Influence of Planting *Xanthoceras Sorbifolia* Bunge on Bacteria and Fungi Diversity of Fly Ash

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Abstract

Background: Fly ash is the product of coal combustion, and a large amount of fly ash accumulation is of great harm to the environment. The yellow horn (*Xanthoceras sorbifolia* Bunge) is a unique edible oil tree species in China. Yellow horn has developed root system and can survive in soil contaminated with heavy metals. Thus, it could be used for phytoremediation in fly ash.

Results: In this study, high-throughput 16S rRNA and ITS rDNA gene Illumina sequencing technology was used to analyze the microbial community diversity in fly ash before (CK group) and after (S group) planting yellow horn. The abundance and diversity of microorganisms in fly ash were changed by planting yellow horn. The dominant bacterial phyla: Proteobacteria (CK-24% *vs* S-42%), Firmicutes (CK-23% *vs* S-10%), Actinobacteria (CK-15% *vs* S-11%). The dominant phyla in fungi: Ascomycota (72% for CK, 69% for S), Mortierellomycota (4% for CK, 3% for S).Some beneficial bacteria that could degrade heavy metals increased in proportion, including Betaproteobacteriales (4% for CK *vs* 10% for S group), Burkholderiacae (1% for CK *vs* 6% for S groups), Nitrospirae (0.3% for CK *vs* 0.8% for S groups), Rhizobiales (3% for CK *vs* 6% for S groups) and Sphingomonadaceae (2% for CK *vs* 4% for S groups).

Conclusion: These results indicate that the planting of yellow horn can increase the abundance of heavy metal-degrading bacteria in rhizosphere fly ash, which is of great significance for the biological remediation of fly ash.

Background

The situation of more coal, less oil and less gas in China determines that thermal power generation is still the main way of power generation in China at present and for a long time in the future [1]. Fly ash is a kind of solid waste produced after coal combustion, and 1 t coal combustion can produce about 250–300 kg fly ash, mainly composed of Al_2O_3, SiO_2, Fe_2O_3, CaO, TiO_2, MgO, Na_2O, etc. [2]. In addition, the main problem of fly ash is the presence of As, Cr, Cd, Pb, Hg, Se and other toxic metals particles [3]. In 2018, China consumed up to 1.8 billion tons of coal for thermal power generation, and about 450 million tons of fly ash was produced in the process. In China, the comprehensive utilization rate of fly ash is 70%, which is mainly used in cement production for building materials, and then used to extract valuable elements, improve soil and treat water pollution [1, 4–7].

Yellow horn (*Xanthoceras sorbifolia* Bunge) is a kind of economical oil tree endemic to China [8–10]. Yellow horn also is cold resistant, drought tolerant, salinity tolerance, wind resistance, and suitable for growing in the Rocky Mountains, loess hills, calcareous alluvial soil, fixed or semi-fixed sand area. Due to the developed roots with taproot up to 25 m long, more than 20 large laterals root, abundant capillary root, yellow horn is very widely distributed in our country, and has a huge ecological effect [11–14]. Therefore, the developed root system of yellow horn can be planted in the fly ash for soil remediation and secrete organic matter to slowly change the microbial composition in the fly ash, thus achieving the purpose of repairing the pollution of fly ash and protecting the environment.

Soil rhizosphere microbial diversity increases with the increase of plant diversity, which can fully degrade pollutants in the soil and convert them into nutrients beneficial to plant growth, thus promoting more plant diversity. Therefore, phytoremediation is a feasible and environmentally friendly way for people to treat solid waste [3, 6, 15–22]. Saravanan et al. believed that during the growth process of plants, root exudates promoted the growth and activity of rhizosphere microbial community to form a plant-microbial interaction model to remediation soil pollution [23]. Zdenek et al. studied the long-term remediation of polycyclic aromatic hydrocarbons (PAHs) by willow (*Salix x smithiana Willd*) from straw burning fly ash contaminated soil, and found that the total removal rate of PAHs by phytoremediation was 50.9%, while the total removal rate by natural decay was 9.9% [24]. Moreover, the removal amount of PAHs by willow itself was less than 1%, indicating that the remediation of PAHs occurred in the soil [24]. This study was designed to study the changes of microbial diversity (bacteria and fungi) before and after the planting of yellow horn in fly ash, and to seek a method to change the microbial community structure in fly ash by planting plants in fly ash and then dealing with the pollution of fly ash.

Methods

Experimental design

The experiment was carried out in the Institute of Carbon Materials Science of Shanxi Datong University, and the fly ash samples were collected from Datong thermal power Plant. Yellow horn seeds of the same size were planted in POTS on June 15, 2019, and watered once every two weeks with 500ml distilled water. 30 groups of parallel experiments were conducted.

Sampling of fly ash sample

Carefully dig yellow horn trees, the shading soil that is not closely attached to the roots of yellow horn trees is sampled as loose soil, and the fly ash remaining attached to the roots of yellow horn trees is sampled as rhizosphere fly ash. Carefully remove root fragments and fallen leaves. Three replicates were made of fly ash samples from the rhizosphere of the yellow horn. The rhizosphere fly ash samples were immediately frozen with liquid nitrogen and stored in a refrigerator at -80℃ for later analysis. The fly ash samples without planting yellow horn were in group CK, and the fly ash samples with planting yellow horn were in group S.

Microorganism analysis in rhizosphere fly ash

Experimental process

Library construction and sequencing: after extracting the total DNA of the sample, primers were designed according to the conserved region [For bacteria it will be 16S region, for fungi it will be ITS region (Internal Transcribed Spacer)], sequencing adaptors were added to the end of primers; The target sequences were amplified by PCR and its products were purified, quantified and homogenized to get a sequencing library. Then library QC was performed for constructing libraries, qualified libraries were sequenced on Illumina HiSeq 2500. The original image data files obtained by high-throughput sequencing (such as Illumina

HiSeq and other sequencing platforms) were converted into Sequenced Reads by Base Calling analysis. The results were stored in FASTQ format file, which contains sequence information of reads and their corresponding sequencing quality information.

Quality screening of sequencing data

PE reads merge: FLASH [52] v1.2.11 software was used to assemble the reads of each sample according to the minimum overlap length of 10bp and the allowable maximum error ratio of overlap area of 0.2 (Default), and the obtained Mosaic sequence was Raw Tags; Tags filtering: Use Trimmomatic [53] v0.33 software to filter the Raw Tags obtained by Mosaic, remove the low quality readings whose average quality score is less than 20, filter the Tags whose length is less than 75% of the Tags length after quality control, and get high quality Tags data (Clean Tags). Remove Chimera: UCHIME v4.2 software was used to identify and remove chimeric sequences to obtain the final Effective Tags[54].

Species annotation and taxonomic analysis

Clean tags were clustered into OTU by USEARCH [55] (version 10.0)at 97% similarity levels. The OUT(Operational Taxonomic Units) was filtered when reabudance less than 0.005% [56]. Based on the bacteria Silva database (Release132,http://www.arb-silva.de) use RDP Classifier v2.2 under the condition of confidence threshold of 0.8 species of bacteria OTU annotations [57]. Species annotation of fungal OTU was performed with a confidence threshold of 0.8 using RDP [58]. Classifier V2.2 based on the Fungi Unite database (Release 8.0,https://unite.ut.ee/) [59]. To get the corresponding species classification information of each OTU, the OTU representative sequences can be aligned to microbial reference database, then the community composition of each sample was counted at each level (phylum, class, order, family, genus, species). Use QIIME software to generate species richness table at different taxonomic levels, then use R language tool to draw community structure graph of samples at different taxonomic levels [25].

Using PyNAST software (version1.2.2, http://biocore.github.io/pynast/) than the bacteria sample sequences series, using the method of Neighbor - Joining producing bacteria system evolutionary tree [60]. Using ClustalW2 software (http://www.ebi.ac.uk/Tools/msa/clustalw2/) used after a sequence of fungal samples sequences than Neighbor - Joining method fungi system evolutionary tree [61].

Diversity analysis

The alpha diversity index (Chao1 index, Ace index, Shannon index and Simpson index) of the samples was evaluated using Mothur v.1.30 software [29]. Beta diversity analysis was performed using QIIME software to compare the degree to which different samples were similar in terms of species diversity [30]. Lefse analysis was used to screen the Biomarker and compare the p and q values to find the significance of differences between the two groups at each classification level [62].

Results

Sequencing data and sequence OTU analysis

A large number of fungi and bacteria exist in fly ash, and the planting of yellow horn alters the diversity of rhizosphere microorganisms. The results of Rarefaction Curve analysis showed that the sequence of bacterial samples was sufficient for data analysis (Additional File 1: Figure S1). A total of 478,559 pairs of reads were obtained from the bacterial analysis of fly ash samples by sequencing, and a total of 464,013 Clean Tags were generated after the splicing and filtration of double-ended reads, and 77,049-77611 Clean Tags were generated for each sample (Additional File 2: Table S1). A total of 8,603 bacterial Operational Taxonomic Units (OTUs) were identified. There are 1,528 bacterial OUT overlapped in CK and S (Planting yellow horn) groups, 557 OUTs unique to CK group and 655 unique to group S (Fig. 1a). The bacteria came from 37 phyla and 896 genera (Additional File 3: Table S2).

The results of Rarefaction Curve analysis showed that the sequence of Fungal samples was sufficient for data analysis (Additional File 4: Figure S2). In the fungal analysis of fly ash samples in this study, 479,959 pairs of Reads were obtained by sequencing. A total of 449,696 Clean Tags were generated after splicing and filtering of double-ended reads, and 72,409-77101 Clean Tags were generated for each sample (Additional File 5: Table S3). A total of 611 fungal OTUs were identified. There are 230 fungal OUTs shared by CK and S groups, 182 OUTs unique in CK group and 199 OUT unique in S group (Fig. 1b). The fungi came from 13 phyla and 170 genera (Additional File 6: Table S4).

Species annotation and taxonomic analysis

High quality OTU sequences from two groups of fly ash samples were aligned to the microbial reference database (Release132, http://www.arb-silva.de and Release 8.0, https://unite.ut.ee/) to annotate the OTU corresponding species classification information, including phylum, class, order, family, genus and species. Then, QIIME software was used to generate different classification level of species abundance table [25], and various taxonomic level of community structures were drawn by using R language(https://www.r-project.org). Figure 2 shows the species distribution of bacteria and fungi at the phylum level. According to the species distribution of bacteria (Fig. 2a), the top eight phyla of bacteria in group CK and group S accounted for more than 90% of the total bacteria. The first dominant phylum, Proteobacteria, accounted for 24% in the CK group and 42% in the S group. Firmicutes, the second dominant phylum, accounted for 23% in CK and 10% in S group. The third dominant phylum, Actinobacteria, accounted for 15% in the CK group and 11% in the S group. The fourth dominant phylum, Bacteroidetes, accounted for 10% in CK group to 6% in S group. The fifth dominant phylum, Cyanobacteria, accounted for 10% in CK group to 4% in S group. The sixth dominant gate, Acidobacteria, accounted for 5% of the CK group to 8% of the S group. The seventh dominant phylum, Chloroflexi, accounted for 3% in CK group to 6% in S group. The eighth dominant phylum, Gemmatimonadetes, accounted for 2% in group CK to 3% in group S. In conclusion, the proportion of gram-positive bacteria decreased and the proportion of gram-negative bacteria increased after planting yellow horn (Additional File 7: Figure S3; Additional File 8: Figure S4). According to the distribution of fungal species (Fig. 2b), the dominant phylum of fungal samples in this experiment was Ascomycota(72% for CK, 69% for S),

Basidiomycota (9% for CK, 9% for S), Mortierellomycota (4% for CK, 3% for S).the dominant genera of Cladosporium (7% for CK and 8% for S), Mortierella (4% for CK and 3% for S), Penicillium (3% for CK and 4% for S).

Figure 3 is a cluster heat map of species abundance of bacteria and fungi at the phylum level. Clustering heat map of bacterial species abundance showed that CK-1 and CK-3 could be classified as one group, while S-2 and S-3 could be classified as one group. The Fungal species abundance cluster heat map showed that S-1 and CK-2 could be grouped together. In Fig. 3a, it is obvious that in group S: Elusimicrobia, Omnitrophicaeota, Chlproflexi, Gemmatimonadetes, Dependentiae, Acidobacteria, Nitrospirae, Proteobacteria, Patescibacteria, FCPU426, Fibrobacteres, Verrucomicrobia, Armatimonadetes and WPS-2 14 phyla were significantly up-regulated. As can be clearly seen from Fig. 3a, CK group: Planctomycetes, Rokubacterta, Thaumarchaeota, Entotheonellaeota, Synergistetes, Kiritimatiellaeota, Fusobacteria, Firmicutes, Epsilonbacteraeota, Spirochaetes, Actinobacteria and Bacteroidetes 12 phyla were significantly increased. In Fig. 3b, in the fungal sequence, it was obvious that the expression of Chytridiomycota and Rozellomycota in group S was up-regulated.

Each branch in the evolutionary tree represents a species. The length of the branch represents the evolutionary distance between two species, that is, the degree of species difference. The OTUs of bacteria and fungi were made phylogenetic trees at the taxonomic level of genus (Additional File 9: Figure S5). In the phylogenetic tree, the ring diagram showed the species evolution tree, and the genus names with the same color represented the same phylum. As shown in Figure S5a, 27 OTU sequences with the highest taxonomic abundance belong to Proteobacteria, and 16 OTU sequences with the highest taxonomic abundance belong to Actinobacteria. According to the results of Figure S5b, 51 genus OTU sequences with the highest taxonomic abundance belong to Ascomycota, and 26 genus OTU sequences with the highest taxonomic abundance belong to Basidiomycota.

Diversity analysis

Table 1
Alpha diversity index of bacteria and fungi in fly ash before and after planting yellow horn

Classification	Sample ID	Ace	Chao1	Simpson	Shannon
Bacteria	CK	1517.57 ± 21.90	1535.24 ± 22.56	0.0075 ± 0.0040	6.2065 ± 0.1390
	S	1445.85 ± 255.37	1508.86 ± 287.23	0.0050 ± 0.0001	6.2892 ± 0.0531
Fungi	CK	244.24 ± 24.80	245.64 ± 27.95	0.0212 ± 0.0056	4.4514 ± 0.0672
	S	222.31 ± 36.83	214.11 ± 41.43	0.0200 ± 0.0006	4.5215 ± 0.1660

Alpha diversity reflects species abundance and species diversity of fly ash samples without and after planting yellow horn [26, 27]. There are four Alpha diversity measures: Chao1, Ace (Abundance-based Coverage Estimator), Shannon, and Simpson. These results are compared and presented in Table 1. Chao1 and Ace index measure species richness, i.e. the number of species. Among the bacteria, Chao1 and Ace indexes of group S were 0.95 and 0.98 times of those of group CK, respectively, indicating that the abundance of bacteria in fly ash decreased slightly after planting yellow corn, showing no statistical difference. In terms of fungi, the results showed that the abundance of fungi in fly ash decreased slightly after planting yellow horn, and there was no statistical difference. Shannon and Simpson indexes are used to measure species diversity [28]. The larger Shannon index and smaller Simpson index indicate that the species diversity of the sample is higher [29]. Among the bacteria, compared with the CK group, the Simpson index of group S decreased by 33.3%, and the Shannon index increased by 1.01 times, indicating that the bacterial diversity in fly ash increased slightly after the planting of yellow horn, but there was no statistical difference. Among the fungi, compared with the CK group, the Simpson index of group S decreased by 5.6%, and the Shannon index increased by 1.02 times. There was also no statistical difference, indicating that the diversity of fly ash fungi increased by a smaller extent after the yellow horn was planted.

The OTU composition of fly ash samples without and after planting yellow horn was analyzed by QIIME software, which could reflect the difference and distance of samples [30]. Principal component analysis (PCA) used variance decomposition to reflect the difference of multiple data groups on the two-dimensional coordinate chart, and the two characteristic values that could reflect the maximum variance were selected for the coordinate axis [31]. The R language tool was used to draw PCA diagrams of bacteria and fungi, respectively, and the PCA analysis results between groups were shown in **Fig. 4.** It can be seen from Fig. 4A that the bacterial community of fly ash without planting yellow horn is significantly different from that after planting yellow horn, and the planting of yellow horn significantly changes the diversity of bacterial community of fly ash. As can be seen from Fig. 4B, there are slight difference in the fungal community and fungal diversity between the fly ash without planting and the fly ash after planting.

According to the biomarker screening criteria with Line Discriminant Analysis (LDA) score > 4, LEfSe (Line Discriminant Analysis (LDA) Effect Size) was used to identify eligible biomarkers [32]. Lefse evolutionary branching diagrams of bacteria and fungi in fly ash samples without yellow horn planting (CK group) and after yellow horn planting (S group) were shown in Fig. 5. Figure 5A showed that the high abundance of o- Bacteroidales, o- Clostridial, f- Enterobacteriaceae and o-Enterobacteriales was observed in CK group. However, the high abundance of o- Rhizobiales, f- Sphingomonadaceae, s- unculted- bacterium g- Limnobacter, g- Limnobacter, f- Burkholderiacae, o- Betaproteobacteriales, f- Xanthomonadaceae, and o- Xanthomonadales were existed in S group, and most of them could be used as phytoremediation. Compared to the CK, the high abundance of o- Eurotiales was identified in fungi of S group (Fig. 5b).

Discussion

Plant growth can change a series of soil environmental factors and affect the composition and function of microbial community [21, 33, 34]. In this paper, the microbial diversity of fly ash before and after the growth of yellow horn was studied. There was no significant difference in the abundance and diversity of bacteria and fungi in rhizosphere fly ash. The results showed that planting yellow horn slightly decreased the abundance of bacteria and fungi in the rhizosphere fly ash, and mildly increased the diversity of bacteria and fungi in the rhizosphere fly ash. The results showed that the effect of planting yellow horn on bacteria in fly ash was much greater than that on fungi. Bang-Andreasen et al. also found that the influence of wood ash application on the abundance and diversity of bacteria in agricultural and forest soil was greater than that of fungi[35].

The heavy metals in fly ash can stress the growth of microorganisms, and planting yellow horn will increase microbial diversity, which is more helpful to the adsorption and degradation heavy metal in fly ash.Through the study on the microbial community in the fly ash before and after the growth of yellow horn, Proteobacteria was significantly increased in S group compared with the CK group (CK- 42%*vs* S-24%). Proteobacteria can degradeheavy metals, which is conducive to the removal of heavy metals from fly ash [28]. In addition, S-uncultured -bacterium- G-limnobacter (CK-0.003% vs S-3%) is a thiosulfate oxidizing bacterium. The stoichiometric formula of thiosulfate oxide in this bacterium is:$S_2O_3^{2-}$ $+2O_2+H_2O2SO_4^{2-}+2H^+$,$\Delta G_0{}'$= 818.42 kJ·$mol^{-1}$. the increase of limnobacter contentindicates that the acid content of fly ash is increasing [36].Soil acidification makes it easier for heavy metals to enter the soil and migrate to plant roots, where they can be absorbed by plants[37, 38], further to the promotion the degradation of heavy metals by Proteobacteria.The bacteria of Gammaproteobacteria (CK-12%*vs* S-21%) have been repeatedly found in nutrient-rich sites such as the rhizosphere, which also proves that the soil is nutrient-rich after the planting of yellow horn[39, 40].

The dominant phylum in the healthy soil bacterial library are Proteobacteria, Acidobacteria, Actinobacteria, Verrucomicrobia, Bacteroidetes, Chloroflexi, Planctomycetes, Gemmatimonadetes, and Firmicutes [41]. In our fly ash samples, the second dominant phyla is Firmicutes (23% for CK *vs* 10% for S groups), which decreases in proportion, while Proteobacteria and Acidobacteria increase in proportion, which is consistent with the result that plant growth promotes the rapid transformation of bacterial community to Proteobacteria and Acidobacteria [42]. It is also consistent with the conclusion of Wang et al that gram-negative bacteria are more resistant to metal contamination than gram-positive bacteria [43].

Fly ash is short of nitrogen and organic matter. The proportion of Acidobacteria (5% of CK *vs* 8% of S groups) in the microbial community was increased in the fly ash after the planting of yellow horn. Acidobacteria participates in the carbon cycle and degrades plant polysaccharides, such as cellulose and lignin [44]. The content of beneficial microorganism in the fly ash of root of yellow horn increased.For example, Nitrospirae (0.3% for CK *vs* 0.8% for S groups) participates in the second stage of nitrification: nitrite oxidation, which provides nitrogen to plant roots to promote plant growth [45, 46], was increased (0.3% for CK *vs* 0.8% for S groups). Betaproteobacteriales (4% for CK *vs* 10% for S groups) and Burkholderiacae (1% for CK *vs* 6% for S groups) play a role in nitrogen fixation in plant roots to promote plant growth [47–49]. In addition, Rhizobiales (3% for CK *vs* 6% for S groups) is the predominant bacteria

OUT in fly ash samples planted with yellow horn. It is a typical beneficial bacteria for plant symbiosis and can significantly increase the accumulation of heavy metals [50]. Sphingomonadaceae (2% for CK *vs* 4% for S groups) was found by Baraniecki, C.A. et al to have the function of absorbing heavy metal Cd and degrading a variety of aromatic compounds, showing great potential for environmental protection [51]. These microorganisms' diversities elevated indirectly and indirectly acidify, chelate, precipitate and fix the heavy metals in fly ash, and finally repair the fly ash.

In this study, the dominant fungal phyla were Ascomycota, Basidiomycota, and Mortierellomycota. Dominant fungal genera: Cladosporium, Mortierella, Penicillium. The abundance of most of the bacteria decreased, which may be caused by the increase of heavy metals in the rhizosphere fly ash, resulting in a slight decrease in the abundance of the fungal community.

Conclusion

The microbial diversity of fly ash before and after yellow horn growth was studied. The results showed that planting yellow horn had effect on the abundance and diversity of bacteria and fungi in the rhizosphere fly ash. In terms of microbial community structure, the proportion of proteobacteria (24% for CK groups *vs* 42% for S groups) and acidobacteria (5% for CK groups *vs* 8% for S groups), which are conducive to soil carbon cycling, increased greatly. The abundance of some beneficial bacteria also increased, such as Nitrospirae, Betaproteobacteriales, Burkholderiacae, Rhizobiales, Sphingomonadaceae. The functions of these bacteria are closely related to nitrogen cycling, nitrogen fixation, and environmental protection. This study provides important perspective for the phytoremediation of fly ash.

Abbreviations

ITS: Internal Transcribed Spacer; PAHs: polycyclic aromatic hydrocarbons; OTUs: Operational Taxonomic Units; Ace: Abundance-based Coverage Estimator; PCA: Principal component analysis; LEfSe: Line Discriminant Analysis Effect Size; LDA: Liner discriminant analysis

Declarations

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Authors' contributions

ZHL, ZWC, JXH and JGZ conceived and designed this experiment. ZHL, ZWC, HFM collected samples and performed the study. ZHL, ZWC, JXH, JGZ, HFM, XLB, JQ, WJL, JWL and SG participated in the acquisition and analysis of the data. ZHL and ZWC wrote the manuscript. JXH and JGZ participated in

the discussion draft of the manuscript. XLB and JQ revised the manuscript finally. All authors read and approved the final manuscript.

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Availability of data and materials

Not applicable.

Ethics approval and consent to participate

Not applicable.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

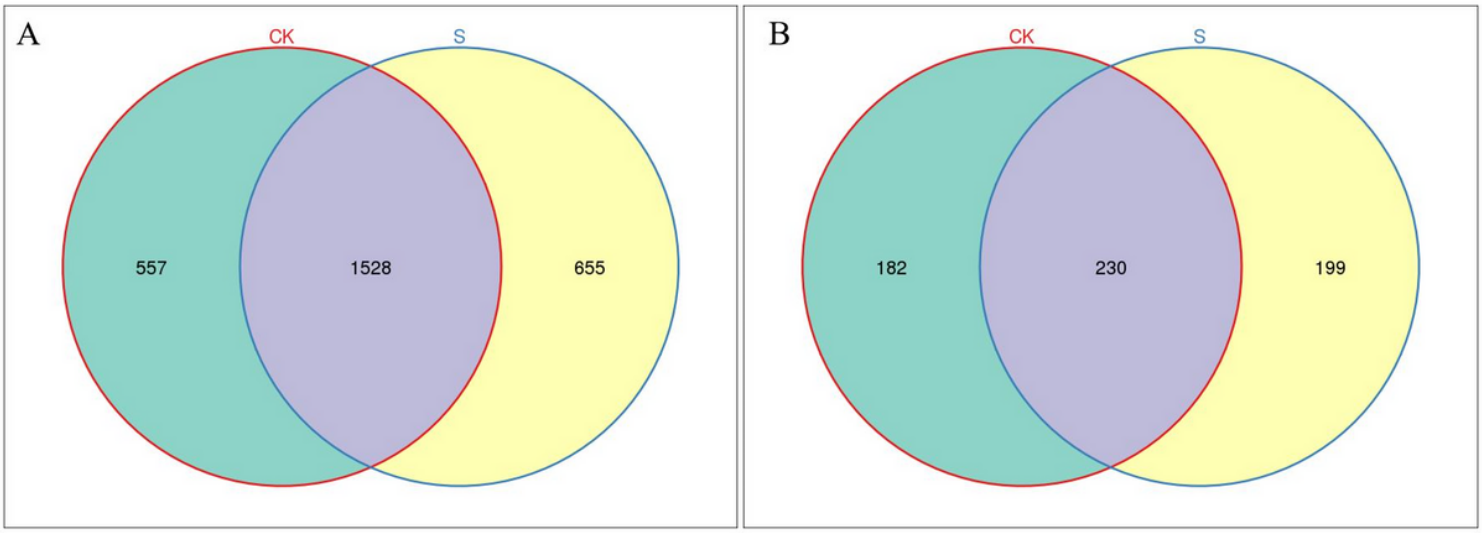

Figure 1

OTU-Venn diagrams of bacteria (A) and fungi (B).

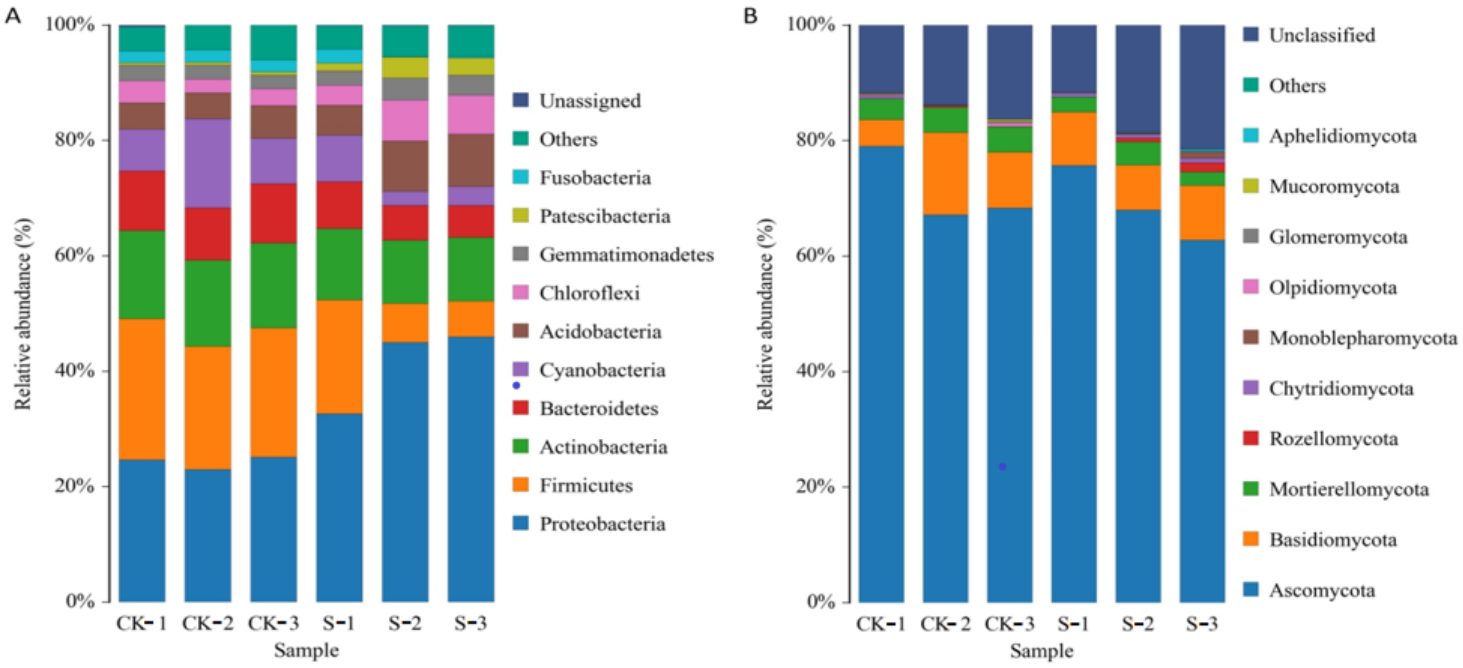

Figure 2

Species distribution at bacterial (A) and fungal (B) phylum levels.

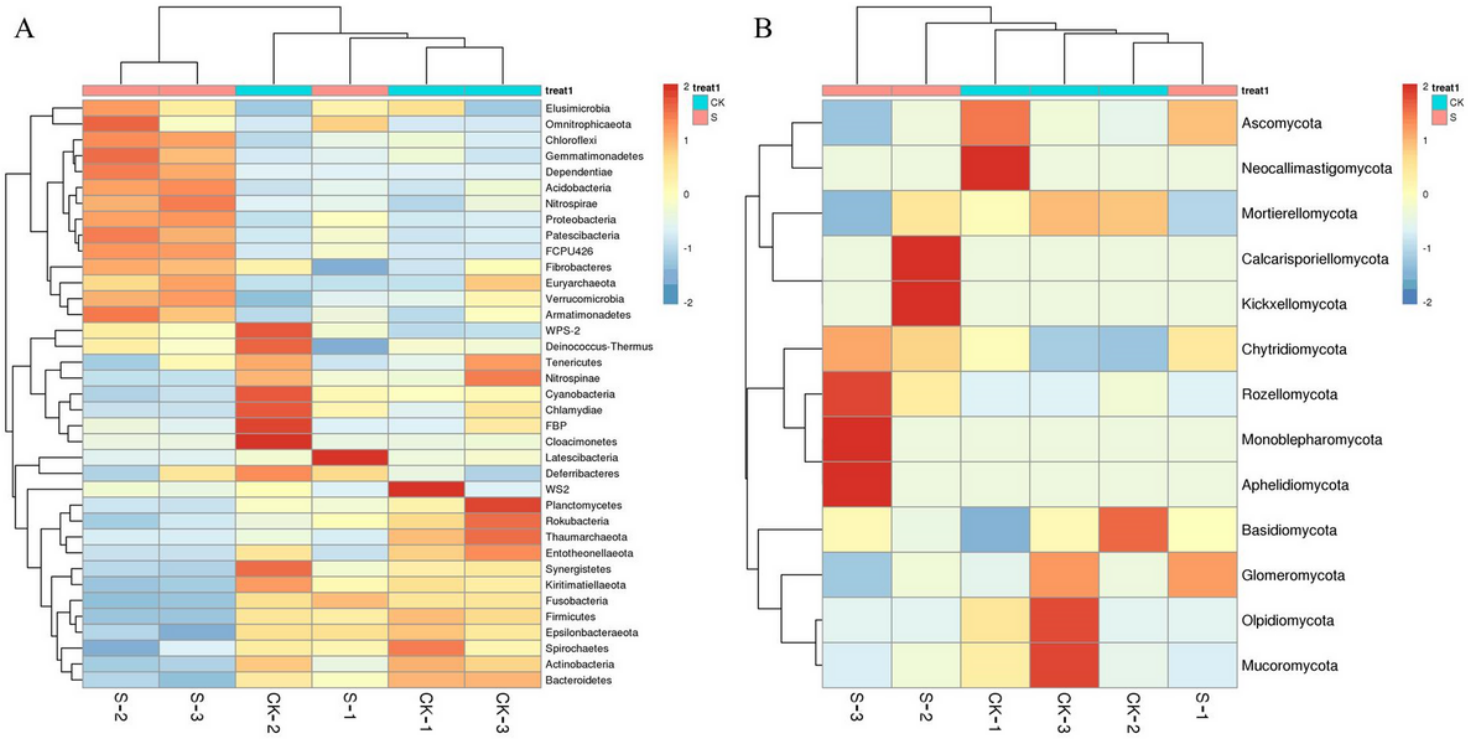

Figure 3

Clustering heat maps of species abundance at the bacterial (A) and fungal (B) phylum levels.

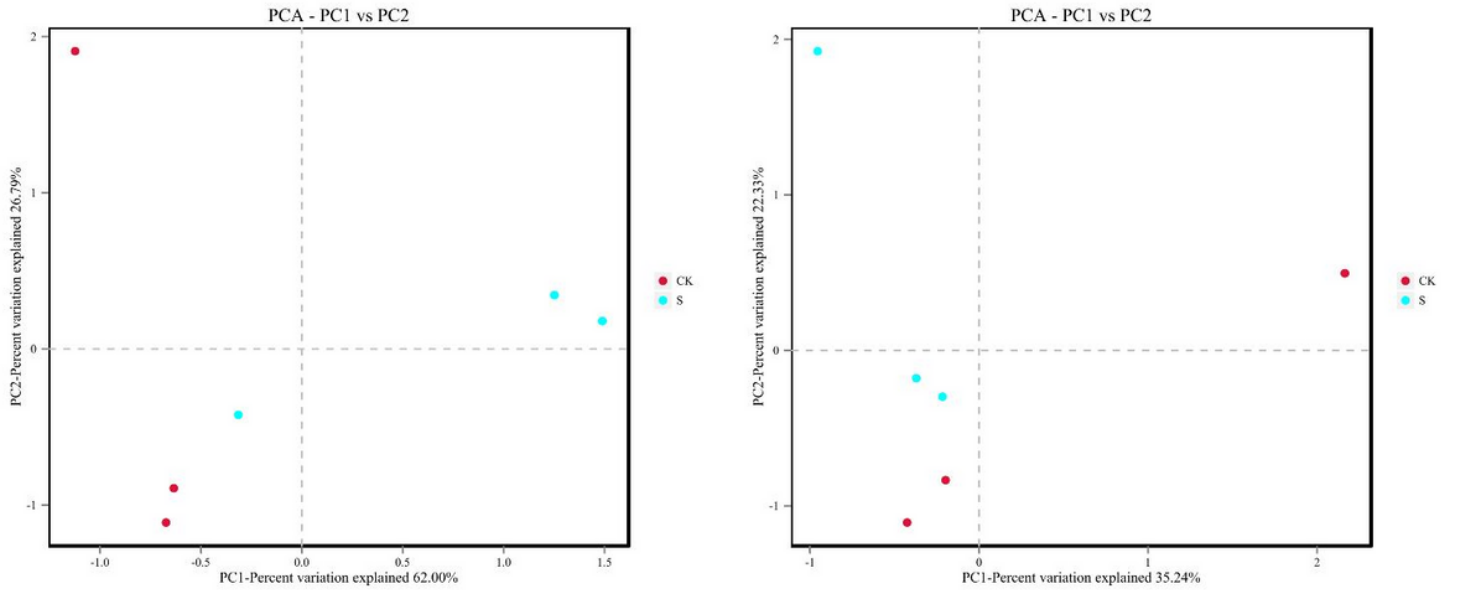

Figure 4

Principal component analysis diagrams of bacteria (A) and fungi (B).

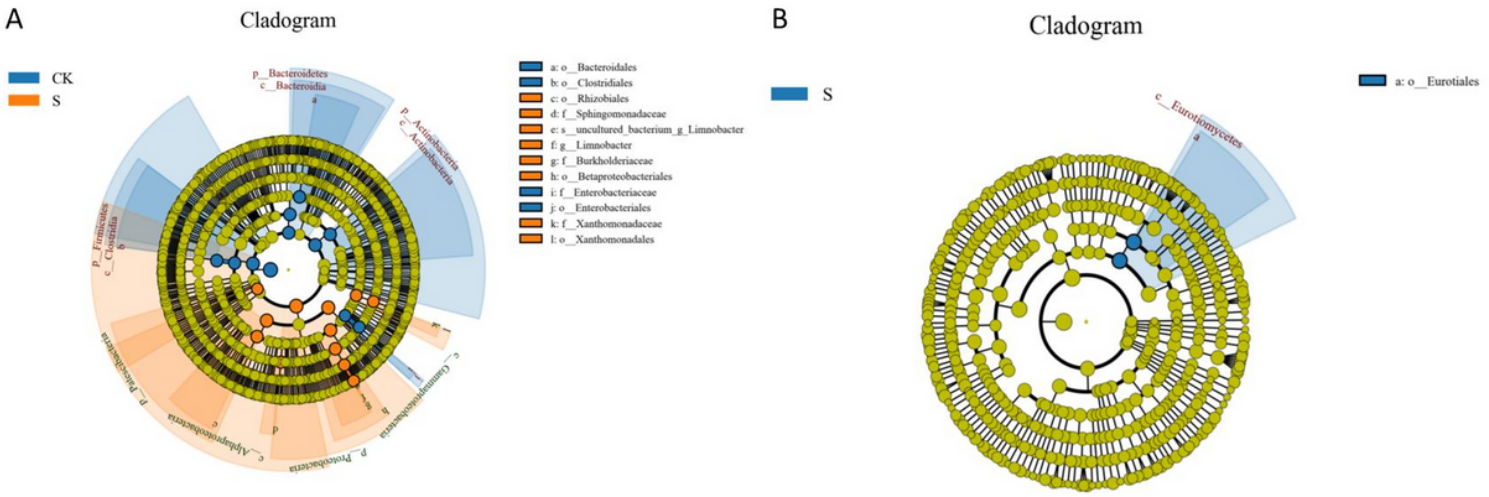

Figure 5

LEfSe evolutionary branching of bacteria (A) and fungi (B).

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