Bacteria Community Structure Analysis of Daqu in Different Storage Periods of SheDian Liquor

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Abstract

[Purpose] In order to study the diversity of bacterial communities in different storage periods of Daqu of Shedian liquor.

[Methods]We probed into the changes of bacterial community compositions in Daqu (yeasts for making hard liquors) of Shedian liquor after different storage periods and identified 20 phyla and 268 genera of bacterial communities in the bacterial 16S rDNA V3-V4 zone by using high- throughput sequencing.

[Result]It was found the absolutely dominant phylum was Firmicutes in both the 3- and 6-month-old Daqu, accounting for 62.66% and 63.53% respectively, but was Proteobacteria (80.86%) in the 9-month-old Daqu. The absolutely dominant genera were *Lactococcus* (27.90%) and *Enterobacter* (23.14%) in the 3-month-old Daqu, were the *unclassified f Peptostreptococcaceae* (24.64%) and *Escherichia-Shigella* (11.80%) in the 6-month-old Daqu, and was *Enterobacter* (79.80%) in the 9-month-old Daqu.

[Conclusion]From the perspective of bacterial community composition, the Daqu after 6 months of storage is most favorable for white liquor production and fermentation.

Introduction

Daqu, a saccharification yeast starter used in fermentation of Luzhou-flavor liquors(Zou et al. 2018), not only is the enzyme needed by white liquor fermentation, but also offers abundant microorganisms for fermentation and fragrance formation(Wang et al. 2019). Daqu is involved in many biochemical reactions during the storage period in yeast masses, so the enzymatic system, substance system and bacterial system are all changing continually(Yang et al. 2017). Particularly, the key influence factor on the quality of Daqu and liquors is microorganisms(Du et al. 2019). New Daqu contains diverse bacteria and large biomass, which easily lead to rapid temperature rise and excessive total acids in fermented grains(Song et al. 2017). Moreover, too long storage period will result in severe loss of starches from Daqu masses(Ståhl et al. 2012). So far, the maturity of Daqu during production is mainly decided according to operating experience, which is limited by certain risks and is unfavorable for the prediction of subsequent production.

The Daqu at different storage periods has been mainly studied by traditional isolated culture or polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE). However, the microbial strain isolation is feasible only for research on separable species in Daqu(Pace et al. 1997). The PCR cloning library, PCR-DGGE, and single-strand conformation polymorphism amplification are limited by huge workloads and low sensitivity and thus cannot correctly reflect the varying trends of microbial communities(Dalmasso et al. 2016). In comparison, high-throughput sequencing can rapidly analyze the diversity of complex microbial communities and detect the unculturable microorganisms with low existing rates(Tang et al. 2017). This technique has been extensively applied to research on biological community compositions in soils (Bell et al. 2013), foods(Polaet et al. 2017), and medicine (Soon et al. 2017).

In recent years, high-throughput sequencing has been gradually used into research on liquor production, but relevant research on bacterial community variation during storage period of Daqu is concentrated in Sichuan, but not in Henan. In fact, the Daqu from different regions is largely different in microorganism species and composition owing to the discrepancy in raw material composition, technology and climate. Hence, in this study, Daqu in Shedian liquor at different storage periods was studied. Total DNA was extracted from Daqu microorganisms and after PCR amplification, the bacterial sequences of Daqu were analyzed via high-throughput sequencing. The differences in bacterial community compositions at different storage periods were investigated. From the perspectives of basic composition and abundance variation of bacteria, the findings will theoretically underlie the reasonable judgement of optimal Daqu storage time and Luzhou-flavor liquor starter propagation process.

Materials And Methods

Materials and reagents

Daqu was sampled from SheDianLaoJiu Co. Ltd. (Henan Province, China). Other materials or reagents used here included a FastDNA SPIN kit for soils (MP Biomedicals, USA); a DNA polymerase AP221-02 kit, a Trans DNA 15KMarker (both TransGen Biotech, Beijing); a product recovery kit (Tiangen Biotech Beijing Co., Ltd).

Instruments and facilities

The instruments used here included a Julabo TW12 thermostat water bath (JULABO Technology); a NanoDrop 2000 ultraviolet-visible pectrophotometer, an ST16R high-speed freezing centrifuge, a PICO17 minitype desk centrifuge (all Thermo Fisher Scientific, China); a GeneAmp® 9700 PCR analyzer (ABI, USA); a QuantiFluor™-ST DNA quantification analyzer (Promega, USA); an Illumina Miseq high-throughput sequencing analyzer (Illumina, USA).

Daqu collection

The samples were divided into 3 storage periods, including 3-month (A), 6-month (B) and 9-month (C). From Daqu of the same quality and different batches, a five-point sampling method was adopted, and then the samples were crushed, mixed, sealed and stored at -20°C.

DNA extraction

Total DNA was extracted by using a FastDNA SPIN Kit for Soil according to the manual. DNA concentration and purity were detected on a NanoDrop2000 instrument, and DNA quality was analyzed via 1% agarose gel electrophoretic analysis.

PCR amplification and high-throughput sequencing

The primers used in PCR amplification of V3-V4 variable regions were 338F (5'-ACTCCT ACGGGAGGCAGCAG-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3'). The conditions were: an

amplification system of 20 μL, a buffer solution of 4 μL 5*FastPfu, 2 μL 2.5 mM dNTPs, 0.8 μL of primers (5 uM), 0.4 μL of FastPfu polymerase, and 10 ng of a DNA template. The amplification program was: pre-denaturation at 95°C for 3 min, 30 cycles (denaturation at 95°C for 30 s, annealing at 55°C for 30 s, extention at 72°C for 45 s), extention at 72°C for 10 min. The PCR products were recovered by 2% agarose gel, purified by an AxyPrep DNA gel extraction kit, eluted by Tris-HCl, and detected by 2% agarose electrophoresis. The gel electrophoresis results of the PCR products were shown below.

Quantitative analysis was conducted via QuantiFluor™-ST. The library establishment and high-throughput sequencing were finished by Sangon Biotech (Shanghai) Co., Ltd. (China).

Data processing

The original sequences were sent to quality control on Trimmomatic and spliced on Flash. The operational taxonomic units (OTUs) were clustered on Uparse at the similarity of 97%, and chimaeras were deleted on Uchime. The species of each sequence were classified and annotated with an RDP classifier and compared with the Silva database (SSU123) at the threshold of 70%.

Results

Sequence number and diversity indices

The PE reads obtained from Miseq sequencing were first spliced as per the overlap relationship, and the sequences were quality-controlled and filtrated. After that, the sequences were sent to OTU clustering and species taxonomic analysis. Based on OTUs, diversity indices and sequencing depth were analyzed.

Table 1
OTUs and diversity index analysis

Sample	Effective	OTU	Shannon	Chao1	Coverage
A	75402	458	3.46307	361.5	0.998803
B	47143	469	3.843066	414.1	0.996669
C	49840	237	1.909372	153.8	0.998335

As the sequence number increased, the Shannon index dilution curves of all samples became flattened (Fig. 2). The Coverage rates of all samples exceeded 99% (Table 1), indicating the sequencing depth of this study was enough for analysis of bacterial community composition and diversity. After quality control, 75402, 47143 and 49840 sequences were identified from the 3-, 6- and 9-month-old Daqu respectively. OTU clustering analysis at the similarity above 97% returned 458, 469 and 237 OTUs respectively. The Alpha diversity indices usually consist of Shannon index and Chao1 index. A larger Shannon index indicates a higher diversity of the tested community, and the Chao1 index often estimates total species number, and a larger Chao1 means higher species abundance. Results showed the Shannon index and Chao1 both maximized in the 6-month-old Daqu (3.843066 and 414.1 respectively), indicating

the diversity of bacterial communities was the highest in the 6-month-old Daqu compared with the other two types of Daqu.

OTU Venn diagram

Venn diagrams can statistically reflect the numbers of shared OTUs and exclusive OTUs in a certain sample and visually display the similarity and overlap of OTUs.

From the 3-, 6- and 9-month-old Daqu, 458, 469 and 237 OTUs were identified, and the three types of Daqu shared 54 OTUs, indicating bacterial species of the shared OTUs coexisted at different storage periods of Daqu (Fig. 3). The 3-, 6- and 9-month-old Daqu possessed 189, 221 and 12 exclusive OTUs respectively, and particularly, nearly half of OTUs in either the 3- or 6-month-old Daqu were exclusive, but the 9-month-old Daqu possessed very few exclusive OTUs. Venn diagrams showed the bacterial community compositions of Daqu slightly differed among different storage periods.

Bacterial community level analysis

Taxonomic analysis identified 20 phyla, 38 classes, 84 orders, 138 families, 268 genera and 396 species. The results of species diversity analysis at the phylum level and genus level were illustrated in Figs. 4 and 5 respectively.

With the threshold at relative abundance > 0.5% at the phylum level, 6 predominant bacterial phyla were identified from the 3-month-old Daqu, including Firmicutes (62.66%), Proteobacteria (27.15%), Bacteroidetes (4.40%), Cyanobacteria (1.89%), Actinobacteria (1.30%) and Chloroflexi (0.70%) (Fig. 4). Six predominant phyla were screened from the 6-month-old Daqu, including Firmicutes (63.53%), Proteobacteria (15.26%), Bacteroidetes (14.87%), Spirochaetae (3.08%), Fusobacteria (2.06%) and Actinobacteria (0.65%) (Fig. 4). Four dominant phyla were found in the 9-month-old Daqu, including Proteobacteria (80.86%), Firmicutes (17.39%), Bacteroidetes (0.63%) and Cyanobacteria (0.61%) (Fig. 4).

With the threshold at relative abundance > 1% at the genus level, 10 predominant bacterial genera were recognized from the 3-month-old Daqu, including *Lactococcus* (27.90%), *Enterobacter* (23.14%), *Lactobacillus* (14.81%), *Weissella* (7.12%), *Bacillus* (2.32%), *Pediococcus* (1.97%), *Staphylococcus* (1.61%), *norank c Cyanobacteria* (1.48%), *Enterococcus* (1.48%), and *norank f Bacteroidales S24-7 group* (1.00%) (Fig. 5). Eight predominant genera were recognized from the 6-month-old Daqu, including *unclassified f Peptostreptococcaceae* (24.64%), *Escherichia-Shigella* (11.80%), *Terrisporobacter* (6.47%), *Clostridium sensu stricto 1* (5.68%), *Lactobacillus* (4.61%), *norank f Bacteroidales S24-7 group* (3.65%), *Ruminococcaceae UCG-005* (2.88%), and *Rikenellaceae RC9 gut group* (2.63%). Four predominant genera were recognized from the 9-month-old Daqu, including *Enterobacter* (79.80%), *Lactobacillus* (6.32%), *Weissella* (4.59%) and *Pediococcus* (1.59%) (Fig. 5).

Phylogenetic tree analysis

The sequences corresponding to the top 50 species in terms of abundance were selected and thereby a phylogenetic tree as per the maximum likelihood method was built on FastTree. The phylogenetic tree

was plotted with R-language (Fig. 6).

Based on the phylogenetic tree, the biological evolution can be deduced so as to uncover the evolutionary history and mechanism.

Discussion

The bacterial communities of Daqu at different storage periods were analyzed with high- throughput sequencing, which uncovered totally 20 phyla, 38 classes, 84 orders, 138 families, 268 genera and 396 species. With the threshold at relative abundance > 0.5% at the phylum level, 6 predominant bacterial phyla were identified from the 3-month-old Daqu, including Firmicutes (62.66%), Proteobacteria (27.15%), Bacteroidetes (4.40%), Cyanobacteria (1.89%), Actinobacteria (1.30%) and Chloroflexi (0.70%). Six predominant phyla were found in the 6-month-old Daqu, including Firmicutes (63.53%), Proteobacteria (15.26%), Bacteroidetes (14.87%), Spirochaetae (3.08%), Fusobacteria (2.06%) and Actinobacteria (0.65%). Four predominant phyla were identified from the 9-month-old Daqu, including Proteobacteria (80.86%), Firmicutes (17.39%), Bacteroidetes (0.63%) and Cyanobacteria (0.61%). With the prolonging of storage period, the abundance levels of Firmicutes and Bacteroidetes both first rose and then declined, while those of Proteobacteria and Cyanobacteria first dropped and then increased. At the relative abundance > 0.5%, both Actinobacteria and Chloroflexi were only detected in the 3-month-old Daqu. As reported, predominant phyla including Proteobacteria, Firmicutes, Bacteroidetes and Actinobacteria were detected from Daqu using high-throughput sequencing (Liang et al. 2017), which are very similar to our results, but we also detected Spirochaetae and Fusobacteria.

With the threshold at relative abundance > 1% at the genus level, 10 predominant bacterial genera were recognized from the 3-month-old Daqu, including *Lactococcus* (27.90%), *Enterobacter* (23.14%), *Lactobacillus* (14.81%), *Weissella* (7.12%), *Bacillus* (2.32%), *Pediococcus* (1.97%), *Staphylococcus* (1.61%), *norank c Cyanobacteria* (1.48%), *Enterococcus* (1.48%), and *norank f Bacteroidales S24-7 group* (1.00%). 8 predominant genera were recognized from the 6-month-old Daqu, including *unclassified f Peptostreptococcaceae* (24.64%), *Escherichia-Shigella* (11.80%), *Terrisporobacter* (6.47%), *Clostridium sensu stricto 1* (5.68%), *Lactobacillus* (4.61%), *norank f Bacteroidales S24-7 group* (3.65%), *Ruminococcaceae UCG-005* (2.88%), and *Rikenellaceae RC9 gut group* (2.63%). 4 predominant genera were recognized from the 9-month-old Daqu, including *Enterobacter* (79.80%), *Lactobacillus* (6.32%), *Weissella* (4.59%) and *Pediococcus* (1.59%). At the genus level, the bacterial community compositions of Daqu varied largely among different storage periods. The absolutely dominant genera in the 3-month-old Daqu included *Lactococcus* and *Enterobacter*. The absolutely dominant genera in the 6-month-old Daqu included *unclassified f Peptostreptococcaceae* and *Escherichia-Shigella*. The absolutely dominant genus in the 9-month-old Daqu was *Enterobacter*. At the relative abundance > 1%, *Terrisporobacter*, *Clostridium sensu stricto 1* and *Ruminococcaceae UCG-005* were found only in the 6-month-old Daqu.

Studies have shown that Coccus and Bacillus are the main force of bacteria in Daqu.The dominant genera identified from the 3- or 9-month-old Daqu mostly can produce lactic acid, but excessive ethyl

lactate concentrations will cause inharmonious fragrance in liquors(He et al. 2018; Zheng et al. 2013). The exclusive genera found in the 6-month-old Daqu were *Terrisporobacter*, *Clostridium sensu stricto 1*, and *Ruminococcaceae UCG-005*, which were caproic acid bacteria, metabolites of which are the major fragrant substances of Luzhou-flavor liquors. The Lactobacillus, Lactococcus and Weissella identified from the 3-month-old Daqu were all lactic acid bacteria(Horvath et al. 2009). During the production of white liquors, lactic acid bacteria can ferment carbohydrates, produding abundant lactic acids, but excessive lactic acids will lead to a bitter taste of liquors and decrease the liquor quality. Moreover, the acid environment will produce bioamine(Linares et al. 2011), but the intake of excessive bioamine into the body will cause adverse reactions(Mah et al. 2002). The *Clostridium sensu stricto 1* identified from the 6-month-old Daqu belongs to Clostridia, which is a dominant class of caproic acid bacteria during the production of Luzhou-flavor liquors(Hu et al. 2015). *Terrisporobacter* and *Ruminococcaceae* are also caproic acid bacteria(Zheng et al. 2013). The *Enterobacter* found in the 9-month-old Daqu can ferment glucoses to form lactic acid(Li et al. 2015) and is also a bioamine-producing bacterium in Daqu (Curiel et al. 2011). At the same time, the high content of ethyl lactate formed by lactic acid can inhibit the main aroma of liquor(Li et al. 2015).Based on the characteristics of the bacteria mentioned above and the diversity and abundance of bacterial communities in the 6-month-old Daqu, we suggest the storage period of Daqu for fermentation is 6 months. Liu Junhong et al. studied the growing rules of microorganisms in Daqu after different storage periods by using the traditional isolating culture and found the 4- to 6-month-old Daqu masses were suitable for fermentation(Liu et al. 2009). With high-throughput sequencing, Liang et al.(2017)explored the prokaryotic microoganism composition and flavor compositional variation of 0- to 6-month-old Daqu and found the 4- to 6-month-old Daqu was more suitable for fermentation of high-quality liquors. Our findings are basically consistent with these studies.

Moreover, the species identified from Daqu of Luzhou-flavor liquors for the first time are *norank c Cyanobacteria*, *Escherichia-Shigella*, *Terrisporobacter*, *norank f Bacteroidales S24-7 group*, *Ruminococcaceae UCG-005*, and *Rikenellaceae RC9 gut group*. With high-throughput sequencing, Hu et al. detected *norank c Cyanobacteria* from ensilage corn straws at the genus level(Hu et al. 2018). Dai Yijie et al. found with high-throughput sequencing that the dominant genus in the Daqu of Maotai-flavor liquors was *Escherichia-Shigella* (Dai et al. 2019). *Terrisporobacter* was found in the washes of Luzhou-flavor liquors(Li et al. 2018). High-throughput sequencing also identified *norank f Bacteroidales S24-7 group* in the intestinal tracts of mice(Li et al. 2018). Moreover, high-throughput sequencing identified *Ruminococcaceae UCG-005* and *Rikenellaceae RC9 gut group* in the microbial communities of milch cow claw corners (Bay et al. 2018). Nevertheless, the functions of bacteria in the fermentation of Luzhou-flavor liquors should be further investigated.

Totally 396 bacterial species were identified through high-throughput sequencing, and a resource library of Daqu bacteria from Luzhou-flavor liquors after different storage periods was preliminarily established. The information of bacteria was richer compared with traditional isolating culture. This study enriches the knowledge on the diversity of bacterial communities in the Daqu of Luzhou-flavor liquors and will theoretically underlie the rational judgement of Daqu storage time during practical productions and promote the standardized and scientific Daqu production.

Declarations

Ethics approval and consent to participate: This article does not contain any studies with human participants or animals performed by any of the authors.

Consent for publication: Not applicable.

Availability of data and materials: The datasets used or analysed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare that they have no competing interests.

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Authors' contributions: Chunmei Pan contributed to the conception of the study;Shangping Jin, Zhijun Zhao performed the experiment;Junying Fu,Xian Wang contributed significantly to analysis and manuscript preparation;Yanbo Liu performed the data analyses and wrote the manuscript;Xiyu Sun, Suna Han helped perform the analysis with constructive discussions.

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Figures

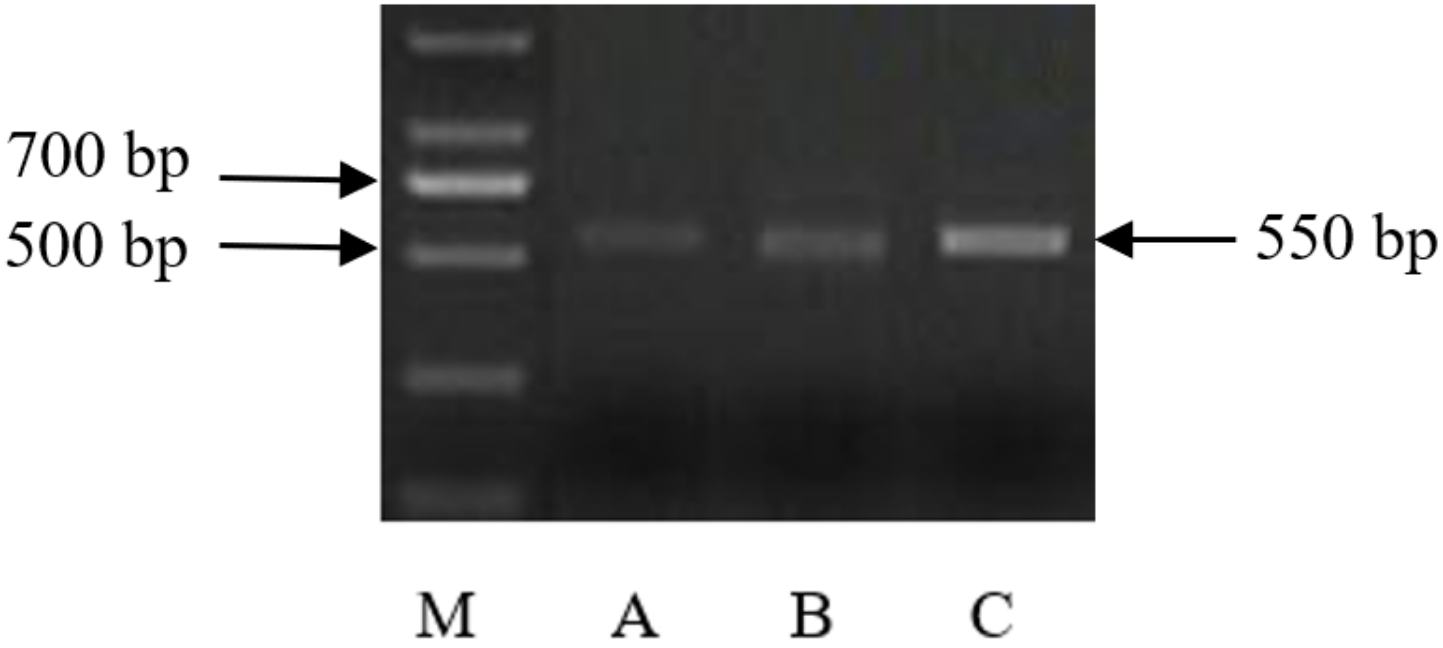

Figure 1

PCR products of Daqu at different storage periods

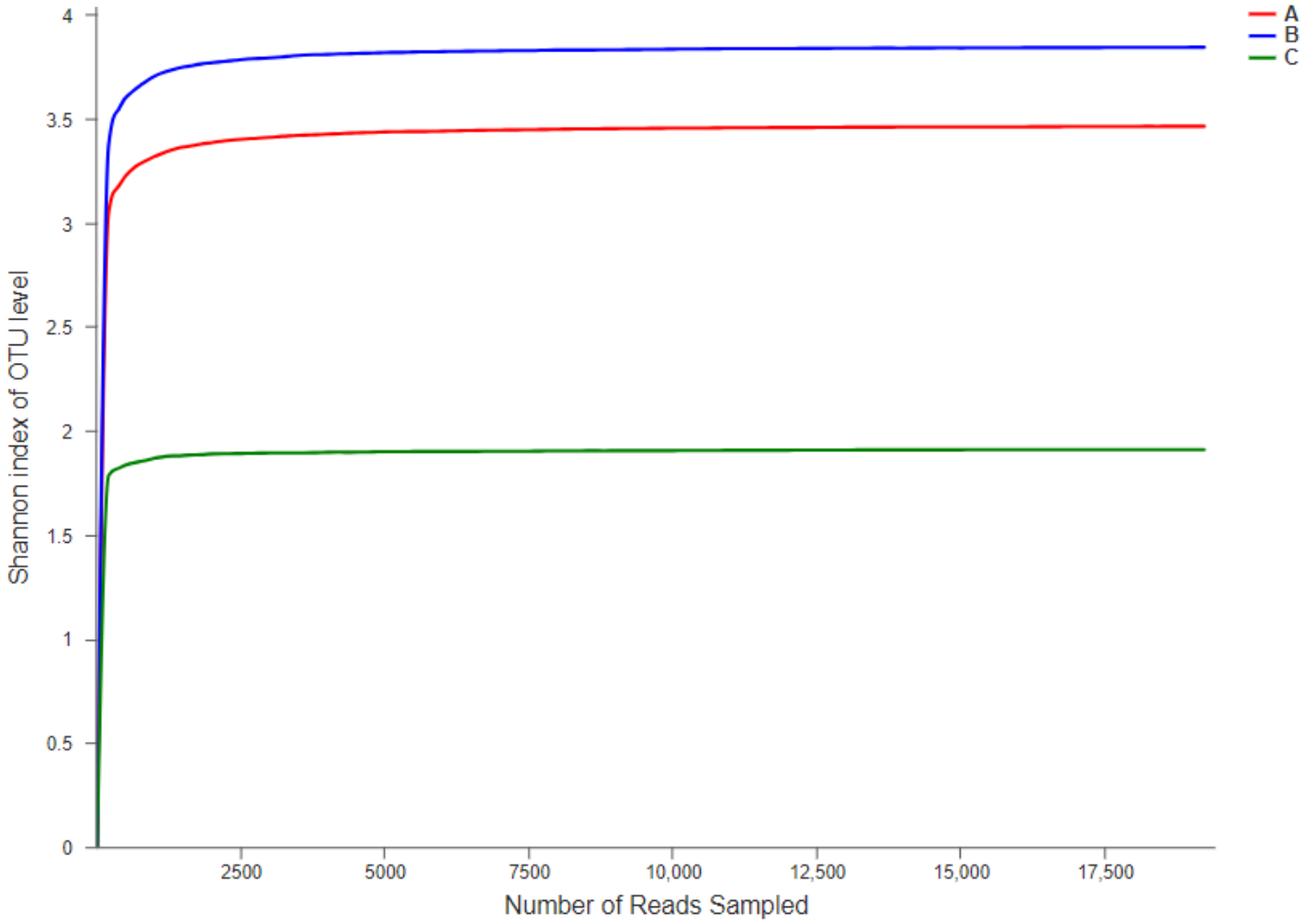

Figure 2

Sample Shannon index dilution curve

Figure 3

Venn diagram of Daqu in different storage periods

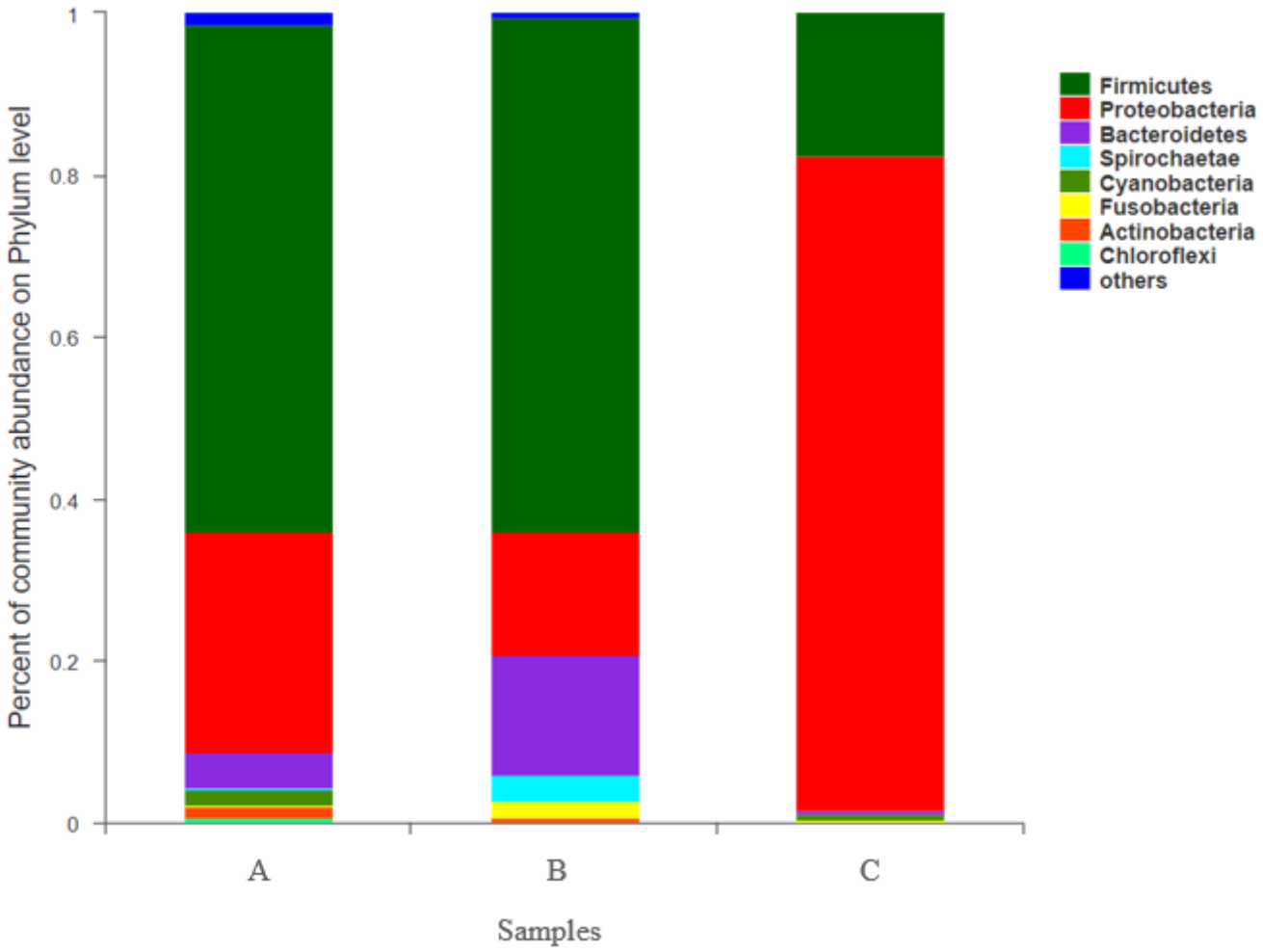

Figure 4

Phylum classification of Bacteria phylum of Daqu in different storage periods

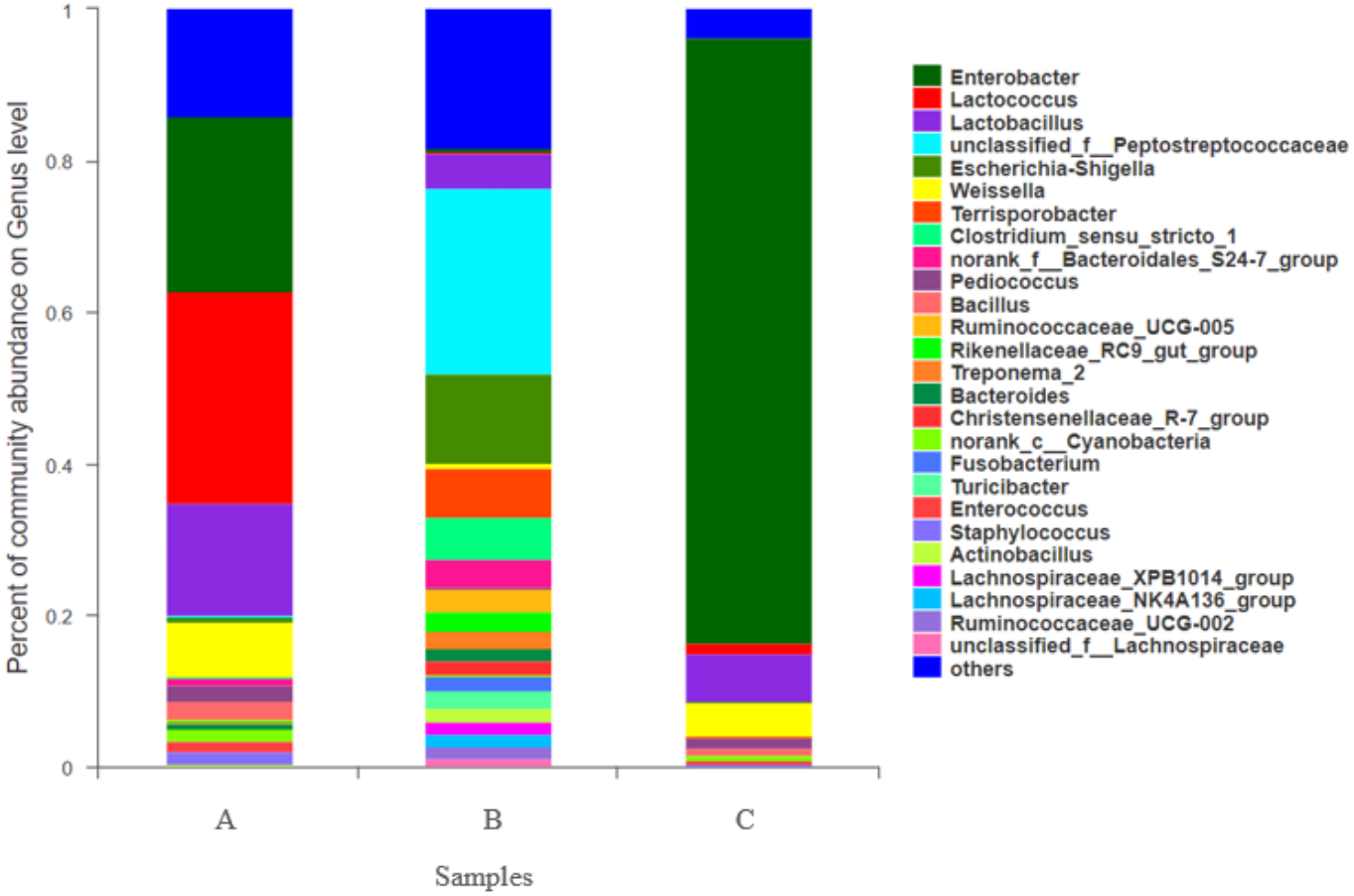

Figure 5

Genus classification of Bacteria phylum of Daqu in different storage periods

Figure 6

Phylogenetic tree