Interaction with Endogenous Microorganisms, Comamonas testosteroni Enhanced the Degradation of Polycyclic Aromatic Hydrocarbon in Soil

Xueting Sun
Harbin Normal University
Xin Li
Harbin Normal University
Yue Cui
Harbin Normal University
Ziwei Jiang
Harbin Normal University
Qiao Wang
Harbin Normal University
Qian Lu (lq87618@163.com)
Harbin Normal University
Jizhe Cui
Harbin Normal University

Research Article

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Abstract

This study was to explore the functional role of *Comamonas testosteroni* (*Ct*) on soil indigenous microorganisms, and analyze the effect of *Ct* on PAHs degradation in PAH-contaminated soil. Results showed that inoculation of *Ct* could degrade naphthalene (Nap), phenanthrene (Phe), and benzo [α] pyrene (BaP) significantly. The degradation rate of Nap, Phe and BaP was 81.18%, 63.38% and 37.98% on day 25, respectively, suggesting that the low molecular weight of Nap and Phe were easier to be degraded by microorganisms than BaP. Network analysis showed that inoculation of *Ct* significantly increased the bacteria closely related to PAHs. Structural equation models confirmed *Steroidobacter* as functional bacteria could affect the degradation of Nap and BaP. Inoculated *Ct* could effectively enhance the synergy among indigenous bacteria to degrade PAHs. This would be helpful to understand the function of inoculated strains in PAH-contaminated soil and identify functional microorganisms of PAHs remediation.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are a class of fused-polycyclic aromatic ring compounds that are mainly discharged from the incomplete combustion of fossil fuels or other organic materials (Humel et al., 2020). PAHs are of great concern because of their lipophilic, mutagenic, and carcinogenic properties. The low aqueous solubility and the high solid-water distribution ratios promote PAH accumulation in the solid phases of the terrestrial environment (Lu et al., 2019a). With the development of industry, the concentration of PAH in the soil is typically detected at a relatively high-level in recent years (Sun et al., 2018). Long-term PAH pollution disturbs the soil ecological environment, causing severe potential risks to soil organisms and humans (Li et al., 2019a). Previous studies have found that naphthalene (Nap), phenanthrene (Phe) and pyrene (Pyr), etc. were predominant PAHs in 16 precedence-controlled PAHs on the United States Environmental Protection Agency (USEPA) priority pollutant list (Patel et al., 2018; Zhang and Chen, 2017). Nap is the lowest molar mass of the PAHs and it can be adsorbed onto the soil due to its adsorption capacity and high-water solubility (Mendes et al., 2020). Benzo [α] pyrene (BaP) is considered a priority control pollutant because of its carcinogenic, teratogenic and mutagenic effects (Bhattacharya et al., 2014). Therefore, it is necessary to find suitable remedial technology to alleviate the possible environmental risk posed by PAHs in soils.

In recent years, several physical, chemical, and biological technologies have been established and applied to remediation of PAH-contaminated soil (Varjani et al., 2017). Compared to the high cost and potential secondary contamination incurred by physical and chemical methods, bioremediation, especially microbial remediation, is considered a cost-effective remediation technology (Vila et al., 2015). Many microorganisms including bacteria and fungi have been isolated from PAH-contaminated sites with the ability to degrade PAHs efficiently. PAH-degrading bacteria can metabolize PAHs as a source of carbon and energy in soils (Aranda et al., 2016), and they play an important role in the degradation of PAHs. Some bacteria such as *Pseudomonas*, *Sphingomonas*, *Bacillus*, *Sphingobacterium* and *Mycobacterium* have been widely recognized and used to degrade PAHs (Venkateswar et al., 2015; Mallick, et al., 2011). A

previous study showed that a mixed inoculation of *Bacillus subtilis* and *P. aeruginosa* removed 82.2% of Phe within 30 days under laboratory conditions (Zhao et al., 2011). The isolated strain *Comamonas sp. ZF-3* had efficient biodegradability of phenolic compounds and heterocyclic compounds in coking wastewater (Yuan et al., 2020). It can be seen that bacteria have the ability to degrade PAHs with complex structures very efficiently. However, the biodegradation of PAHs solely utilizing native bacteria under natural conditions is a very slow process. The efficiency of bacteria to degrade PAHs are related to many factors, mainly including the concentration of effective bacteria, environmental factors, and the degree of soil PAHs pollution. Therefore, finding the optimistic degradation conditions is the key to improve the degradation efficiency.

Comamonas testosteroni (*C. testosteroni*) is a gram-negative bacterium belonging to the class of β-proteobacteria, which is widely found in soil, activated sludge and seabed sediments. *C. testosteroni* can degrade many sterols and PAHs, and even use these complex organic compounds as the sole carbon source (Zalesak et al., 2017). Moreover, *C. testosteroni* can degrade heavy metal pollutants in the environment (Zheng et al., 2014). It has a strong adaption to change environments and xenobiotics. With these attractive features, researchers have begun to explore *C. testosteroni* strains as environmental bioremediation hosts (Tang et al., 2018). However, due to the complexity of the natural ecosystem, finding the optimal inoculation conditions for inoculants is crucial to maximizing the degradation of pollutants. In this study, we inoculated *C. testosteroni* into PAHs (Nap, Phe, BaP) contaminated soil under an optimal system. The principal goals of this study were: (1) to determine the optimal degradation system through the orthogonal analysis to maximize the degradation of PAHs inoculated with *C. testosteroni*, (2) to analyze the effect of inoculation of *C. testosteroni* on the degradation of PAHs in the soil under the system, (3) to assess the relationship among inoculants, indigenous microbes and the degradation of PAHs. This research will provide useful perspectives and methods for solving PAHs soil pollution by inoculating *C. testosteroni*.

2. Material And Methods

2.1 Preparation of inoculant

C.testosterone strain (NR029161.2) were selected as inoculum. The inoculum was prepared by growing in lysogeny broth (LB) agar plates at 28°C for 14 h. The independent colony was inoculated into LB liquid medium and incubated at 28°C for 14 h on a rotary shaker (180 rpm). Exponential growth-phase cells were then harvested by centrifugation at 4°C for 15 min (4500 rpm), discarded the supernatant and resuspended individually in sterile water.

2.2 Experimental design and sample collection

Soils were collected from topsoil at a depth of 0–20 cm from Harbin Normal University, China. They were air-dried at room temperature (around 25℃) and then passed to a 2 mm sieve. Contaminated soil was prepared by adding Nap, Phe and BaP (> 98% purity; Sigma-Aldrich, Shanghai, China) stock solution (in acetone) to sieved soil with different concentrations (Table 1).

Table 1
The combination of parameters based on orthogonal table with 3 factors and 3 levels.

	Factors		
Level	A Concentration of total PAHs (μg/kg) (Nap/Phe/BaP)	B Concentration of bacteria (DW, %)	C Temperature (℃)
1	4800 (2400/800/1600)	0	30
2	2400 (1200/400/800)	0.01	20
3	1200 (600/200/400)	0.05	10

The orthogonal design was used to find the optimal PAHs degradation system. They were designed with 3 factors and 3 levels of the orthogonal table as shown in Table 1. PAHs concentration (Factor A), bacteria concentration (Factor B) and temperature (Factor C) were chosen as three factors. The levels of total PAHs concentrations were 4800 μg/kg, 2400 μg/kg and 1200 μg/kg, respectively. The levels of *C.testosterone* concentrations were 0, 0.01% and 0.05%. The levels of temperature were 30 ℃, 20 ℃ and 10 ℃. Samples of the soils, each weighing 200 g (dry weight, DW) was placed in a glass container with 500 g capacity and covered with aluminum foil.

According to the results of the orthogonal experiment, the optimal conditions were selected to study the changes in the degradation process of soil PAHs. Two groups of the experiment were carried out, including soil with Nap, Phe and BaP (CK), and inoculated of *C.testosteroni* in soil with Nap, Phe, BaP (CT). The experiment continued for 25 days and each experimental treatment was repeated three times. The samples were collected on days 0, 2, 4, 6, 10, 15, 20 and 25. Each sample was divided into two parts, one part was sealed with tinfoil bags, and quick-frozen with liquid nitrogen, then stored at -80℃ for microbial community and molecular biological analysis, the other part was stored at 4℃ for PAHs analysis.

2.3 Soil PAH analysis

Sample extraction and purification were performed following the USEPA Method 3550B (USEPA 1996) with some modifications. Briefly, an amount of 10 g of each soil sample with diatomite mixtures was extracted with the ASE 150 (Dionex Corp, USA) through n-hexane/dichloromethane (1:1, v/v) solvent mixtures. The extraction was evaporated by a rotary vacuum evaporator, concentrated to about 1 mL, and then loaded on a silica gel column for purification, and the residual solvent was removed by nitrogen. The residue was dissolved in 1 mL of n-hexane. PAHs were analyzed by Agilent 7890A gas chromatography system combined with 5975C mass selective detector (GC/MS), and PAHs were separated by HP-5MS (30m×0.32mmi.d.×0.25μm film thickness), details could be found in Li et al. (2019a).

2.4 qPCR and high throughput sequencing

Soil microbial DNA was extracted by using the Ezup column soil genomic DNA extraction kit (Sangon Biotech, Shanghai, China). The genes (*16SrRNA*, *3α-HSD/CR* and *NahAc*) abundance were determined by real-time fluorescent quantitative PCR (qPCR). It was performed on each DNA template by using the SYBR Green Master Mix on the ABI 7500 Real-Time PCR system. The primers and amplification conditions were listed in Table S1. The qPCR reaction conditions as follows: pre-denaturation for 30s at 95℃, denaturation for 10s at 95℃, annealing for 30s at 60℃, 40 cycles. The qPCR reaction mixture consisted of 2μL of DNA template, 10μL of 2×ChamQ Universal SYBR qPCR Master mix, 0.4μL of 50×Rox Reference Dye 2, 0.4μL each forward and reverse primer, to a final volume of 20μL with ddH_2O.

The *16SrRNA* gene comprising V3 and V4 hypervariable regions was amplified by PCR using the forward primer 341F (5'- CCTACGGRRBGCASCAGKVRVGAAT-3') and reverse primer 806R (5'-GGACTACNVGGGTWTCTAATCC-3'). High-throughput sequencing of PCR product was performed on an Illumina MiSeq platform (Illumina, San Diego, CA, USA) at GENEWIZ, Inc. (China).

2.5 Data analysis

SPSS 25.0 and Origin 2018 were used for statistical analysis of the orthogonal experiment and PAHs content. The changes in the microorganism community and the relationships between microbes and functional gene were analyzed by using the nonmetric multidimensional scaling ordination (NMDS) and redundancy analyses (RDA), both of them were conducted by Canoco5.0 software. The relationship between PAHs and microorganisms was analyzed by network analysis (Cytoscape 3.6.1). The structural equation model (SEM) was performed by the SPSS package of Amos 20.0. Functional capacity of the bacteria was predicted based on the bacteria composition using the phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt), functional genes were predicted based on the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (https://www.kegg.jp/).

3. Results And Discussion

3.1 Orthogonal analysis

Inoculation was a favorable way to biodegrade PAHs. However, many factors affect the degradation of PAHs by microorganisms, temperature, indigenous microbial community structure, and inoculum concentration. The orthogonal experiment is a versatile optimization and economical experimental design method for arranging experiments with multiple factors and multiple levels (Mu et al., 2020). To evaluate the effectiveness of environmental factors on PAHs degradation by *C. testosteroni*, the orthogonal test was adopted to three factors and three levels (Table 1), and the results were presented in Table 2. A1B2C2 was the optimal condition of PAHs degradation in this study. The degradation rate of total PAHs reached up to 58.09% on day 25 when the concentration of PAHs (Nap/Phe/BaP) was 4800 (2400/800/1600) μg/kg, the concentration of bacteria was 0.01% and the temperature was 20℃. In contrast, under the conditions of 10℃ with lower PAHs concentration, PAHs was only decreased 9.39% (A3B1C3). The above results indicate that suitable temperature or suitable inoculation concentration of *C. testosteroni* was beneficial to the degradation of PAHs.

Table 2
The contribution of orthogonal experimental factors to the degradation rate of PAHs

Experimental numbers	Factor			Result
	A	B	C	Total PAHs degradation rate(%)
A1B1C1	1	1	1	31.01
A1B2C2	1	2	2	58.09
A1B3C3	1	3	3	24.98
A2B1C2	2	1	2	21.01
A2B2C3	2	2	3	49.52
A2B3C1	2	3	1	18.62
A3B1C3	3	1	3	9.39
A3B2C1	3	2	1	39.03
A3B3C2	3	3	2	26.81
K_1	114.08	61.41	88.65	
K_2	89.15	146.64	105.91	
K_3	75.23	70.41	83.89	
$\bar{K}_1$	38.03	20.47	29.55	
$\bar{K}_2$	29.72	48.88	35.30	
$\bar{K}_3$	25.08	23.47	27.96	
Optimal Level	A1	B2	C2	

$\bar{K}$

K_i represents the value of the i-th level, K_i represents the mean of the i-th level, and R_j represents the range of the factor *j*.

Experimental numbers	Factor			Result
	A	B	C	Total PAHs degradation rate(%)
R_j	12.95	28.41	7.34	

$\bar{}$

K_i represents the value of the i-th level, $\bar{K}_i$ represents the mean of the i-th level, and R_j represents the range of the factor *j*.

In order to analyze the influence of various factors on the degradation efficiency of PAHs, the range analysis was performed on the orthogonal experiment results. Base on the results of range analysis, the order among the factors was arranged according to the magnitude of the range (Table 2). The greater the range, the greater the influence of factor *j* on the test index. For the PAHs degradation efficiency, the order of significant influence of various factors was as follows: bacteria concentration > PAHs concentration > temperature. These results revealed that the inoculation level of *C. testosteroni* had significant influences on the degradation of PAHs.

3.2 Concentrations and degradation of PAHs

Based on the optimal system in the orthogonal experiment (A1B2C2), the dynamic characteristics of Nap, Phe and BaP degradation in the soil were further analyzed. Figure 1 showed the concentration changes of Nap, Phe and Bap at different times after inoculation during 25 days in CT. The concentration of these PAHs was showed a downward trend and the degradation trends of Phe and BaP were similar (Fig. 1a). Initially, Nap, Phe and BaP were 2315μg/kg, 770μg/kg and 1529μg/kg, respectively. At day 25, these PAHs decreased to a minimum value, and there were significantly lower than the initial ones ($p < 0.05$).

The degradation rates of Nap, Phe and BaP were analyzed in CT. Figure 1b showed the average PAHs degradation in the order of Phe (81.2%) > Nap (63.4%) > BaP (38.0%) at day 25. The degradation rate of Nap was rapidly increased to 54.01% on the 10th day and then increased slowly by only 9.4% on the 25th day compared with that on the 10th day. At the end of the experiment, the accumulation of Nap degradation was 1467μg/kg. Phe and BaP were effectively degraded and the degradation rate was 43.8% and 21.0% during 10 days, respectively. Phe had a high degradation rate in the following 15 days (37.8%), however, the degradation rate of BaP was lower than that of Phe. BaP was composed of 5-ring polyarenes, which belongs to high molecular weight PAHs (HMW-PAHs, ≥ 5 rings). The solubility of PAHs was one of the limiting factors for PAHs degradation. The increases in the molecular weight of PAHs lead to a decrease in their biodegradability (Ghosal et al., 2016). In the first 10 days, the degradation rate was Nap > Phe > Bap, and Nap was significantly higher than other PAHs in CT ($p < 0.05$). However, in the following 15 days, Nap had the lowest degradation rate (9.4%) compared with other PAHs. Previous research has shown that HMW-PAHs were converted into low molecular weight PAHs (LMW-PAHs, < 4 rings) and then degraded (Minkina et al., 2019). We speculated that BaP and Phe might be converted into

Nap for further degradation, which should be the main reason for the low Nap degradation rate detected from 10-25days. The degradation rate of Phe was the highest (81.2%) within 25 days, indicating that inoculation of *C. testosteroni* effectively promoted the Phe degradation. In other words, under the condition of A1B2C2, inoculation of *C. testosteroni* could rapidly reduce Nap in a short time, and effectively decline the concentration of Phe and BaP. The degradation of PAHs might be due to the inoculation of *C. testosteroni* or it was promoting the synergism of indigenous microorganisms.

3.3 Microbial performance under PAHs expose in soil

The microbial richness could be reflected by the Ace index (Sengupta et al., 2015). The microbial richness of CK and CT under PAHs expose was decreased, however, it was generally higher in CT than that in CK except on the 6th day (Fig. 2a). The main reason might be related to the relative abundance of *C. testosteroni*, which was significantly increased on day 6 (> 60%) and then species richness was reduced. The diversity of microorganisms was investigated according to the Shannon index (Lu et al., 2018). In this study, the diversity of the CT was different compared with CK (Fig. 2a). Inoculation of *C. testosteroni* could decrease the bacterial diversity during 0–6 days and then increased in the later period (10–25 days). Inoculation of *C. testosteroni* could change the bacterial diversity and richness. NMDS was used to identify differences in the bacterial community for each group (Fig. 2b), which further showed that the bacterial community was different between the inoculated group (CT) and non-inoculated group (CK). Under PAHs exposure, bacterial community composition changed rapidly during 25 days. Moreover, the microbial community structure of the CK from 0–6 d was relatively similar, however, it was opposite in CT, which might be caused by the inoculation of *C. testosteroni*. The bacterial community structure was changed continuously in CK, suggesting that the PAHs might be harmful to bacterial metabolism and survival and then bacterial community succession. Notably, samples from CK and CT were grouped in diverse polygonal regions and no intersection of the bacterial communities between the two groups, suggesting that *C. testosteroni* inoculant affected bacterial communities due to its synergistic effect on indigenous bacteria in the soil.

High-throughput sequencing results showed that the soil bacterial community mainly originated from 25 phyla. The bacterial community composition of the two groups was similar at the phylum level. The dominant phylum consisted of Proteobacteria, Acidobacteria, Actinobacteria, Bacteroidetes, Chloroflexi, Gemmatimonadetes, and the relative abundances accounted for 21.8–53.5%, 13.94–37.25%, 8.27–35.05%, 5.36–19.51%, 3.71–6.89%, 2.73–6.38%, respectively (Fig. S1). Proteobacteria and Acidobacteria have the highest abundance, accounting for about 60% on average and this phenomenon was similar to the result of Delgado et al. (2018). The abundance of Proteobacteria was higher in CT than that in CK, which might be caused by the inoculants of *C. testosteroni* which belongs to Proteobacteria. The classified dominant genera in the top 30 abundance of the bacterial community composition in soils were plotted as a relative abundance map of the genera level (Fig. 2c). The dominant bacterial genus mainly includes *Comamonas*, *Sphinomonas*, *RB41*, *Pseudarthrobacter*, *Flavobacterium*, *Lysobacter*, with relative abundances ranging from 0 to 37.73%, 1.88 to 15.2%, 3.25 to 7.53%, 0.47 to 18.53%, 0.03 to 11.71%, 0.18 to 4.94%, respectively.

In the CT, *Comamonas* was the main genus, showing a fluctuating change, peaking at day 6 and disappearing at day 25. The abundance of *Sphingomonas* and RB41 showed a gradual upward trend and became the main genus at day 25 (Fig. 2c). *Comamonas* and *Sphingomonas* could effectively degrade PAHs (Smułek et al., 2020). Moreover, the concentration of Nap, Phe and BaP were decreased and reached the minimum at day 25 in this study. It was proved that the inoculants could better adapt to the soil conditions and perform their functions or cooperate with the indigenous microorganism to degrade PAHs. The abundance of *Flavobacterium* was higher at 0-4d and then suddenly decreased from day 6 to day 25 in CT, while it was extremely low in CK (0.03–0.18%). Previous research showed *Flavobacterium* had the potential to degrade PAHs (Ahmad et al., 2019). The microbial community structure of the CK was a small change in the early stage, but the inoculation had a significant effect on the microbial community. We speculated that the degradation of the PAHs in the CT was closely related to the dynamic changes of microorganisms, and *C. testosteroni* might further degrade PAHs by affecting indigenous bacteria. It is worth noting that the abundance of *Pseudarthrobacter* was high at 25d in the CK. The study of Li et al. (2019b) reported that *Pseudarthrobacter* was affected by PAHs exposure, which could be used as an important indicator of soil PAH pollution, indicating that PAHs pollution at day 25 was worse in CK compared with CT.

3.4 Quantitative analysis of gene abundance

16SrRNA gene was present in all bacterial genomes, and it was generally used as a marker gene for bacterial classification. In this study, the *16SrRNA* gene copy number was measured to determine the changes in bacterial quantity in the soil. As shown in Fig. 2d, the average abundance of *16SrRNA* gene in CT was generally higher than that in CK during 0-25d except for the 15d and ranged from 242.3 to 620.5 log copies/gDNA, indicating that *C. testosteroni* could increase the abundance of bacteria. Moreover, the abundance of the *16SrRNA* gene were obvious fluctuations in CT might due to the impact of PAHs or the competition among microorganisms.

3α-hydroxysteroid dehydrogenase/carbonyl reductase (3α-HSD/CR) from *C. testosteroni* was a member of the short-chain dehydrogenase/reductases (SDRs) superfamily, which was a class of enzymes that metabolize a range of substrates including steroid, monosaccharides and PAHs, etc (Ji et al., 2014). The gene abundance of *3α-HSD/CR* has a certain effect on the degradation of PAHs. In this study, the gene abundance in the CT was significantly higher than that in the CK within 25 days ($p < 0.05$) (Fig. 2e), indicating that inoculation of *C. testosteroni* increased the degradation efficiency of PAHs. On the day of 25, the abundance of *Comamonas* was very low in CT (Fig. 2c), but the gene abundance of *3α-HSD/CR* was still higher in CT than that in CK. This result showed that the presence of indigenous microorganisms who contributed greatly to the *3α-HSD/CR* gene abundance, and their potential to degrade PAHs was activated by *C. testosteroni*.

NahAc was the common initial dioxygenase gene encoding the large (α) subunit of dioxygenase, which was the critical enzyme in the upper pathway of PAHs degradation (Xu et al., 2019). The first step for aerobic bacteria to degrade PAHs was to hydroxylate the aromatic ring through dioxygenase to form cis-dihydrodiol and to reform the diol intermediate by the dehydrogenase. Subsequently, the hydroxylated

aromatic ring was cleaved by the ortho- or inter-cleavage pathway through the diol or outer diol ring cleavage dioxygenase. Finally, it was converted into intermediates of the TCA cycle by intermediates such as catechol (Mallick et al., 2011). Quantification of *NahAc* gene abundance could evaluate the ability of PAHs degradation by indigenous microorganisms. The *NahAc* gene abundance was significantly higher in CT than that in CK ($p < 0.01$), indicating that inoculation of *C. testosteroni* might enhance the synergy of indigenous microorganisms to degrade PAHs (Fig. 2e). The changing trend of *NahAc* abundance was consistent with *16SrRNA* gene from day 0 to day15. At this period, *NahAc* abundance might mostly be affected by the number of bacteria. After 15d, its abundance increased, indicating that the inoculation of *C. testosteroni* gradually changed the microbial community and caused more endogenous microorganisms to participate in the degradation of PAHs. The results of RDA showed that the correlation between bacteria and functional genes in the CK was relatively scattered while concentrated in CT, which might further explain *C. testosteroni* could increase the potential of indigenous bacteria to degrade PAHs (Fig. S2).

3.5 Predicted potential function of bacterial community

PICRUSt was developed to predict the functional characteristics of the microbial community based on *16SrRNA* homology and conserved feature of functional contribution (Li et al., 2019b). All predicted functional genes were mainly associated with six components in KEGG pathways, approximately 51% associated with metabolism, 15% associated with genetic information processing and 12% associated with environmental information processing. Since the highest proportion of predicted functional genes were associated with metabolism, further exploration of the responses of the soil microbial metabolism to PAH exposure is necessary.

Functional genes included the metabolism of amino acid, carbohydrate, lipid, nucleotide, energy, and xenobiotics, which were predicted in large quantities. In CT, more complex substances such as lipid and xenobiotics metabolism were improved after inoculation (Fig. 3a). In other words, *C. testosteroni* improved the ability of soil microorganisms to degrade complex compounds. The subsystem of xenobiotics biodegradation and metabolism involved 12 components associated with PAHs were predicted. These results were analyzed for further exploration of the responses of soil microbial metabolism to PAHs (Fig. 3b). We found that the majority of metabolism was stronger in the CT, such as PAH degradation, especially benzoate degradation and aminobenzoate degradation. Moreover, it was interesting that the change was similar to the abundance of *C. testosteroni*, with the peak at day 6 (Fig. 2d), indicating that *C. testosteroni* had an important impact on the metabolism of degrading PAH for bacterial communities. These metabolisms weakened at the day of 25 in CT, while there were opposite in the CK, indicating that the corresponding substances had been effectively degraded.

3.6 Identifying functional microorganisms for PAHs degradation

The interaction between different genus bacteria in CK and CT were described in Fig. 4a and b, respectively. The 23 nodes and 29 nodes were found in CK and CT respectively. In addition, inoculation

with *C. testosteroni* could significantly enhance the correlation between each bacteria genus in CT, and the positive correlation was significantly increased in CT compared with CK ($p < 0.05$). *C. testosteroni* was only negative correlation with RB41 (Fig. 4b). In order to better understand the effect of microorganisms on PAHs, the network analysis was constructed based on the relationship between PAHs and bacteria at the genus level in CT. The results showed that a total of 13,12, and 16 bacteria genera were significantly negatively correlated with Nap, Phe and BaP, respectively ($p < 0.05$) (Fig. 4c). The negative correlation represented the action of microorganisms on PAHs or the toxicity of PAHs to microorganisms, namely, more related bacteria fewer PAHs, or less related bacteria more PAHs. Microbes multiplication in a contaminated environment were able to use the contaminant as the source of energy because of their genetic adaptability which leads to bioremediation (Gupta et al., 2015). The negative correlations showed that the biodegradation of PAHs by bacteria was strong, moreover, the degradation effect of PAHs in the CT was better than that of uninoculated *C. testosteroni* (Table 2). These results showed that in the complex microbial community structure, *C. testosteroni* was not only degraded PAHs by themselves, but it was mainly strengthening the interaction of endogenous microorganisms to promote the degradation of PAHs.

SEM was a priori approach to infer causality between factors in ecosystems (Lu et al., 2018; Wu et al., 2020; Chen et al., 2020). In order to further identify functional microorganisms for PAHs degradation, SEM was employed to analyze the relationship between bacteria and PAHs, these results were shown in Fig. 5. *Flavisolibacter* (r = -0.195, $p < 0.001$) and *Steroidobacter* (r = -0.818, $p < 0.001$) affected Nap directly (Fig. 5a), moreover, *Flavisolibacter* (r = -0.391, $p < 0.05$) and *Steroidobacter* (r = -0.67, $p < 0.001$) impacted Phe and BaP directly, respectively (Fig. 5b, c). Previous studies reported that *Steroidobacter* and *Sphingomonas* could degrade aromatic hydrocarbons such as PAHs (Yun et al., 2018; Lu et al., 2019b). In this study, *Sphingomonas* did not have a significant correlation with Nap and Phe, but it was significantly positively correlated with *Steroidobacter* (r = 0.823, $p < 0.01$), and *Steroidobacter* were significantly positively correlated with *Flavisolibacter* (r = 0.845, $p < 0.01$). These results illustrated that *Sphingomonas* might indirectly affect the degradation of Nap and Phe by affecting the correlationship of bacteria. It was worth noting that *RB41* and BaP were negatively correlated with each other (Fig. 5c). *RB41* was shown to be important in maintaining the metabolic and biogeochemical functions of soils under low-nutrient or stress conditions (Wang et al., 2019). This result indicated that *RB41* was affected by the exposure of BaP, however, it could degrade BaP. *Comamonas* was significantly negative correlation with *RB41* (r = -0.718, $p < 0.001$). With the decrease of PAHs concentration, *Comamonas* abundance decreased, while *RB41* abundance increased, resulting in a negative correlation between them. In this study, the key microbes closely related to Nap, Phe and BaP were *Steroidobacter*, *Flavisolibacter*, *Sphingomonas*, *Comamonas* and *RB41*. With the inoculation of *C. testosteroni*, the interaction between *Steroidobacter* and other indigenous bacteria was strengthened in CT. *Steroidobacter* had relatively low abundance (< 1%), indicating the role of *C. testosteroni* might be to trigger other bacteria genus to resistance PAHs.

Inoculation was an effective method to degrade PAHs (Yuan et al., 2020). Because of the influence of complex environmental factors, it was difficult to confirm inoculants degrade pollutants or strengthen other microorganisms to degrade pollutants. In this study, we selected *C. testosteroni* as an inoculant

which was originally existed in the soil and inoculated in PAHs contaminated soil. Previous studies were reported that *C. testosteroni* could effectively degrade PAHs (Zalesak et al., 2017). We found the relative abundance of *C. testosteroni* was high in CT. Moreover, the concentration of Nap, Phe and BaP were decreased continuously, which was proved that the inoculants could adapt to the soil conditions and better perform their functions to degrade PAHs. However, according to the results of network analysis and SEM, *C. testosteroni* could degrade PAHs, moreover, it also enhanced the synergistic effect of indigenous microorganisms or changed the soil microhabitat to degrade PAHs. Based on the above results, screening of functional microorganisms not only pays attention to their functions but also needs to focus on the groups of microorganisms with interactive effects.

4. Conclusion

In this study, the optimum inoculated conditions of *C. testosteroni* in soil were revealed by orthogonal test. Inoculation of *C. testosteroni* increased the degradation of PAHs because of inoculant increased degrading gene abundance and stimulated metabolic activities of PAHs degrading microorganisms. *C. testosteroni* inoculated in soil could change the bacteria community structure and enhance bacteria interaction to increase the PAHs degradation. This study would be helpful to understand the function of inoculated strains in PAHs contaminated soil and identify functional microorganisms of PAHs remediation.

Declarations

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Authors' contribution

All authors participated in conceiving the study. Xin Li conducted experiments with assistance from Ziwei Jiang. Xueting Sun conducted all statistical analyses, with comments and suggestions from Xin Li and Qiao Wang. Yue Cui and Qiao Wang conducted partial experimental indexes determination. Xueting Sun wrote the manuscript with signifcant assistance and comments from Qian Lu and Jizhe Cui.

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Declaration

Ethics approval and consent to participate Not applicable.

Consent for publication Not applicable.

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

Competing interests The authors declare no competing interests

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Figures

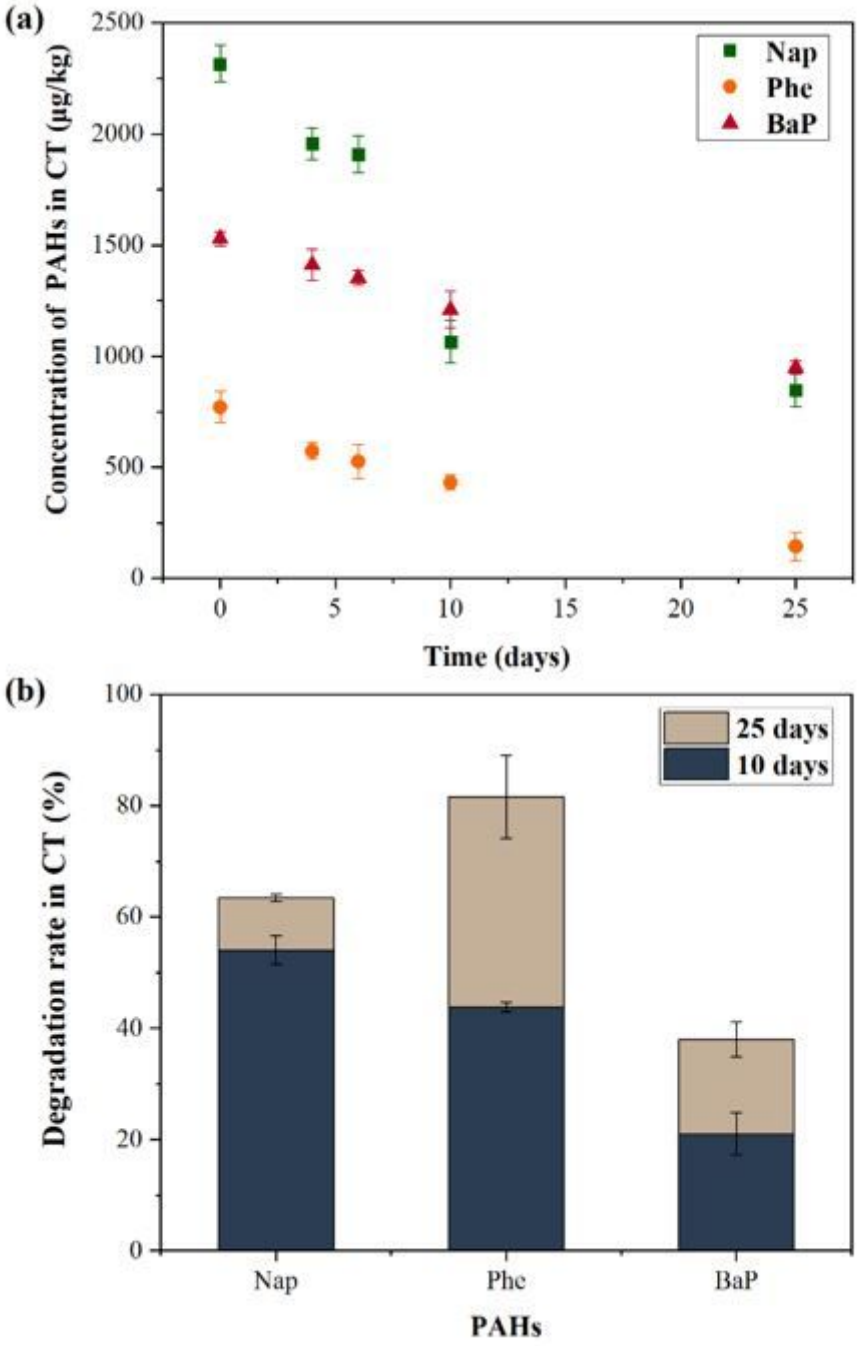

Figure 1

Changes of PAHs concentration (a) and PAHs degradation rate (b) in soil from 0 to 25d. All values represent means ± SD (n = 3).

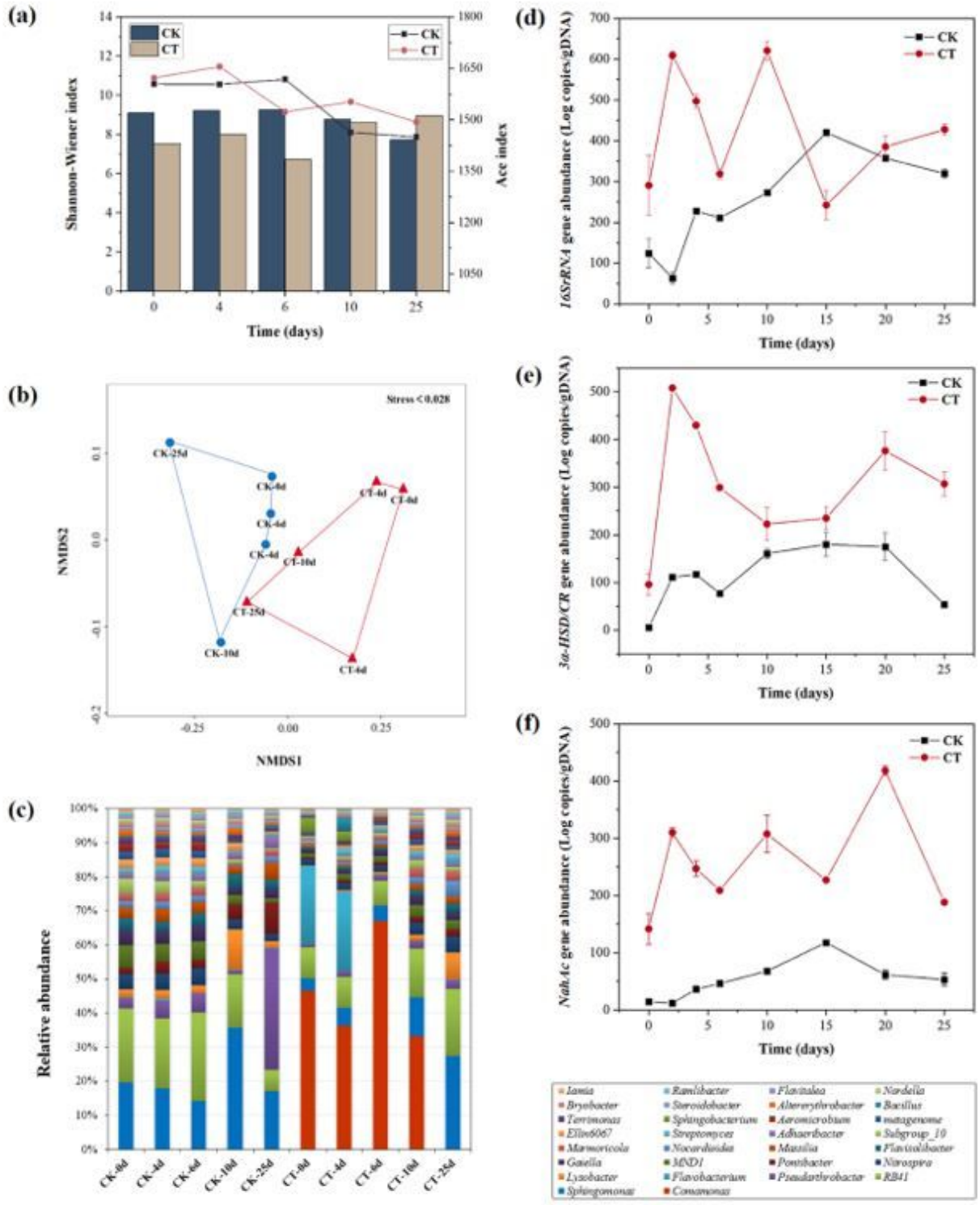

Figure 2

Shannon-Wiener index and Ace index of bacteria for soil samples (a); NMDS ordination of bacterial community from CK and CT (b); Relative abundance of bacteria genera during incubation experiment (c); Relative quantity of functional genes: 16S rRNA (d), 3α- HSD/CR (e) and NahAc (f).

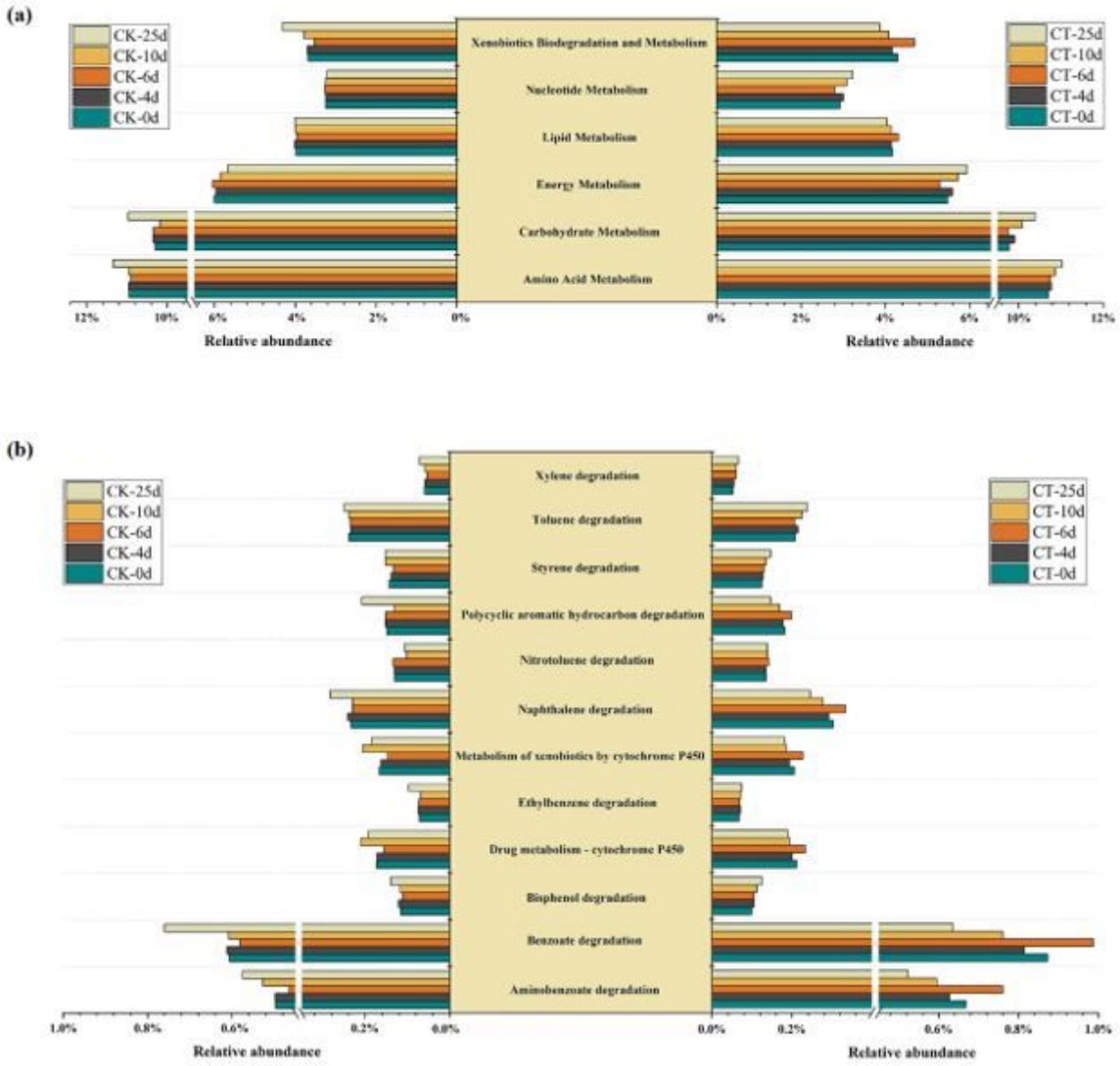

Figure 3

PICRUSt analysis based on KEGG database in CK and CT. Level 2 function categories (a); Level 2 function categories (b).

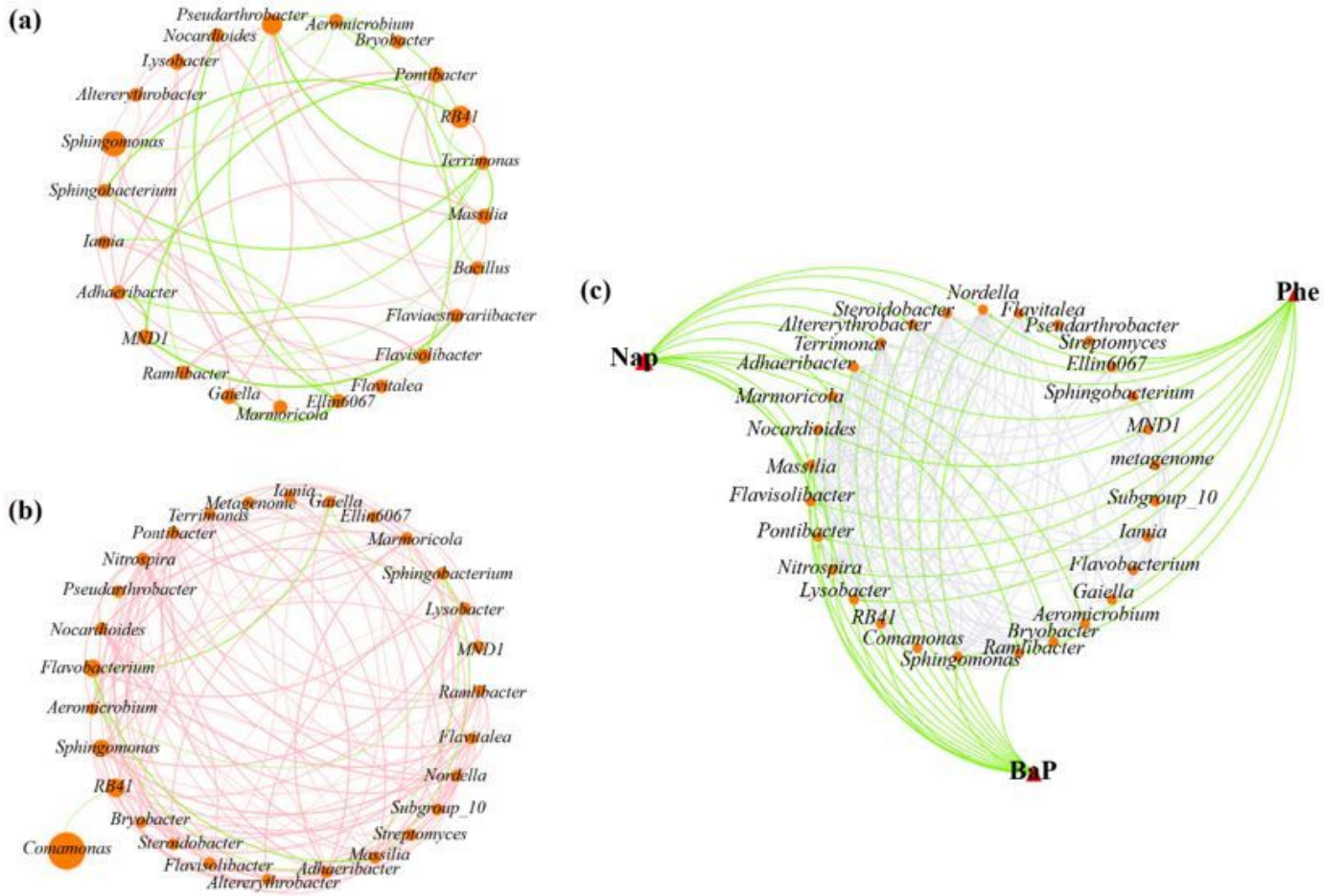

Figure 4

The network analysis of the relationship between bacteria genera in CK (a), CT (b). The network analysis of PAHs and related bacteria in CT (c). Orange nodes represent the related bacteria, and the size represents the abundance. Red triangles represent different types of PAHs. Lines represent the significant correlation ($p < 0.05$). Red lines represent positive correlation. Green lines represent negative correlation. Gray line represents the significant correlation between bacteria genera.

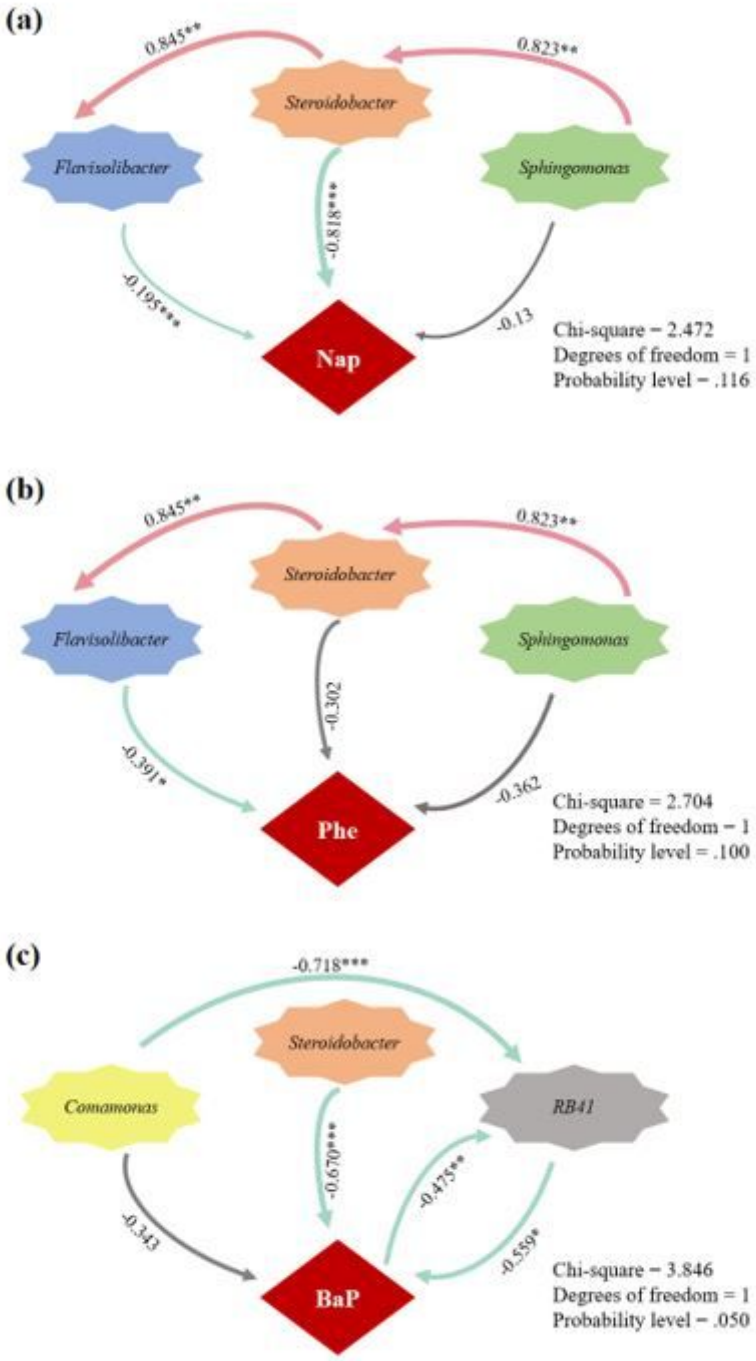

Figure 5

SEMs showed the causeal of relationship between related bacteria genera and Nap (a), Phe (b) and BaP (c) in CT. Arrows depict casual relationships: red lines indicate positive effects, green lines indicate negative effects and gray lines indicate paths with coefficients non-significant different. Arrow widths were proportional to R values. Numbers adjacent to arrows are standardized path coefficients. *p < 0.05, **p < 0.01, ***p < 0.001.

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