

# Continuous cropping of cut chrysanthemum reduces rhizospheric soil microbial populations, diversity and network complexity

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## Research Article

**Keywords:** Continuous cropping, cut chrysanthemum, enzyme activity, bacterial community, co-occurrence networks, diversity

**Posted Date:** November 3rd, 2021

**DOI:** <https://doi.org/10.21203/rs.3.rs-1023064/v1>

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# Abstract

Continuous cropping of cut chrysanthemum causes soil degradation and chrysanthemum quality decline, but the biotic and abiotic mechanisms behind it remain unclear. This impedes our ability to assess the true effects of continuous cropping on agricultural soil functions and our ability to repair impaired soils. Here we examined the impact of different replanting years on microbial communities and enzyme activities in rhizosphere soil of cut chrysanthemum (*Chrysanthemum morifolium*). Our results showed that soil total nitrogen (TN) and organic carbon (SOC) contents were significantly lower in the soil with 12 years of continuous cropping (Y12) than that in the soil with 1 year of cropping (Y1). Compared with Y1, Y12 treatment decreased alkaline phosphatase and  $\beta$ -glucosidase by 12.1 and 24.4%, but increased the activities of soil urease and catalase by 98.2 and 34.8%, respectively. Soil bacterial populations in Y6 (continuous cropping for 6 years) and Y12 treatments decreased by 52.3 and 87.5% compared with that in Y1 treatment. Moreover, the bacterial  $\alpha$ -diversity (Shannon index) significantly decreased by 37.3 and 57.6% over 6 and 12 years of continuous cropping, respectively. Long-term monoculture cropping shifted the bacterial community composition, with decreased abundances of dominant phyla such as *Proteobacteria* and *Acidobacteria*, but with an increase in the relative abundances of *Actinobacteria* and *Chloroflexi*, and *Gemmatimonadetes*. Moreover, Y6 and Y12 treatments harbored less microbial network complexity, lower bacterial taxa, and fewer linkages among bacterial taxa, relative to Y1. Soil pH, SOC, and TN were the main edaphic factors affecting soil bacterial community compositions and diversity. Overall, our results demonstrate that continuous cropping has a significant negative impact on soil microbial diversity and complexity.

## 1. Introduction

Continuous cropping refers to planting a single crop in the same field year after year. This is a vital and common soil management practice in China because of limited arable land resources and lower related agronomic management costs relative to rotation practices [1]. Nevertheless, crop continuous cropping has tremendous pressure on the soil's capacity and negatively affects soil function including serious soil sickness, ecosystem degradation, loss of productivity [2, 3]. Thus, it is crucial to find indicators for early evaluation of soil health decline for the sustainable management under continuous cropping system.

Cut chrysanthemum (*Chrysanthemum morifolium*), which is native to Northeastern Europe and Asia, is the oldest ornamental plant and an important herb and known for a wide range of biodiversity and attractive colors [4]. Cut chrysanthemums are in great demand worldwide and have great export value because of their broad use, such as in tea, medicine, ornaments, and food [5]. China is one of the important exporters of cut chrysanthemums in the world, and the cultivated area is gradually expanding. Continuous cropping of cut chrysanthemum has become the mainstream cultivation practice to obtain higher economic benefits. However, long-term monoculture has negative impacts on soil physical and chemical properties (abiotic factors), thereby threatening chrysanthemum quantity and quality. The soil abiotic characteristics including soil moisture, pH, nutrient, and organic matter (SOM) mediate the alteration of soil microorganisms [3, 6, 7], which are likely to be sensitive to continuous monoculture.

However, the abiotic and biotic mechanism and their interactions behind the adverse effects of continuous monoculture remains unclear.

Soil microbes play an extremely vital role in soil elements cycle and ecosystem functions, regulating the response of soil ecosystems to human disturbance [8, 9]. Healthy soil is the fundamental guarantee for plant growth and food security in agriculture [10]. Given the significance of soil microorganisms for specific soil functions [11], they must be considered when exploring the mechanisms behind the response of agricultural soil systems to continuous cropping cultivation. Increasing numbers of studies have shown that soil enzyme activities and soil microbial composition maintain a certain relationship in response to continuous cropping [12, 13]. The effects of continuous cropping on soil microbial community have been assessed in crops and vegetables [14, 15, 16]. For example, Ali et al. [16] reported that rhizospheric soil microbial community diversity significantly reduced during the cucumber continuous cropping. Nevertheless, the dynamic successions of microbial communities of facility horticultural plant rhizosphere soil are less understood [4]. To date, it remains unknown how the response of rhizospheric soil microbial community to long-term cut chrysanthemum continuous cropping.

There are two relationships between species in the microbial community, either competition for resources and space [17] or mutualism [18]. Microbial co-occurring network can reveal the relationships between microbial species and explain the assembly of complex microbial communities in various environments such as oceans and sandy land [19]. Network analysis can also reveal why certain groups of microbes appear together consistently, or whether some groups of microbes are more important to keep the network stability in response to environmental disturbances [20]. In addition, the complexity of the network is an important indicator of the stability and the function of the ecosystem [21]. Therefore, an unknown question is how the complexity of soil microorganism, as expressed by network connectivity, changes with continuous cropping.

Here, this study focused on how the abundance, diversity, composition, and network complexity of rhizospheric soil bacteria change in response to cut chrysanthemum continuous cropping. Moreover, we determined whether alterations in bacterial community caused by continuous cropping are linked with soil properties. We hypothesized that continuous cropping would adversely affect soil bacterial abundance, diversity, and network complexity. The diversity and composition of soil bacteria were quantified by using 16S rRNA gene amplicon sequencing. The total amounts of bacteria were applied using the dilution-plate method. The objectives of this study were to (1) evaluate the changes of enzymatic activities to continuous cropping; (2) compare the bacterial population, composition, and diversity in different continuous cropping years; and (3) assess the correlations between the bacterial community and soil properties. These results provided a comprehensive understanding of the response of soil microbial community to cut chrysanthemum continuous cropping, and may conducive to improving agricultural strategies by regulating the community function of soil microbiome to reduce the adverse impacts of long-term monoculture. These findings provided a scientific basis for a comprehensive understanding of the effects of chrysanthemum continuous cropping on soil bacterial

communities, and could conducive to lower continuous cropping obstacles by regulating the soil microflora.

## 2. Materials And Methods

### 2.1 Experimental site

The experimental field was located in Zhejiang Haifeng Biotechnology Co., Ltd., Shaoxing city, Zhejiang province, China (29°58' N, 120°34' E) at an altitude of 24 m. This area belongs to a subtropical monsoon climate. The mean annual rainfall is about 1400 mm, and the mean annual temperature is 16.9°C. The soil type is fluvo-aquic soil. Some soil basic physicochemical properties were as follows: total soil nitrogen (TN) 3.30 g kg<sup>-1</sup>, soil organic matter (SOM) 33.95 g kg<sup>-1</sup>, total soil phosphorus (TP) 0.27 g kg<sup>-1</sup>, total soil potassium (TK) 74.6 g kg<sup>-1</sup>, ammonium (NH<sub>4</sub><sup>+</sup>-N) 0.55 mg kg<sup>-1</sup>, nitrate (NO<sub>3</sub><sup>-</sup>-N) 22.01 mg kg<sup>-1</sup>, available P 14.90 mg kg<sup>-1</sup>, and pH 5.14.

### 2.2. Experimental design

The experimental field contained 12 greenhouse plots of three monoculture years of treatment with four replicates, each 20.0 × 10.0 m in size. The greenhouse plots of different treatments were separated by at least 2 m. Three treatments were selected: planting for only 1 year in 2020 (Y1); continuous cropping for 6 years from 2015-2020 (Y6); and continuous cropping for 12 years from 2009-2020 (Y12), respectively. The cut chrysanthemum seeds (*Dendranthema morifolium* (Ramat.) Tzvel. cv. Golden fan) were planted in March and September every year. Cut chrysanthemum in this area have always adopted the double cropping system pattern. The N, P<sub>2</sub>O<sub>5</sub>, and K<sub>2</sub>O application rates were 200, 200, and 400 kg ha<sup>-1</sup>, respectively, which were split 6 times for application through fertigation during the one growing season. The special water-soluble fertilizer for flowers with N of 15%, P<sub>2</sub>O<sub>5</sub> of 15%, and K<sub>2</sub>O of 30% was applied in the history of cultivation. Cut chrysanthemum seeds were first sown in the nursery, and then transplanted to the greenhouse plot for growth after growing seedlings. Each greenhouse had 5,000 chrysanthemums planted in 28 rows, and a drip irrigation line was installed between two planted rows. The irrigation amount throughout the chrysanthemum growing season is 4500 m<sup>3</sup> ha<sup>-1</sup>. Cut chrysanthemums were harvested at the maturity stage by hand.

### 2.3 Rhizospheric soil samplings and chemical analyses

Rhizospheric soil were sampled at the maturity stage of chrysanthemum in 2020. Totally 10 chrysanthemum plants were collected using the "S" pattern in each plot. The 10 plant rhizosphere soils were mixed into a composite sample. The collection method of rhizosphere soil was as follows: the plants were carefully dug out of the ground, and then gently shook the soil attached to the roots and collected, which was the rhizospheric soil. The collected rhizospheric soils were placed in an ice box and transported to the laboratory, part of which was kept in -80°C for molecular analysis, and the other part was dried naturally at room temperature, and the passed through a 100-mesh sieve for chemical analysis.

Soil mineral N ( $\text{NH}_4^+\text{N}$ ,  $\text{NO}_3^-\text{N}$ ) was determined by the continuous flow injection analyzer (AA3, SEAL, Germany). Soil pH was measured with the soil: water ratio of 1:2.5 using a pH meter. SOC and TN were measured by the TOC/TN analyzer (Analytikjena, Multi N/C 3000). TP was determined by molybdenum blue colorimetry. Available P was measured by the Spectrophotometer with 0.5M  $\text{NaHCO}_3$  extraction. All the methods mentioned above were followed by [22].

## 2.4 Measurement of soil enzymatic activity and soil bacterial populations

Alkaline phosphatase activity was measured following the method of [23].  $\beta$ -glucosidase was measured by the method of [24]. Soil catalase was measured using the method of potassium permanganate titration [25]. Soil urease was incubated with urea as substrate for 5 h at 37°C, and then measured by spectrophotometry.

Bacterial populations were enumerated by the method of plate culture count with the beef extract peptone medium [26]. Each count was repeated in 4 independent soil dilutions (repetitions), and expressed in the colony forming unit per gram of dry soil weight ( $\text{CFU g soil}^{-1}$ ).

## 2.5 High throughput sequencing of 16S rRNA genes

Soil DNA was extracted from 0.3 g soil by the Powersoil™ DNA isolation kits (MoBio, San Diego, CA, USA) followed by the instructions. DNA quality and quantity were checked using NanoDrop 2000 (Thermo Scientific, Wilmington, DE, USA). The extracted DNA was stored in a refrigerator at -20°C until use.

The V4–V5 regions of the bacterial 16S rRNA was amplified using the primers 515F and 907R [27]. PCR reactions were performed in triplicate in a 25- $\mu\text{L}$  mixture containing 2 $\mu\text{L}$  of 10  $\times$  Fast Pfu buffer, 0.5  $\mu\text{L}$  of each primer (10  $\mu\text{M}$ ), 2  $\mu\text{L}$  of 2.5 mM dNTPs, 0.5  $\mu\text{L}$  of Fast Pfu polymerase, and 2  $\mu\text{L}$  of purified template DNA (10 ng), and 17.5  $\mu\text{L}$  PCR-grade water. The PCR reaction conditions were: denaturation at 95°C for 4 min, annealing at 95°C for 30 sec, 55°C for 30 sec, 72°C for 30 sec,, and extension at 72°C for 6 min for 30 cycles. The PCR products were purified and subjected to emulsion PCR, and then sent for Illumina MiSeq sequencing at the Personal Biotechnology Co., Ltd. (Shanghai, China).

Overlapping raw paired-end reads were merged with FLASH (v 1.2.11) [28]. Merged sequences were processed using the QIIME toolkit (v 2018.6) [29]. Chimeric sequences were excluded by using USEARCH. The high-quality sequences were clustered into operational taxonomic units (OTUs) at 97% sequence identity by the UPARSE [30]. The taxonomic identity of 16S rRNA sequences was performed using BLAST (Basic Local Alignment Search Tool) of NCBI (National Centre for Biotechnology Information).

## 2.6 Microbial co-occurrence network construction

Network analysis was conducted to reveal the bacterial taxa co-occurrence patterns. To decrease the complexity of the datasets, OTUs presenting in more than 3 samples were reserved for the network construction. Similarity matrices were assessed using Spearman rank correlation. The one node in

networks expressed individual OTU. The edges in the networks represented statistically significant ( $P < 0.01$ ) Spearman correlations with  $R^2 > 0.8$ . The topological features were estimated by degree, average degree, betweenness, modularity, clustering coefficient, etc. Networks were visualized by the Gephi software [31, 32] by using the Fruchterman–Reingold layout.

## 2.7 Statistical Analysis

The  $\alpha$ -diversity (richness and Shannon index) of bacterial community was calculated to evaluate their differences among treatments with different continuous cropping years via the “vegan” package of R software. The relative abundances of bacterial community species were visualized using Circos software online (<http://circos.ca/images/>). For comparing the difference of bacterial community compositions,  $\beta$ -diversity was measured via PCA (Principal Component Analysis). Multiple regression model (“stats” package) and variance decomposition analysis (“relaimpo” package in R) were applied to assess the importance of soil chemical properties in explaining the dissimilarities in enzymatic activities and the abundance and diversity of bacterial community. The associations between enzymatic activities and community ( $\alpha$ -diversity,  $\beta$ -diversity, and populations) of soil bacteria were evaluated by Random Forest analysis. The relationships among the top 20 genera of *bacteria* community in different cropping year treatments were evaluated by the correlation heatmaps in R.

A one-way analysis of variance (ANOVA) was carried out to examine the effect of continuous cropping treatments on the enzymatic activity and abundance and  $\alpha$ -diversity (species richness and Shannon index) of bacteria.  $P$  value  $< 0.05$  was defined as statistically significant. These graphs and statistical analyses were performed by the R 3.14 Software.

## 3. Results

### 3.1 Soil chemical characteristics

Continuous cropping treatments significantly affected soil chemical properties (Table S1). Continuous cropping resulted in a nearly 1 unit increase of soil pH after 12 years ( $P < 0.05$ ). The SOC content and total nitrogen were significantly decreased as the age of continuous cropping years increases ( $P < 0.05$ ). By contrast, the total P and available P were significantly greater in the Y6 and Y12 treatments than in Y1 treatment ( $P < 0.05$ ). Continuous cropping had negative effects on  $\text{NO}_3^-$ -N content but had almost no effects on  $\text{NH}_4^+$ -N. Overall, continuous cropping adversely affected most of the soil chemical properties in the cut chrysanthemum field.

### 3.2 Soil enzymatic activity

Soil enzymatic activities of alkaline phosphatase and  $\beta$ -glucosidase were significantly lower in the continuous cropping treatments (Fig. 1). For example, compared with Y1 treatment, the activities of these two enzymes were 12.1 and 24.4% lower in the Y12 treatment, respectively. Furthermore, with increasing cropping years, alkaline phosphatase activity decreased (Fig. 1b). By contrast, soil enzyme activities of

urease and catalase were significantly higher ( $P < 0.05$ ) in the Y12 treatment than in the Y1 treatment (Fig. 1c, d). Therefore, alkaline phosphatase and  $\beta$ -glucosidase can be used as indicators of continuous cropping obstacles in cut chrysanthemum soil.

### 3.3 Populations and diversity of soil bacteria

The soil bacterial populations gradually decreased in the soils with continuous cropping of cut chrysanthemum (Fig. 2a). Compared to Y1 treatment, Y12 years decreased soil bacterial populations by 87.5%. The  $\alpha$ -diversities of soil bacteria (Shannon index) were significantly lower in Y6 and Y12 treatments than in Y1 treatment (Fig. 2a). Compared with Y1 and Y6 treatments, Y12 treatment decreased Shannon index by 10.44 and 6.49%, respectively. However, the Chao1 index did not change during the 12 years of continuous cropping ( $P > 0.05$ , Fig. 2b). Overall, continuous cropping resulted in significant decreases in soil bacterial populations and diversity.

The phylum compositions of bacteria in the three treatments are shown in Fig. 2d. The top five phyla significant statistically were *Proteobacteria* (34.03%-41.59%), *Actinobacteria* (11.5%-23.73%), *Chloroflexi* (9.95%-13.67%), *Acidobacteria* (6.58-18.63%), and *Gemmatimonadetes* (6.38%-13.34%). These five phyla comprised over 88% of the overall relative abundance. The *Proteobacteria* was the dominant phylum in all plots, with the percentage highest for Y1 (41.59%) and lowest for Y6 (34.03%). Moreover, continuous cropping treatments had lower *Acidobacteria* percentages compared to only cropping 1-year. By contrast, continuous cropping led to the significant increase of the relative abundance of *Actinobacteria* and *Chloroflexi*, and *Gemmatimonadetes*.

### 3.4 $\beta$ -diversity and keystone species identify

Venn diagram was used to distinguish the difference of bacterial community based on unique and shared OTUs across three treatments (Fig. 3a). A total of 25221 OTUs were observed in Y1, Y6 and Y12 treatments (Fig. 3a), there was 886 shared OTUs (3.5% of the total). The number of shared OTUs was 2,103 in the group of Y1 and Y6 (8.3% of the total), while the number of shared OTUs was 1,368 in the group of Y1 and Y12, (5.4 % of the total) (Fig. 3a), indicating that the bacterial community changed more significantly after 12 years of continuous cropping than after 6 years of continuous cropping.

PCA analysis showed that the community structure of bacteria was differentiated among different continuous cropping treatments, axis1 and axis2 explained 43.2% and 34% of the total variation (Fig. 3b). Heatmap showed that the community composition of bacteria based on top 20 genus was similar to that in Y6 and Y12 and significantly differentiated from that in Y1 (Fig. 3c). Continuous cropping lowered the genus of *Pseudolabrys*, *Gemmatimonas*, *Subgroup\_6*, *Haliangium*, *Devosia*, *SC-I-84*, *Bryobacter*, *Candidatus\_Solibacter*, and *Bradyrhizobium* were enriched in AD3, KD4-96, Ellin6067, *Sphingomonas*, *Streptomyces*, *Luteimonas*, *Nocardioide*. Random forest indicated that the genus of *Stenotrophomonas*, *CL500-29\_marine\_group*, *cvE6*, *Bradyrhizobium*, *OM27\_clade*, and *Mycobacterium* made more contributions to the change of bacterial community composition caused by continuous cropping (Fig. 3d).

### 3.5 The microbial network complexity

The soil bacterial co-occurrence networks were built under different continuous cropping systems (Fig. 4). The network of the Y1 treatment consisted of 595 nodes linked by 2,352 edges, which were significantly higher than those in Y6 (428 nodes, 1,468 edges) and Y12 treatment (381 nodes, 1,096 edges), suggesting the strong co-occurrence patterns of soil bacteria under the only one-year cropping system. Moreover, according to the topological properties of the networks, the modularity and average degree of the network in the Y12 treatment were the lowest among three treatments, suggesting that continuous cropping leads to the simplification of the bacterial community.

### 3.6 The correlations between soil enzymatic activities and bacterial biological traits

Soil bacterial populations were significantly correlated with catalase ( $P = 3.4e-05$ , Fig. 5c) and urease activities ( $P = 0.0011$ , Fig. 5d), but not with the activities of  $\beta$ -glucosidase and alkaline phosphatase ( $P > 0.05$ , Fig. 5a, b). There are significant positive correlations between bacterial community composition and catalase ( $P = 1.7e-05$ , Fig. 5g) and urease ( $P = 0.0012$ , Fig. 5h). Soil  $\alpha$ -diversity (i.e., Shannon index) significantly and positively correlated with  $\beta$ -glucosidase ( $P = 0.016$ , Fig. 5i) and negatively correlated with catalase ( $P = 6.6e-05$ , Fig. 5k). These results suggested that the activities of soil  $\beta$ -glucosidase and catalase were closely linked with the change of bacterial community under a continuous cropping system.

### 3.7 The associations between soil chemical properties and soil biological traits

The relationships among soil properties, bacterial community (populations and diversities), and soil enzymatic activities were shown in Fig. 6. pH, SOC, total N, and available P were strong soil factors for dissimilarities of the  $\beta$ -diversity, populations, and Shannon index of bacteria. The selected soil chemical properties had the greatest explanation for the  $\beta$ -diversity of soil bacteria (93.62%), followed by the populations of bacteria (87.40%). The explanations of selected soil properties for three types of enzymes (i.e., alkaline phosphatase, catalase and  $\beta$ -glucosidase) were significantly higher than those for urease. a positive correlation between pH and the, with the importance coefficient of 3.34, and a negative correlation between pH and the number of bacteria and Shannon index, with the importance coefficient of 2.02 and 1.83, respectively. pH was the negative factor that significantly affected  $\beta$ -diversity of bacteria (importance = 3.34,  $P = 0.0099$ ), whilst positively affected populations (importance = 2.02) and Shannon index of bacteria (importance = 1.83). In addition, SOC and total N significantly and negatively affected  $\beta$ -diversity of bacteria, with the importance coefficients of 6.85 and 7.46, respectively, whilst SOC and total N positively correlated with soil bacterial populations (importance coefficients of 7.23 and 7.56, respectively) and Shannon index (importance coefficients of 7.19 and 6.03, respectively). Interestingly, the effect of soil phosphorus on biological traits were opposite to that of SOC and TN.

## 4. Discussion

### 4.1 Continuous reduces microbial diversity and populations

Bacterial diversity is a key component of soil biodiversity. Soil biodiversity is positively related to the sustainability of the ecosystem [33], however, which is sensitive to the change in farmland management strategies (e.g., cropping systems, tillage). In this study,  $\alpha$ -diversities (Shannon index) of bacterial communities in Y6 and Y12 were significantly lower than that in Y1 treatment. Moreover, rhizosphere soil bacterial  $\alpha$ -diversity decreased with the extension of continuous cropping time. A reduced diversity of soil bacteria may result in an incompact ecosystem [34], as such, continuous cropping of cut chrysanthemum destroyed the functional stability of the soil. Our finding was in line with the findings of [3], who stated that the  $\alpha$ -diversity of the bacterial community in soybean corn rotation system was higher than that in monocrop continuous cropping treatment. The rhizospheric soil Shannon index was significantly declined over time, possibly because of the accumulation of a large number of pathogenic bacteria in the rhizosphere, thereby generating continuous cropping obstacles. Moreover, the simplification of plant species and root exudates type (e.g., carbohydrates and amino acids) caused by continuous cropping would decrease the diversity of soil microorganism [35]. This viewpoint was also evidenced by [36], who reported that plant roots in monocropping would repeatedly release the same exudates, which may lead to a significant increase in the number of pathogens that use these substrates. However, our result is inconsistent with the finding of [3], Who found that continuously planting soybeans for 13 years led to the increases of soil bacterial diversity and abundance. The possible reason for this difference is that rhizosphere soil and bulk soil respond differently to tillage. Bulk soils in the continuous cropping system may be more susceptible to other agricultural management practices such as fertilization. Schmidt et al. [37] demonstrated that agricultural managements had different effects on root and soil bacterial communities. In our study, the richness (Chao1 index) was not affected by the continuous cropping (Fig. 2b). This is consistent with a previous observation that the richness was less variable in responses to environmental change than the diversity [38]. Changes in the composition of microbial communities do not necessarily result in changes in richness or diversity, due to the existence of functional redundancy [39].

In the present study, our results showed that continuous cropping of cut chrysanthemum led to a significant reduction in rhizosphere soil bacterial populations. Our finding is consistent with the result of prior study [40], indicating that long term continuous planting of soybean reduced the rhizospheric soil bacterial counts. In a previous study, long-term continuous cropping caused microflora changing from “bacterial-type” to “fungal-type”, and bacterial abundance showed a downward trend [41]. Similarly, with the increase of the consecutive monoculture years, the total amount of soil bacteria in the sweet potato field decreased significantly [42].

Variations in the microbial composition may affect the microbial function [43]. We observed that rhizosphere soil bacterial community of cut chrysanthemum was obviously distinct among different cropping years (Fig. 2d; Fig. 3b). Our finding was similar to the results from the Lanzhou lily continuous

cropping system, where the microbial community compositions were distinguished into three groups during 9 years of continuously replanting [44]: one group (0–3 years), second group (3–6 years), and third group (6–9 years). In addition, a similar result was gained in the monoculture system of soybean [3].

## 4.2. Changes of relative abundance of potentially pathogenic bacterial groups

At the phylum level, the top one phylum *Proteobacteria* were significantly decreased by the continuous cropping (Fig. 2d). *Proteobacteria* have been widely reported as the predominant bacterial phyla in rhizospheric soils because of their rapid growth rates [45]. The phylum *Proteobacteria* includes plentiful beneficial taxa such as plant growth-promoting bacteria, which promote nutrient absorption and prevent diseases, and are closely related to plant disease [46]. A prior study has reported that the antagonistic role of *Proteobacteria* in the plant rhizosphere was diminished during continuous cropping [15]. Our results supported these views that the relative of abundance *Proteobacteria* was suppressed over chrysanthemum monoculture cropping.

Another beneficial predominant phylum *Acidobacteria* (the fourth abundant, average relative abundance = 11.17%) was significantly lower in Y6 and Y12 treatments than that in Y1 treatment. Our result is in agreement with the finding of Yin et al. [47], who demonstrated that lower frequencies in the rhizosphere of diseased plants were found than in healthy plants. In contrast, the phylum *Actinobacteria* was significantly elevated in the soils of Y6 and Y12 treatments relative to that of Y1. This may be due to the increased nutrient availability of soil over many years of fertilization during chrysanthemum continuous cropping leading to an increase in this copiotrophic bacteria [41]. *Gemmatimonadetes* are known to conduce to SOC sequestration and decomposition of cellulose and lignin [48]. The relative abundance of the *Gemmatimonadetes* was reported to be closely linked with soil nutrients [48]. In the present research, the relative abundance of *Gemmatimonadetes* was higher in the rhizospheric soils of Y6 and Y12 treatments than that in Y1 treatment, as higher nutrients contents (e.g. AP,  $\text{NO}_3^-$ ) were detected in the Y12 treatment (Table 1), which supported above viewpoint very well.

Table 1

Changes of soil physical and chemical properties under long-term continuous cropping system

| Treatment | pH     | Total N/<br>(g kg <sup>-1</sup> ) | SOC/<br>(g kg <sup>-1</sup> ) | Total P/<br>(g kg <sup>-1</sup> ) | Avail P/<br>(mg kg <sup>-1</sup> ) | NH <sub>4</sub> <sup>+</sup> -N/<br>(mg kg <sup>-1</sup> ) | NO <sub>3</sub> <sup>-</sup> -N/<br>(mg kg <sup>-1</sup> ) |
|-----------|--------|-----------------------------------|-------------------------------|-----------------------------------|------------------------------------|--|--|
| Y1        | 5.14 b | 3.30 a                            | 33.95 a                       | 0.27 b                            | 12.37 b                            | 5.5 a  | 199 b  |
| Y6        | 5.18 b | 2.59 b                            | 25.22 b                       | 0.34 a                            | 28.49 a                            | 4.5 a  | 149 b  |
| Y12       | 6.11 a | 2.35 c                            | 22.50 b                       | 0.34 a                            | 29.80 a                            | 5.8 a  | 111 a  |

Note: Different letters in the same column indicate significant difference ( $P < 0.05$ )

At the genus level, the genera *Stenotrophomonas*, *CL500-29\_marine\_group*, *cvE6*, and *Bradyrhizobium* played important roles in inducing the changes of community compositions under this continuous cropping system (Fig. 3d). In another continuous cropping system (*Panax notoginseng*), the genus *Stenotrophomonas* has been identified to be a key bacterial pathogen that led to the incidence of soil-borne disease under *Panax notoginseng* monoculture system [49]. The second important species, *CL500-29\_marine\_group* (belong to *Acidimicrobiaceae*), has been shown to play a dominant role in the carbon and nitrogen cycle [50]. Moreover, *CL500-29\_marine\_group*s were often considered as predominant groups in freshwater [51]. In the present study, the higher abundances of the *CL500-29\_marine\_group* were detected in continuous cropping treatments with low contents of TN and SOC, suggesting that this genus exhibits high energy metabolism in C/N-limited soil conditions not only in freshwater conditions. The third important species, *cvE6* belonging to chlamydial, was first detected in freshwater samples and named in 2001 [52]. All the chlamydiae described until now are able to infect vertebrates and induce diseases of the living body, but the target of infection is mainly vertebrates, including humans. Our study is the first-time report that the genus *cvE6* may be a potential cause of plant disease in the soil subject to continuous cropping obstacle. Furthermore, the relative abundance of *Bradyrhizobium* decreased over continuous cropping years, and a similar finding was also reported by Xiong et al. [53] in which the amount of the *Bradyrhizobium* reduced over the years of vanilla continuous cropping. Numerous studies have demonstrated that *Bradyrhizobium* played crucial roles in plant growth [54] and the suppression of soil-borne diseases [55], suggesting that the reduction of beneficial bacterial taxa may be the reason for the outbreak of soil diseases after 12-year continuous cropping of chrysanthemums.

## 4.3 Changes in microbial communities induced by soil factors under continuous cropping

Continuous cropping can cause changes in edaphic factors thereby affecting soil microbial community compositions and diversity. Our results showed that soil factors were significantly changed after 12 years of continuous cropping (Table 1). In this study, the results of soil SOC and TN were significantly lower in continuous cropping treatments than that in a one-year cropping system, which is in line with prior report [53]. Similarly, monocrop continuous cropping resulted in the unbalance of soil nutrients and the deterioration of soil properties [56]. On the contrary, the available nutrient (AP and  $\text{NO}_3^-$ ) and pH increased with continuous cropping years, which is in agreement with the findings of Zhong et al. [57], who demonstrated that long-term continuously cropped banana significantly increased soil available nutrients and soil pH value. In our study, pH exhibited the greatest effects on the composition and diversity of soil bacteria during continuous cropping of cut chrysanthemum even the pH varied < 1.0 units across different treatments (Table 1; Fig. 6). This finding is consistent with those of Degruene et al. [58], who reported that bacterial growth was significantly affected even soil pH varied < 0.5 units. Potentially, most bacterial groups exhibit a relatively narrow pH tolerance threshold [7].

Organic carbon is tightly associated with changes in microbial communities under continuous cropping system. Soil organic carbon significantly decreased with the years of cut chrysanthemum continuous cropping (Table 1), suggesting that the cut chrysanthemum continuous cropping field could be regarded

as a carbon-deficient system, thereby oligotrophic microorganisms were dominant in mono-cropped chrysanthemum soils, presumably because continuous intensive cultivation broke the soil structure of plow layer and in turn accelerated carbon degradation. Davis et al. [59] reported that SOC was associated with reduced disease incidence.

Soil microbes play an important role in soil P transformation [60]. In our study, Random Forest analysis revealed that AP and TP are important factors affecting the bacterial community and enzyme activity. Research by Shen et al. [61] also indicated that soil available P was relative to *Fusarium* wilt disease incidence of banana in soil. In addition, TN was the key factor explaining the decrease in populations and diversity of bacterial community in the rhizosphere during continuous cropping (Table 1). This is in line with a previous report that available N was the main edaphic factor influencing the distribution of the bacterial communities in crop continuous cropping system [62].

## 4.4 Continuous cropping reduces the microbial network complexity

Numerous researches employing microbial co-occurrence network analyses have expanded our understanding of microbial symbiosis patterns in different terrestrial ecosystems [63, 64], nevertheless, relatively little is known about the relationship between the patterns of microbial networks and soil function under continuous cropping system. The numbers of edges and nodes for the bacterial communities in Y6 and Y12 treatments were remarkably lower than those in the Y1 treatment, which indicated that Y6 and Y12 had smaller network sizes and lower taxa involved in the microbial interactions than that in Y1 treatment, suggesting that long-term continuous cropping of cut chrysanthemum led to the relatively simplified bacterial community. In general, microbial communities with greater network modularity are considered as better organized communities, which may lead to a more stable ecosystem. In the present study, the numbers of network modularity were significantly lower in Y6 and Y12 treatments than that in the Y1 treatment, indicating that the occurrence of continuous cropping obstacle may be due to the reduction of specific functional taxa and loose network structures caused by low modularity. Moreover, since complex soil microbial communities are more resistant and/or resilient to environmental disturbances [65] than simple individuals, a reduction in diversity and network complexity of soil microorganisms due to continuous cropping may have permanent negative effects on soil functions. As Morriën et al. [66] reported that soil microbial networks become tighter and absorb more carbon as nature restoration progresses, thus, when repairing the soil with continuous cropping obstacles, we should pay more attention to the improvement of physicochemical properties (e.g., SOC), and thereby effectively increase the complexity of soil microorganisms.

## 5. Conclusions

In summary, continuous cropping significantly decreased the enzymatic activities of  $\beta$ -glucosidase together with alkaline phosphatase, but remarkably increased urease and catalase. Soil bacterial population and diversity (Shannon index) significantly decreased over the years of continuous cropping.

The bacterial community compositions altered significantly through different replanting years, with the decrease of phylum *Proteobacteria* and *Acidobacteria* and the increase of *Actinobacteria* and *Gemmatimonadetes*. In particular, the accumulation of the pathogenic bacterial genera of *Stenotrophomonas* and *cvE6* and the depletion of beneficial bacterial genera *CL500-29\_marine\_group* and *Bradyrhizobium* were the reason for the chrysanthemum continuous cropping obstacle under the 12-year replanting system. Co-occurrence network analyses indicated that long-term monoculture cropping induced a decrease in microbial complexity and stability. Linear regression equation showed that  $\beta$ -glucosidase and catalase activities were significantly correlated with the composition and diversity of rhizospheric soil bacterial community, suggesting that  $\beta$ -glucosidase activity and catalase could be regarded as bio-indicator enzymes in the continuous cropping obstacle soil. Random forest analysis showed that pH, SOC, TN, and soil P availability played crucial roles in inducing the changes of soil enzymes and bacterial community diversity and populations. Overall, our study strongly supports our hypothesis that the soil decline of bacterial community complexity may be a key reason of soil continuous cropping obstacle occurrence after 12-year cut chrysanthemum monoculture.

## Declarations

### Data Availability

The data supporting the findings of this study are available from the corresponding author, Rui Tao, upon request.

### Funding

This work was financially supported by the Basic Public Welfare Research Project of Zhejiang Province (LGN20C150003), P. R. China.

### Conflict of Interest

The authors declare that there is no conflict of interest.

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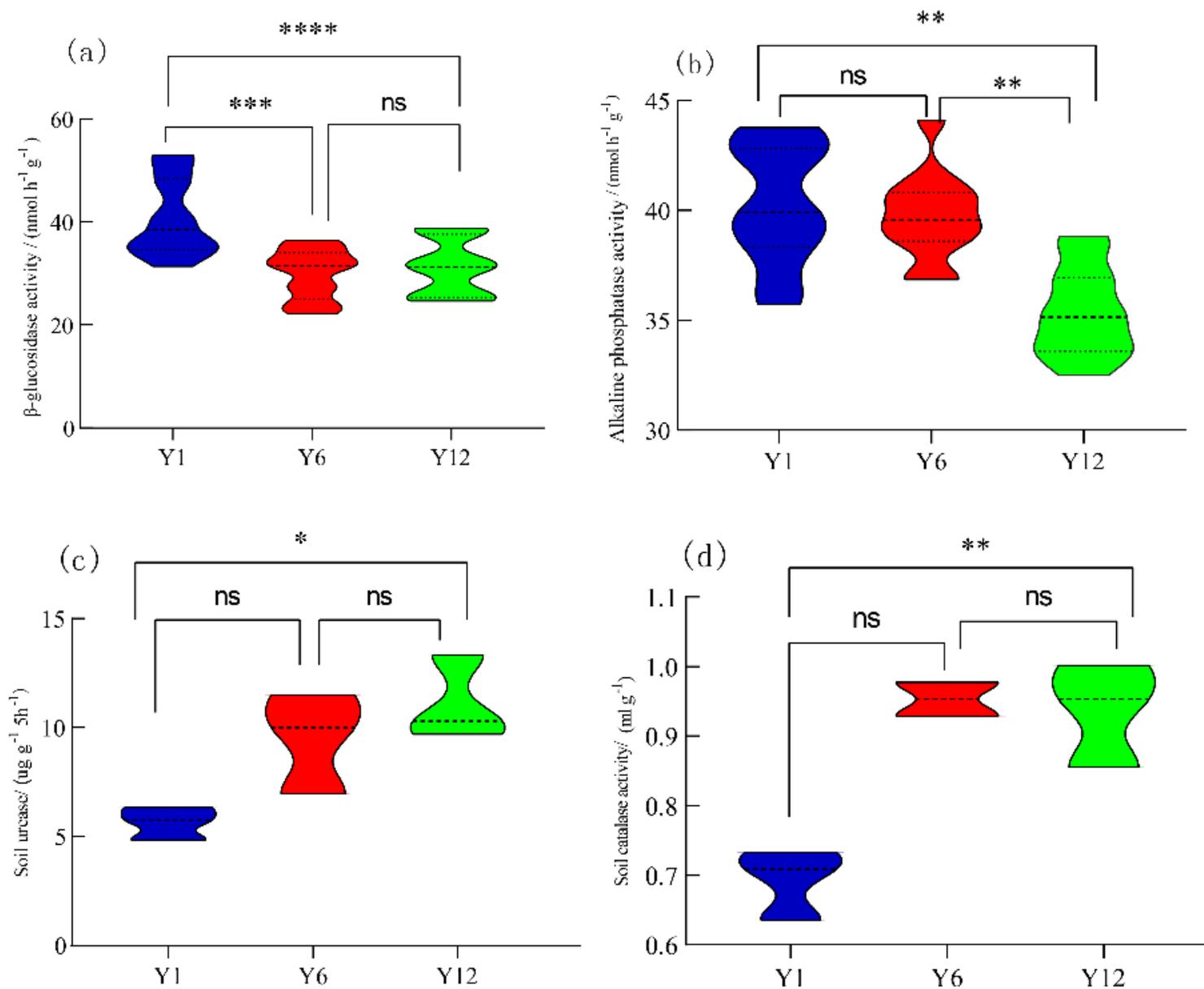
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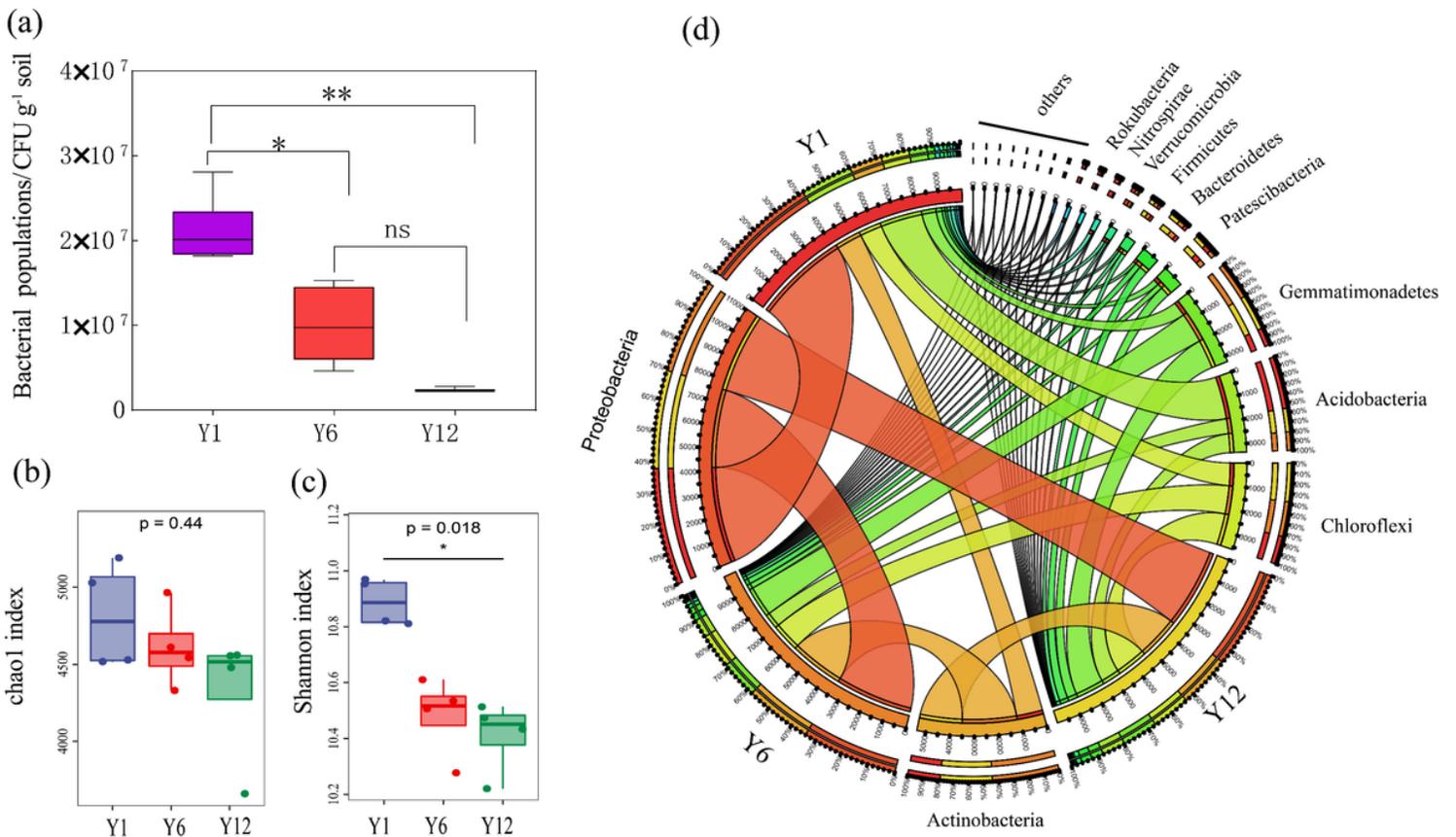
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## Figures



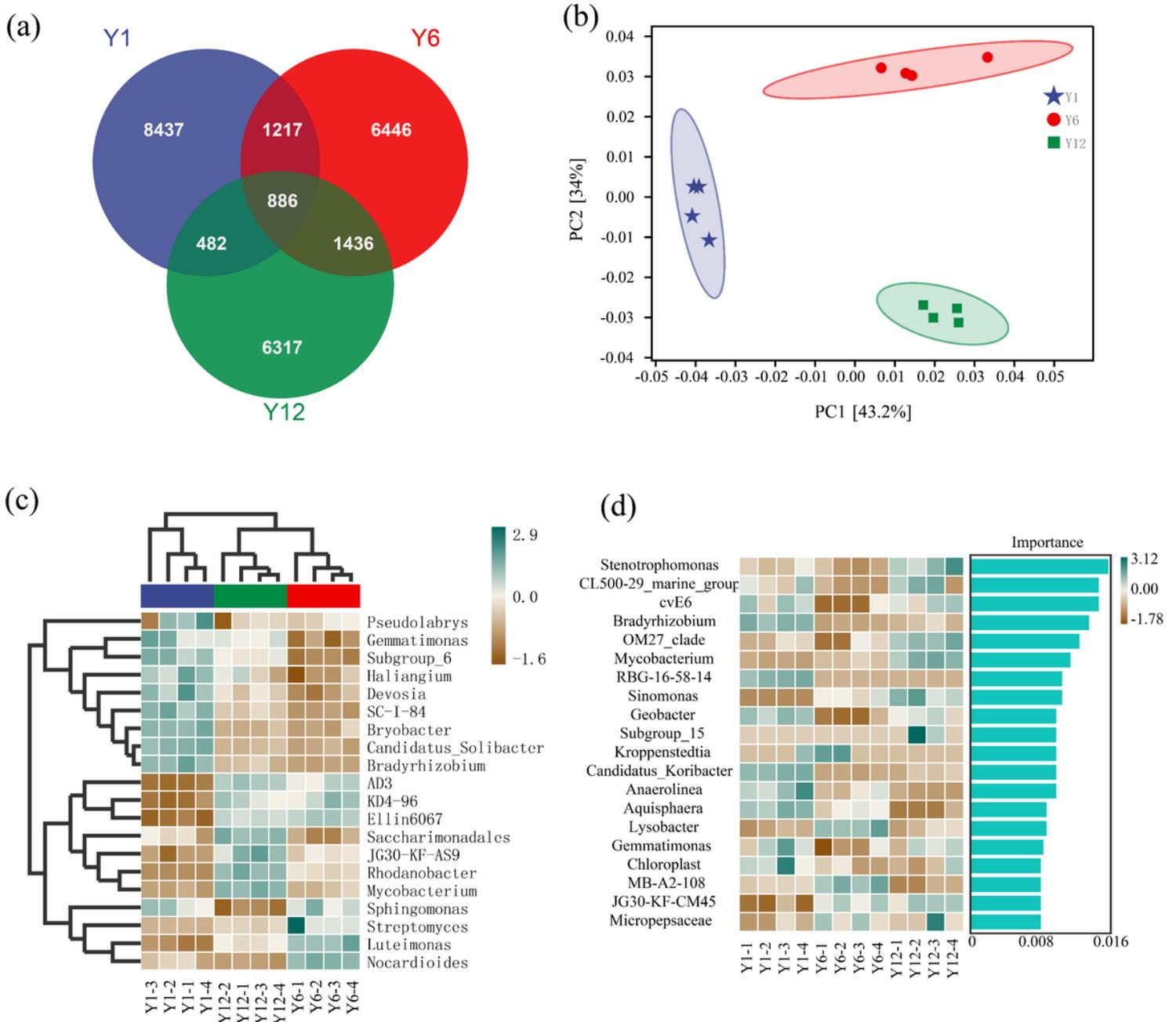
**Figure 1**

Violin plots showing the variance of soil enzyme activities under different continuous cropping treatments. “\*”, “\*\*”, “\*\*\*” and “\*\*\*\*” indicate a significant difference at  $p < 0.05$ ,  $p < 0.01$ ,  $p < 0.001$ , and  $p < 0.0001$  between two groups, respectively. Note: Y1, only planting for one years; Y6, continuous cropping for 6 years; Y12, continuous cropping for 12 years, respectively.



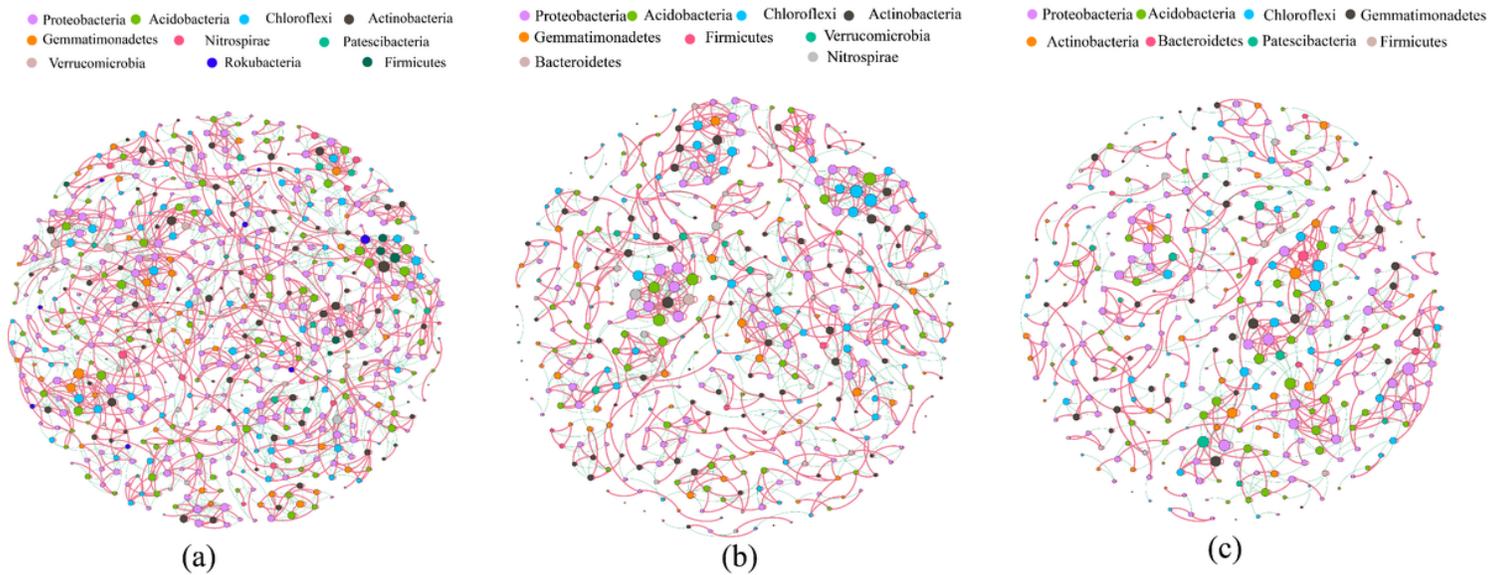
**Figure 2**

The variances of bacterial populations under different continuous cropping treatments were shown in (a). The variances of the  $\alpha$ -diversity of Chao1 index and Shannon index over continuous cropping years were shown in (b) and (c). Circle Diagram analysis showing the changes of relative abundance of bacterial phyla level among different continuous cropping treatments. Asterisks above the columns indicate different significant levels (\* $P < 0.05$ ; \*\* $P < 0.01$ ; \*\*\* $P < 0.001$ , and \*\*\*\* $P < 0.0001$ ). Note: Y1, only planting for one year; Y6, continuous cropping for 6 years; Y12, continuous cropping for 12 years, respectively.



**Figure 3**

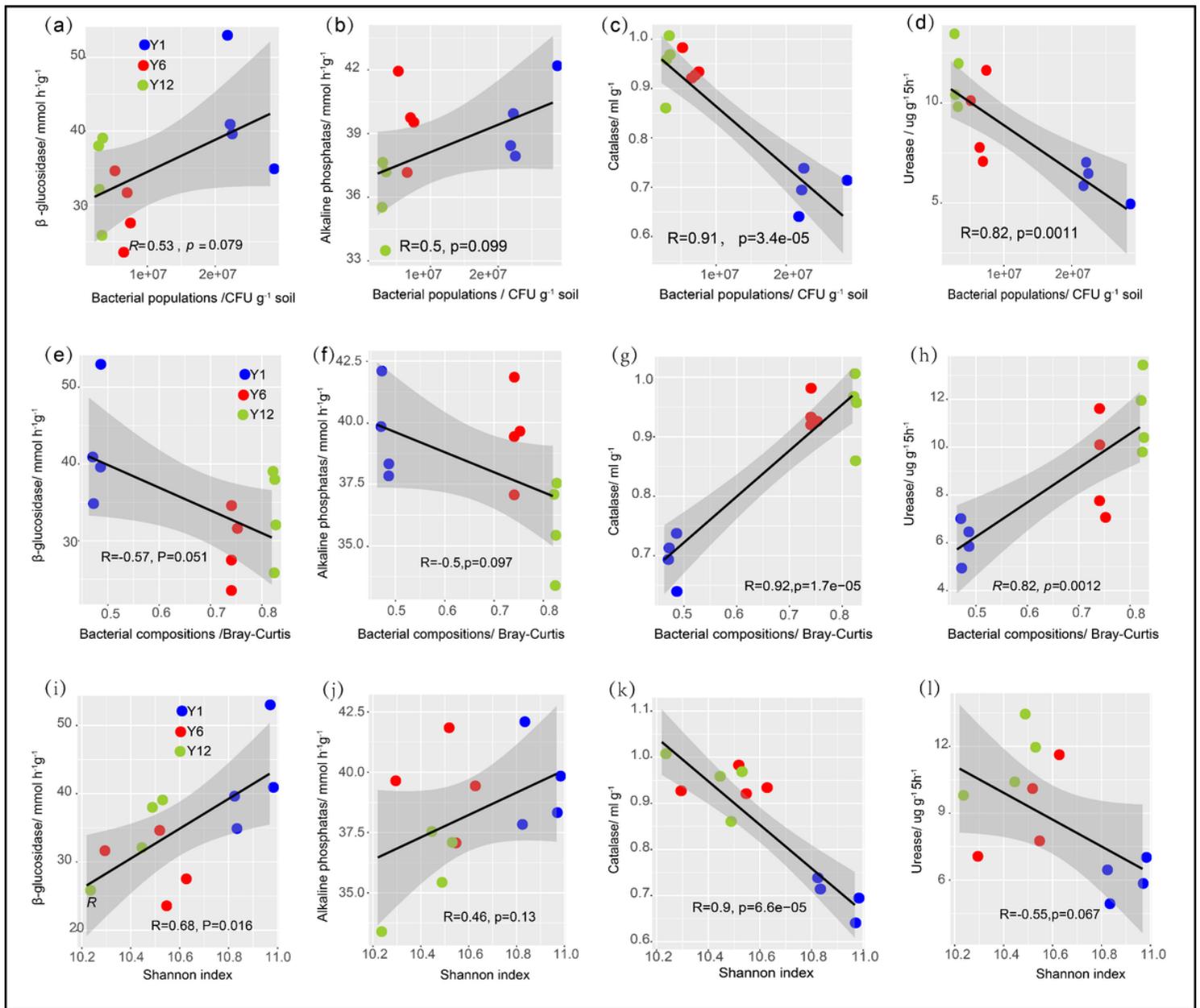
The shared and unique OTUs of AOB (A) and AOA (B) communities among different treatments as assessed by Venn diagram (a). The changes of bacterial community compositions as assessed by PCA (b); Heatmap showing the relative abundance of top 20 genera under different continuous cropping treatments (c); Random forest analysis revealing the key genera of bacterial community structure under continuous cropping(d).



| Topological index | Y1(a) | Y6(b) | Y12(c) |
|-------------------|-------|-------|--------|
| Nodes             | 595   | 428   | 381    |
| Edges             | 2352  | 1468  | 1096   |
| Average degree    | 7.906 | 6.83  | 5.753  |
| Modularity        | 29.64 | 12.27 | 4.614  |

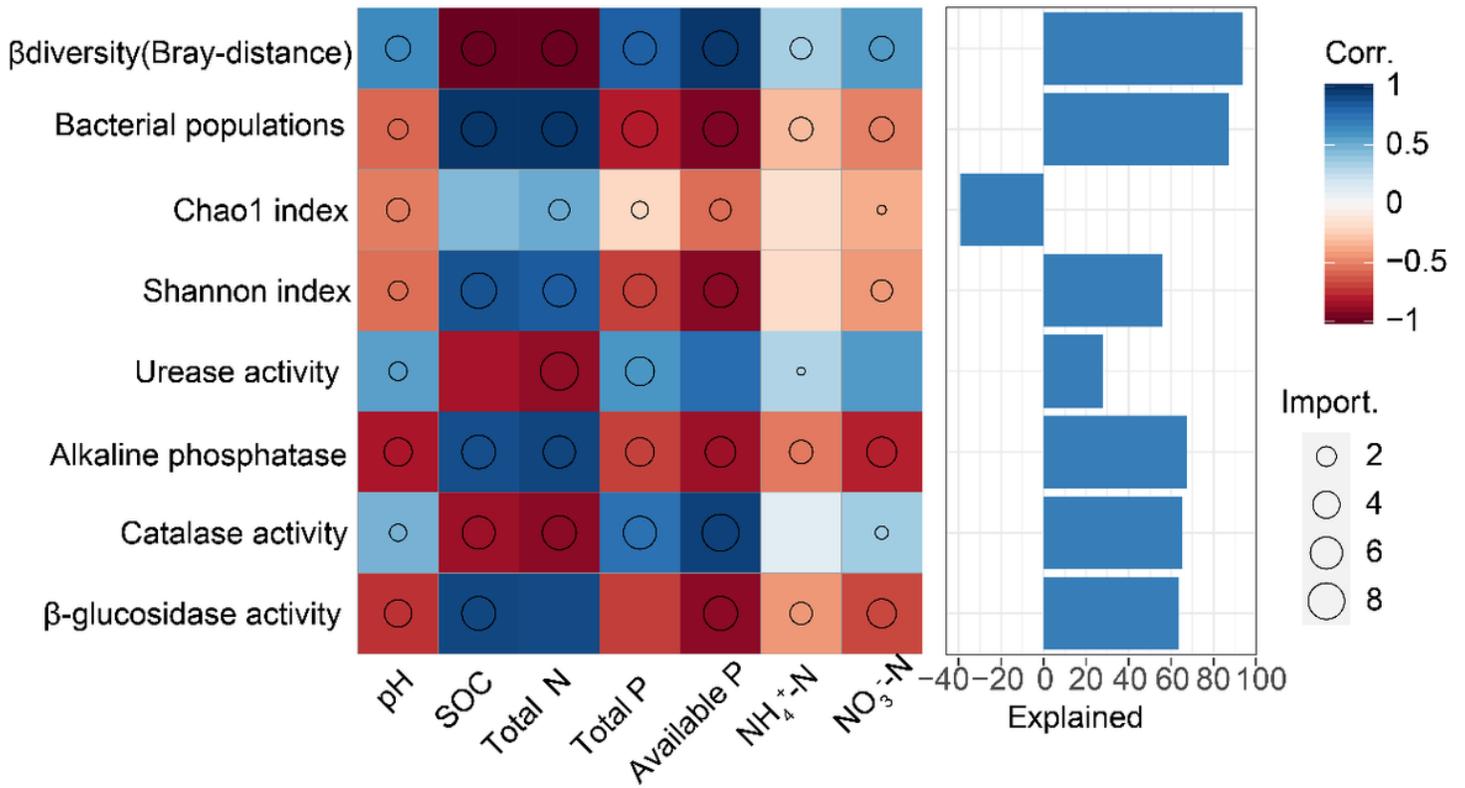
**Figure 4**

Properties of soil microbial correlation-based network under different continuous cropping systems. Network analysis showing the intra-associations inter-associations among different bacterial taxa. Networks were constructed at the operational taxonomic unit (OTU) level. The size of each node is proportional to the number of connections (i.e., degree). Edges between nodes indicate significant correlations among nodes (Spearman's  $r > 0.08$ ,  $P < 0.01$ ). Red and green edges represent T positive and negative associations between taxa.



**Figure 5**

The correlations between soil enzymatic activities and soil bacterial biological indicators.



**Figure 6**

The predictor importance of edaphic factors (pH, SOC, TN, TP, AP, NH<sub>4</sub><sup>+</sup>-N, and NO<sub>3</sub><sup>-</sup>-N) to the enzymatic activities and the populations, composition and diversity of bacteria.