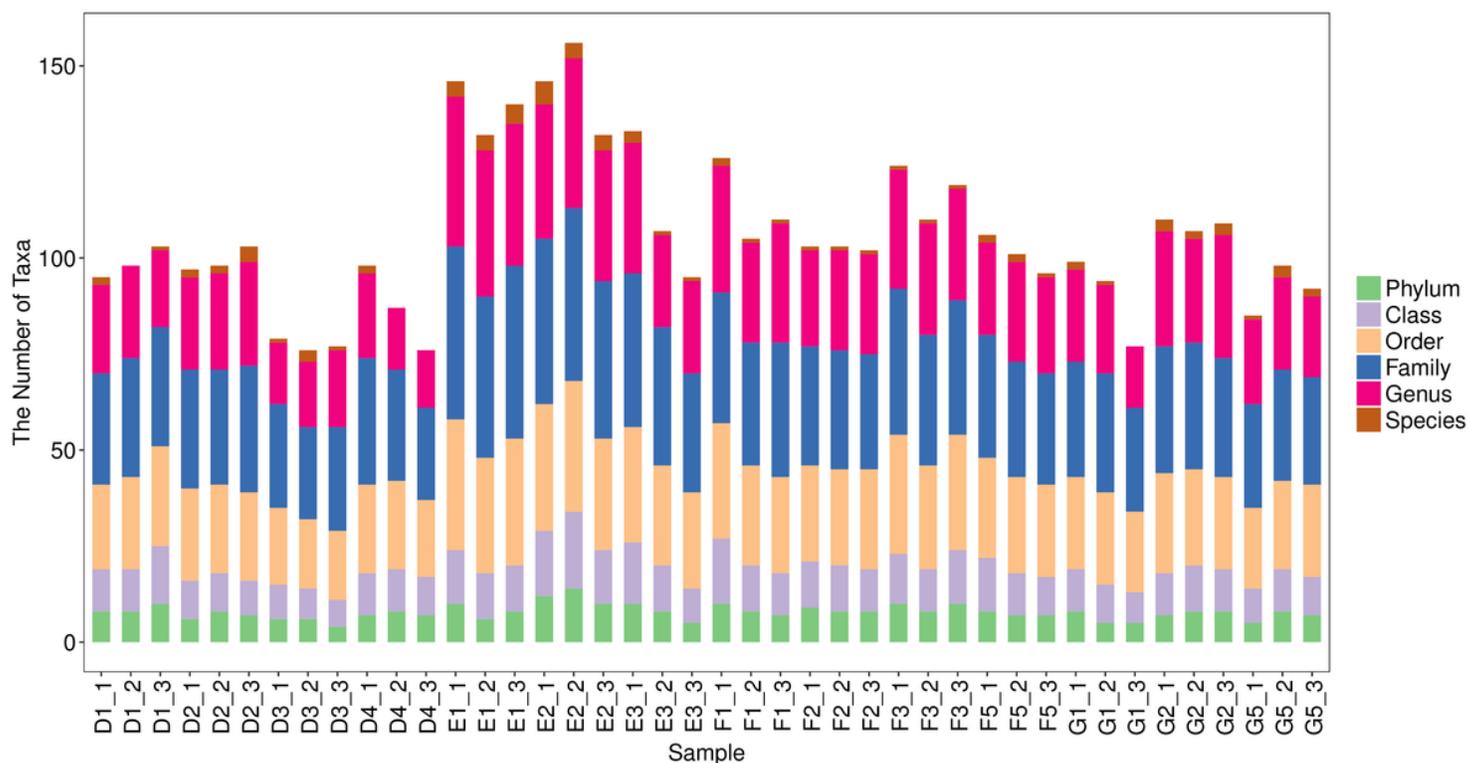


Figure 1

The number of taxa at each classification level for different samples.

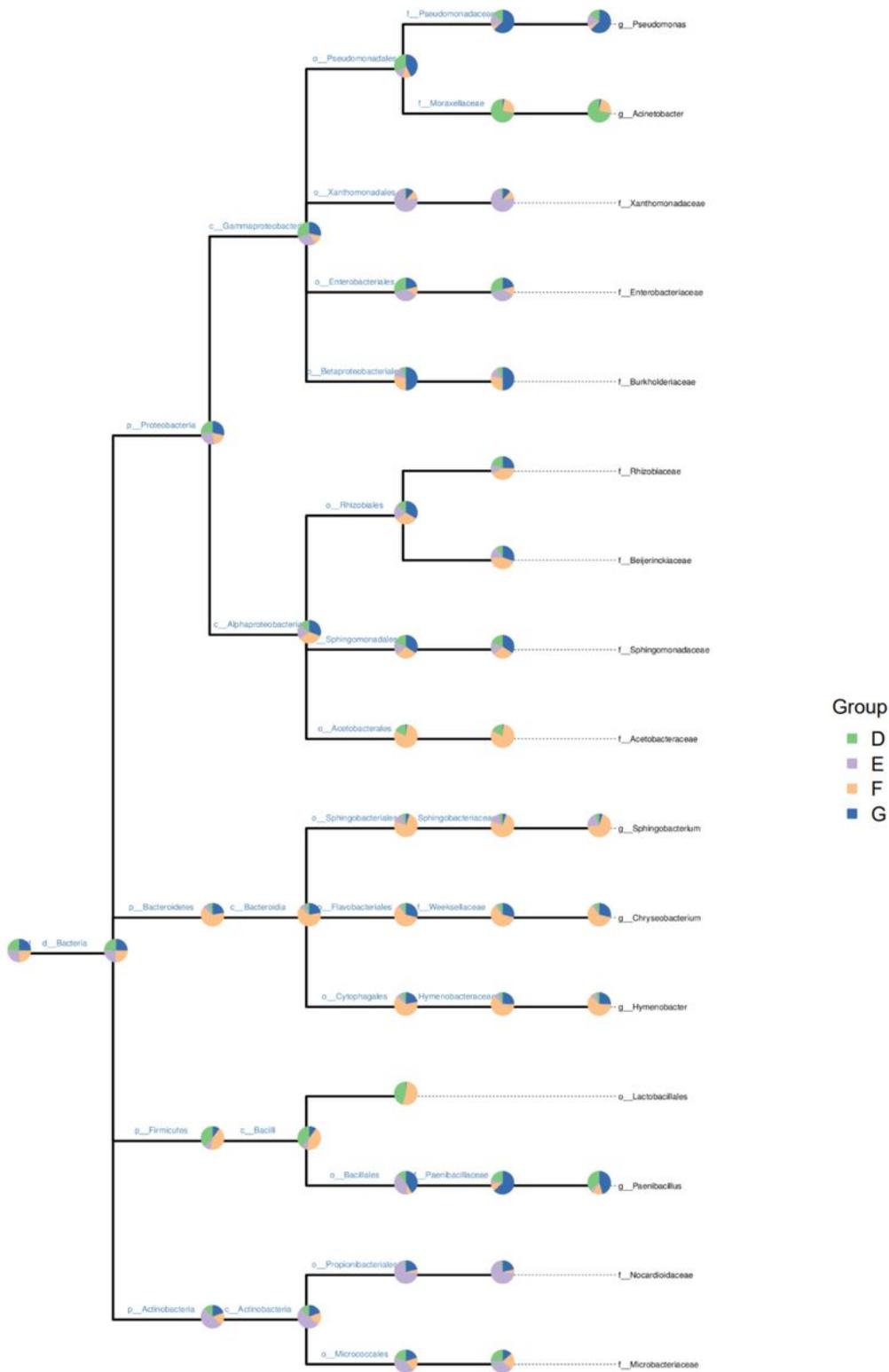


Figure 2

The pie chart (threshold 0.5%) of each branch node in the classification tree shows the proportion of the taxon in each group. The larger the sector area, the higher the abundance of the taxon in the group.

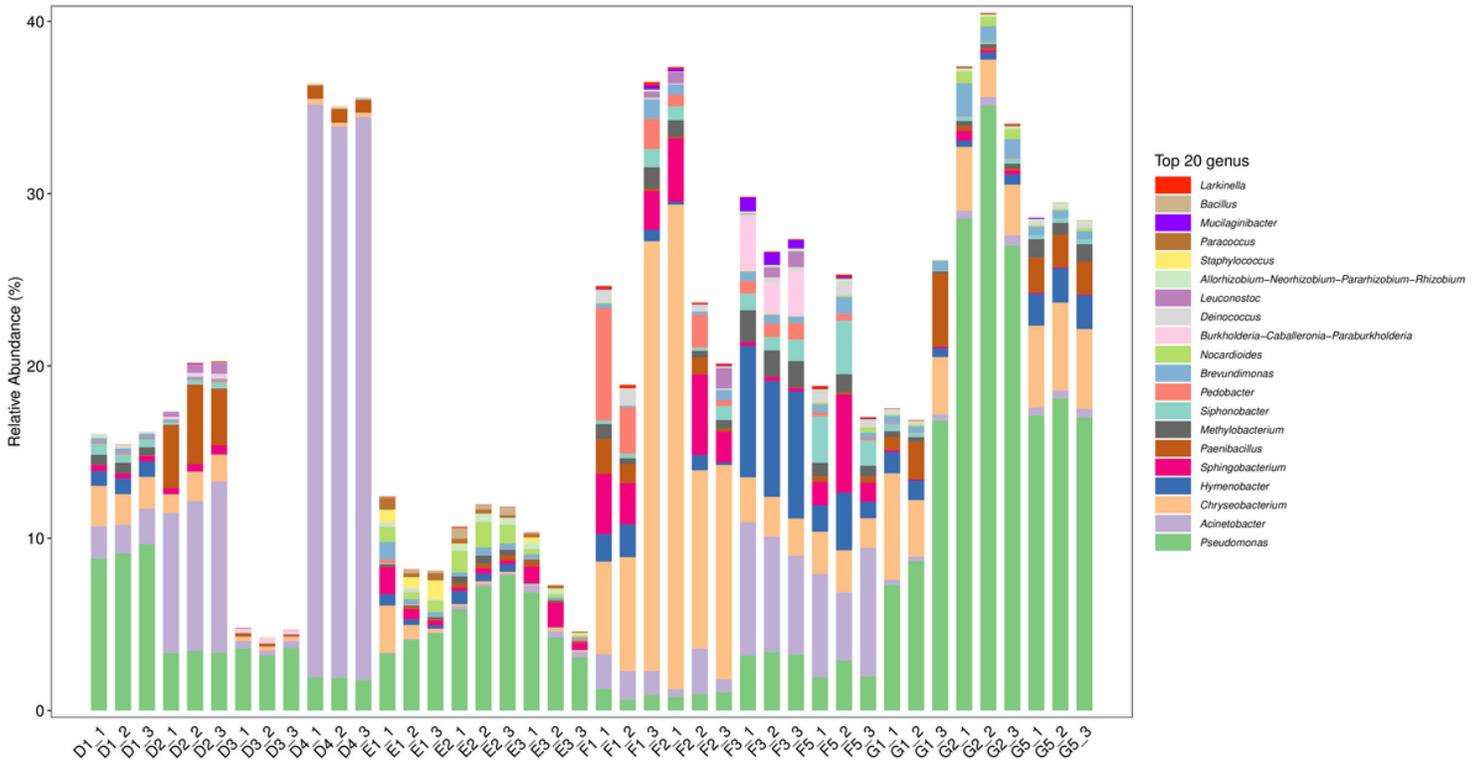


Figure 3

Histogram of phylum-level species composition.

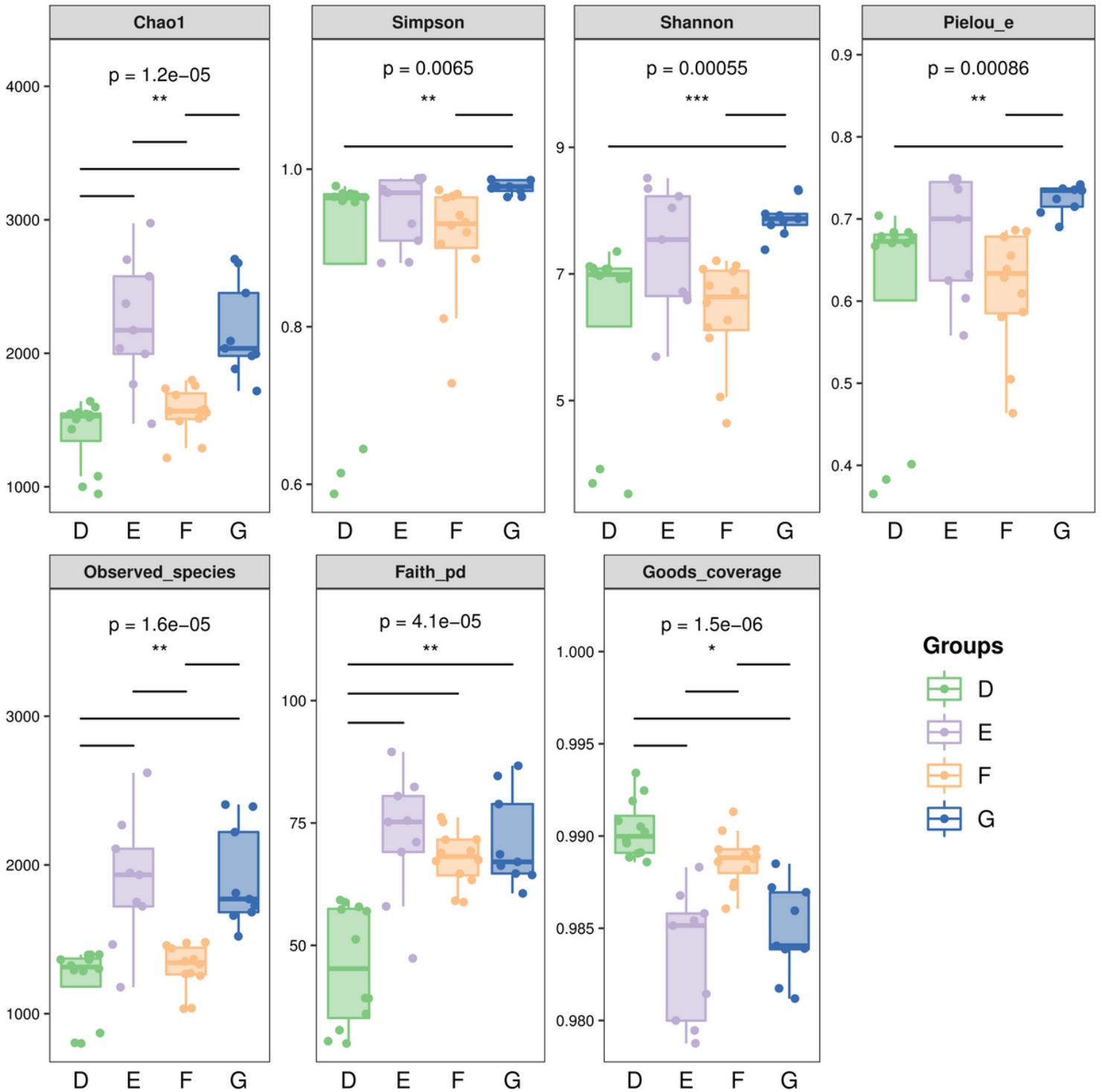


Figure 4

Grouping box plot of Alpha diversity index.

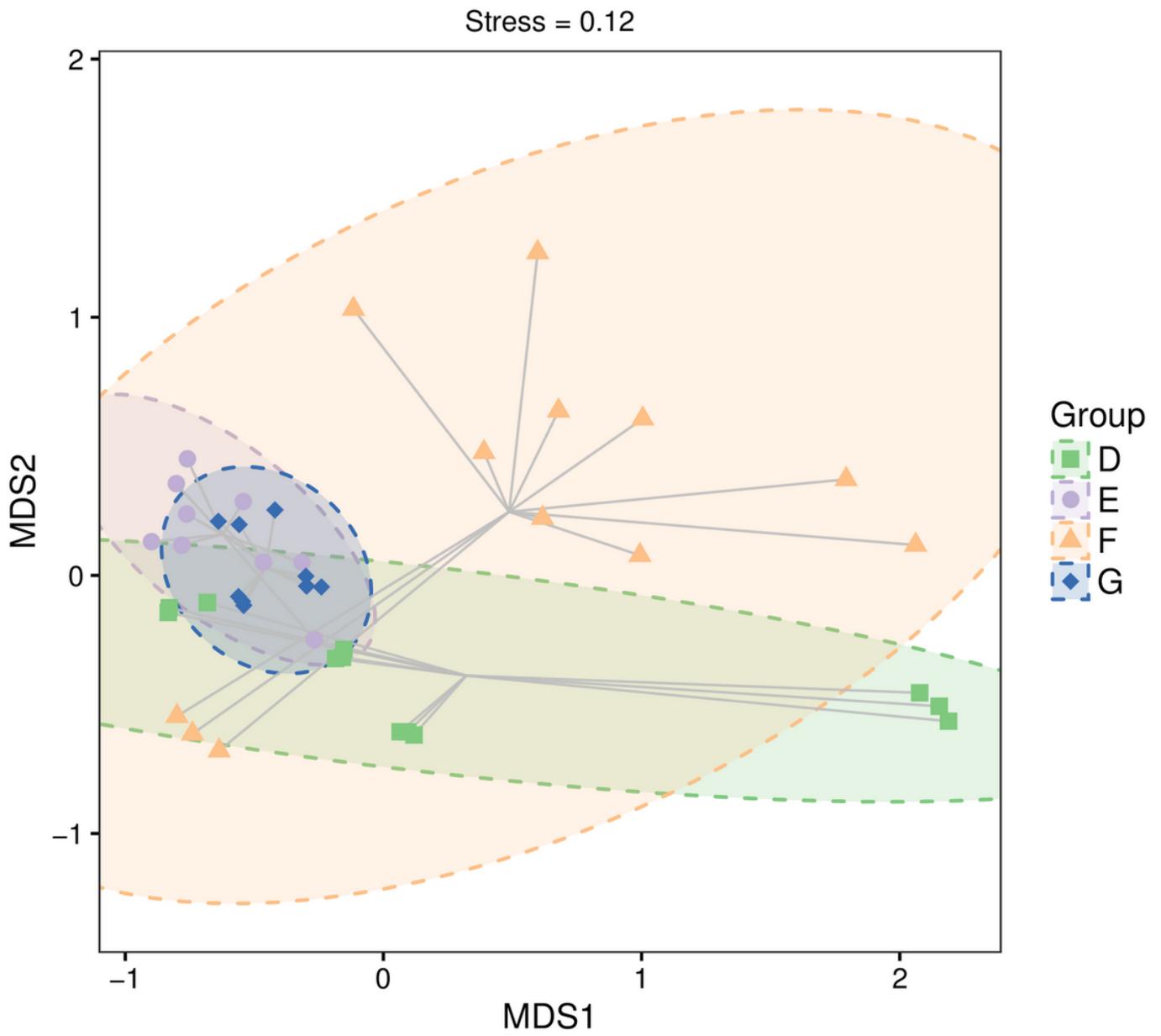


Figure 5

NMDS analysis based on the weighted UniFrac distance.

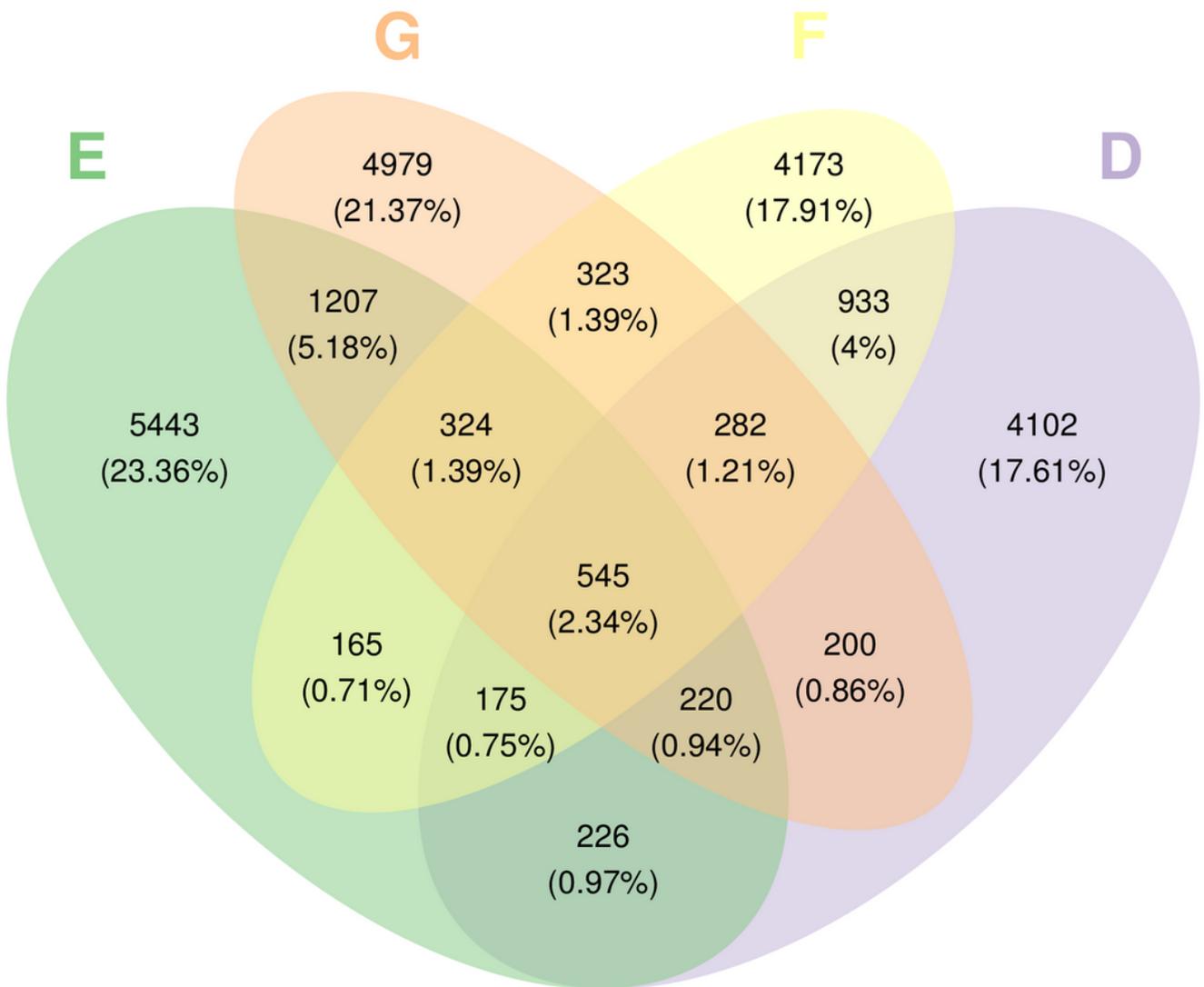


Figure 6

Venn diagram of the sample OTUS distribution.

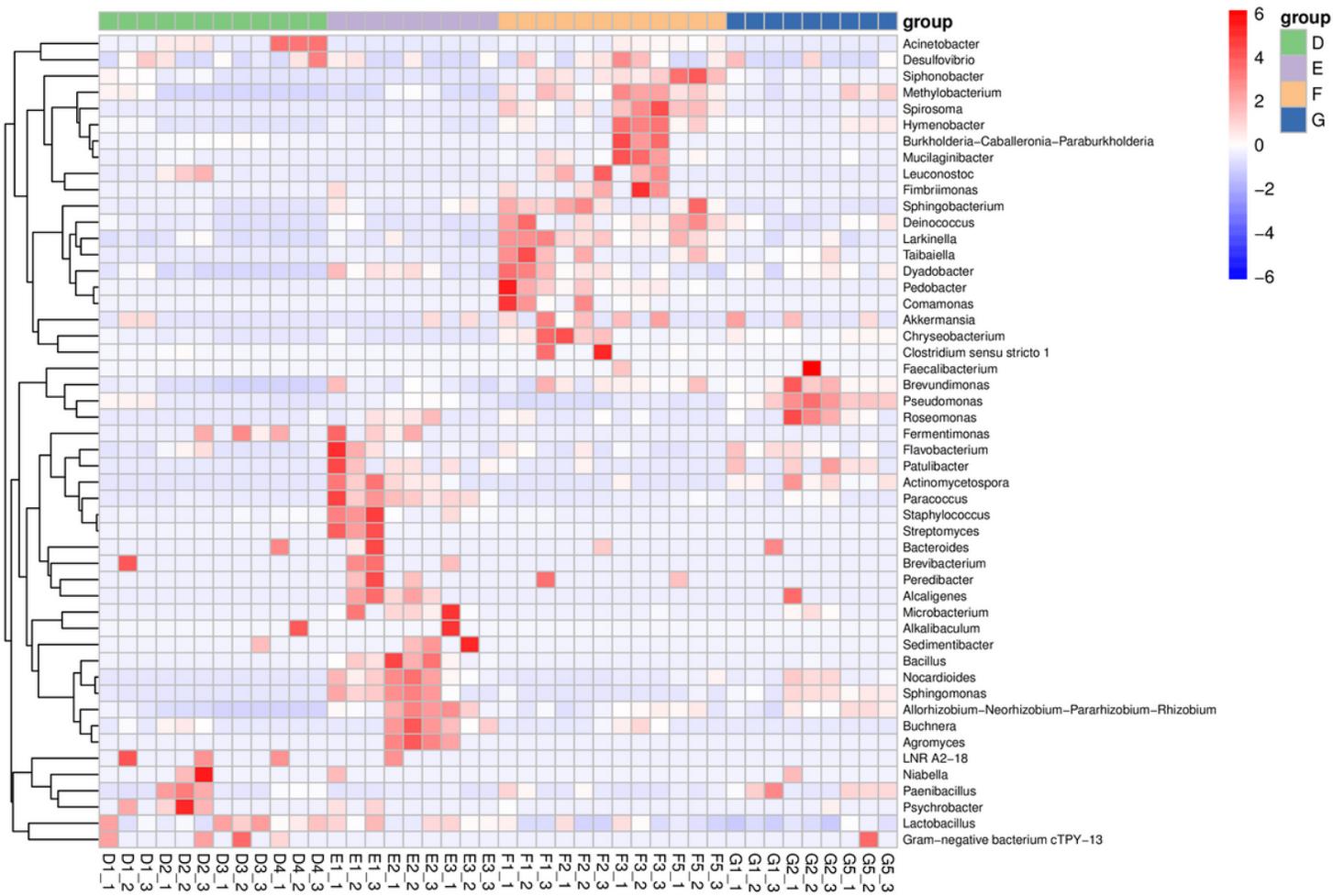


Figure 7

Genus-level species composition heat map for species clustering.

