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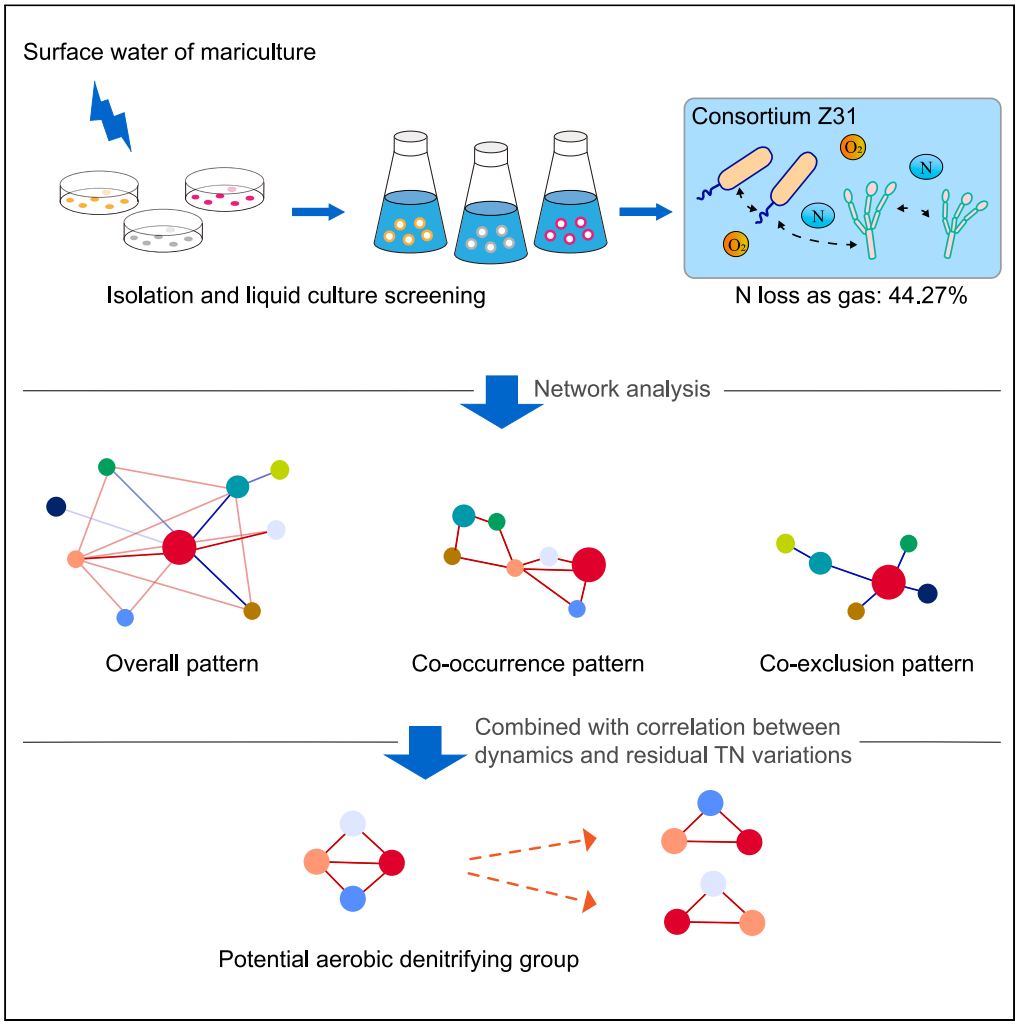

Xiaotian Zuo, Wei Xu, Shiping Wei, ..., Jiege Hu, Hans-Peter Grossart, Zhuhua Luo

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Highlights

Isolation and characterization of a highly active aerobic denitrifying consortium

Nitrate removal and denitrification efficiencies are 100% and 44.27%, respectively

Bacterial-fungal co-occurrence and interactions drive the nitrate removal process

The isolated consortium exhibits a high steady aerobic denitrification performance

Zuo et al., iScience 26, 106824
June 16, 2023
https://doi.org/10.1016/j.isci.2023.106824

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Aerobic denitrifying bacterial-fungal consortium mediating nitrate removal: Dynamics, network patterns and interactions

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SUMMARY

In recent years, nitrogen removal by mixed microbial cultures has received increasing attention owing to cooperative metabolism. A natural bacterial-fungal consortium was isolated from mariculture, which exhibited an excellent aerobic denitrification capacity. Under aerobic conditions, nitrate removal and denitrification efficiencies were up to 100% and 44.27%, respectively. High-throughput sequencing and network analysis suggested that aerobic denitrification was potentially driven by the co-occurrence of the following bacterial and fungal genera: *Vibrio, Fusarium, Gibberella, Meyerozyma, Exophiala* and *Pseudoaltero-monas*, with the dominance of *Vibrio* and *Fusarium* in bacterial and fungal communities, respectively. In addition, the isolated consortium had a high steady aerobic denitrification performance in our sub-culturing experiments. Our results provide new insights on the dynamics, network patterns and interactions of aerobic denitrifying microbial consortia with a high potential for new biotechnology applications.

INTRODUCTION

Nitrogen pollution derived from increasing human activities, mainly owing to excessive input of nitrogenous fertilizers and industry production, raises serious environmental and health issues, e.g., eutrophication of aquatic ecosystems, and impairment of the abilities of aquatic animals to survive, grow and reproduce.[1–3] The majority of nitrogen bio-transforming processes primarily convert nitrogen compounds into other non-gaseous forms,[4] and often couple with denitrification to ensure complete nitrogen removal. Therefore, denitrification is a key biochemical process as it allows for biological nitrogen removal by a stepwise reduction of two of the most important nitrogen pollutants (i.e., nitrate and nitrite) to gaseous nitrogen, commonly nitrous oxide and dinitrogen.[5]

For a long time, denitrification was believed to be sensitive to oxygen and exclusively mediated by anaerobic prokaryotes. Therefore, biological nitrogen removal based on denitrification was assumed to occur exclusively under strict anaerobic conditions. This increased operation cost and impeded full-scale application of anaerobic denitrification in bioremediation efforts, when coupling with traditional nitrification (an aerobic process) for versatile nitrogen removal with distinct requirements of operation conditions, such as dissolved oxygen, pH and organic matter.[6] Serendipitously, the facultative bacterium *Paracoccus denitrificans* can conduct dissimilatory nitrate reduction to dinitrogen under aerobic conditions,[7] which has been termed "aerobic denitrification". It allowed nitrification and denitrification in the same aerated reactor with identical physicochemical conditions, reducing the size of bioreactor, the floor area of infrastructures and hence the construction and operation costs.[8,9] Over the last decade, a few fungal strains capable of aerobic denitrification were identified and received extensive attention, e.g., *Hanseniaspora uvarum*,[10] *Sporidiobolus pararoseus*,[11] *Fusarium solani* RADF-77,[12] *Penicillium tropicum* strain IS0293,[13] and *Barnettozyma californica* K1.[14] The fungal denitrifiers can resist various environmental stressors,[15] especially acidic pH and high temperature,[16] and act as carriers to immobilize bacteria.[17] The aerobic fungal denitrifiers are competent in integrating the advantages of fungal denitrifiers into the aerobic denitrification process, which is beneficial for advancing further technological development of nitrogen removal. However, screening of single strains requires substantial time for their purification regardless of whether bacterial or fungal denitrifiers were applied, and their application requires specific and stable environmental

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https://doi.org/10.1016/j.isci.2023.106824

conditions to sustain purity and high performance.[18] Microbial consortia are regarded as promising alternatives to pure strains, yet, they have been rarely reported in respect to aerobic denitrification.

This is surprising as microbial consortia have been applied in food and drink fermentation for ages, but only recently they have been studied in relation to secondary metabolite production,[19] and a few biodegradation processes, primarily related to their application for more effective pollutant removal.[20] From a bioecological point of view, there are numerous advantages derived from positive interactions in consortia. For instance, mixed microbial cultures provide novel functions and secondary metabolites,[19] stimulate respiratory chain activities,[21] and increase transfer of functional genes.[22] Moreover, network analysis has emerged to identify plausible co-occurrence in consortia, which allows us to better understand microbial interactions.[23] Recently, several studies have gathered in depth knowledge from network patterns of aerobic denitrifying consortia.[24,25] Surprisingly, these studies primarily depicted bacterial networks, and fungal denitrifiers as well as bacterial-fungal interactions have been largely neglected. The strict division of fungi and bacteria in the same microbial community into distinct ecological groups has little empirical support,[26] in particular as ecological functions of bacteria and fungi overlap. Thus, an improved understanding of the role of microbial interactions for biochemical processes such as aerobic denitrification is needed.

As fungi and bacteria comprise complex communities across most shared habitats, they can be assumed to frequently interact.[27,28] A suite of mechanisms to promote metabolic activities possibly underlie bacterial-fungal interactions, including biofilm and fungal highway formation in close physical association, chemotaxis, cooperation for nutrient access, nutrition exchange and complementation in molecular communication.[29] Chen et al. reported that *Citrobacter freundii* can adhere to the mycelia of *Penicillium citrinum*, and nitrate reductase and electron-transmission system activities were enhanced during aerobic denitrification in co-cultures, when compared to single strains.[30] Yet, very little is hitherto known about such cooperative relationships in aerobic denitrification. To better understand aerobic denitrification, especially of mixed microbial cultures, we aimed to refocus on and examine interactions between fungi and bacteria in aerobic denitrifying communities.

Therefore, in the present study, our main purpose was to evaluate dynamics, network patterns and bacterial-fungal interactions of microbial consortia denitrifying under aerobic conditions. Our primary objectives were to (i) screen a natural bacterial-fungal consortium with high aerobic denitrification performance, (ii) demonstrate its nitrate removal characteristics and pathways, and (iii) present community dynamics and cross-kingdom relationships during nitrate removal by exploring network patterns. We offer novel insights into the role of bacterial-fungal interactions for aerobic denitrification, which are crucial for further biotechnological applications including the design of artificial microbial consortia.

RESULTS AND DISCUSSION

Nitrate removal performance and pathways

During cultivation on denitrification medium (DM) (Figure 1), in the first 24 h, the nitrate concentration slightly decreased from 62.83 mg L^{-1} to 57.08 mg L^{-1}. Subsequently, it dropped to 39.67 mg L^{-1} and 0 mg L^{-1} at 32 h and 40 h, respectively. A modest rise in the OD_{600} value from 0.02 to 0.07 occurred in the first 24 h, followed by a peak of 0.50 at 40 h and a decline to 0.39 between 40 h and 72 h. Nitrite was mainly detected at 32 h, while ammonium could not be detected during the incubation. Additionally, total nitrogen (TN) concentration slightly decreased from 87.44 mg L^{-1} to 81.35 mg L^{-1} between 8 h and 16 h. TN was primarily depleted between 32 h and 40 h, with a stepwise decline to 73.60 mg L^{-1} and 48.80 mg L^{-1}, respectively.

Nitrate and TN removal by consortium Z31 were synchronized. Their time courses occurred in three different stages, including a modest growth period, a burst period and a stagnation period, which can be regarded as lag, log and stationary phases of cell growth, respectively. The amount of TN removed was always more than half of the amount of nitrate removed, which indicated that aerobic denitrification was a critical process in nitrate reduction by the consortium Z31. Nitrate removal and denitrification efficiencies under aerobic conditions could reach 100% and 44.27%, respectively. This result suggests a higher performance at an initial substrate concentration within 100 mg L^{-1} compared to many single strains and consortia of previous studies. For instance, nitrate removal and denitrification efficiencies of the bacterium *Pseudomonas mendocina* strain 3-7 at 16 mg L^{-1} were approximately 37.5% and 40.4%, respectively.[31]

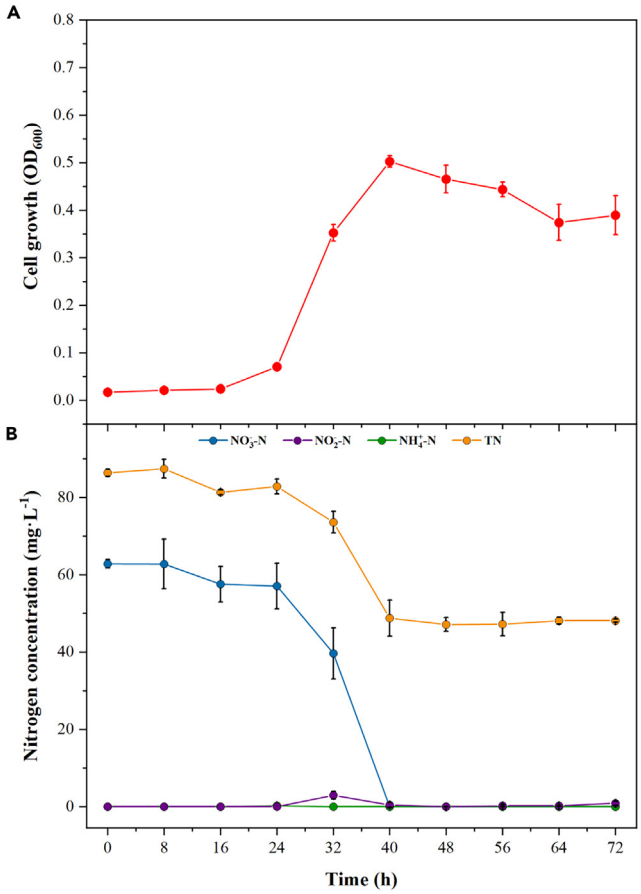

Figure 1. Time course of nitrogen concentrations (nitrate, nitrite, ammonium and TN), and optical density (OD) at 600 nm in aerobic incubations of consortium Z31

(A and B) (A) cell growth, (B) aerobic nitrate removal pattern. Means ± SD (error bars) for triplicate treatments. TN: total nitrogen.

Those of the fungus *P. citrinum* WXP-2 at 75.73 mg L^{-1}, the mix-cultured *Bacillus* consortium at 50 mg L^{-1}, and the bacterial consortia HE1, HE3 and SU4 at approximately 2.4 mg L^{-1} were 51.2% and 33.5%,[30] 80.4% and 33.0%,[32] and 65%–75% and 30%–40%,[33] respectively. The higher nitrogen loss for microbial consortia is ascribed to diverse nitrogen metabolic pathways and intracellular biochemical reactions among different aerobic denitrifiers.[24] Besides, a slight nitrite accumulation in aerobic denitrification at 32 h virtually disappeared toward the end of the experiment. This nitrogen removal pattern is similar to the bacterial strain *Pseudomonas stutzeri* XL-2,[34] and is perceived as a positive case for nitrogen removal as the risk of nitrite toxicity is avoided.[35]

Furthermore, as shown in Table 1, when initial TN was 86.36 mg L^{-1}, the residual concentration of nitrate, nitrite, ammonium and extracellular organic nitrogen at the end of the experiment were 0 mg L^{-1}, 0.88 mg L^{-1}, 0 mg L^{-1} and 19.68 mg L^{-1}, respectively. The amount of nitrogen removed from the ambient media was converted into 27.86 mg L^{-1} of biomass and 38.24 mg L^{-1} of nitrogenous gas. Unfortunately, we could not distinguish between the possible forms of gaseous nitrogen and their corresponding proportion. The nitrogen balance, however, suggests that aerobic denitrification dominated nitrate removal, whereby the proportion of assimilated nitrogen via nitrate reduction was likewise at a high level. Yet, it was lower than the nitrogen assimilation efficiency of the yeast *Rhodotorula diobovata* DSBCA06 which can reach >50% of assimilated nitrogen,[36] but it was higher than that of the aerobic denitrifying bacterium *Pseudomonas balearica* strain RAD-17 with 10.57% of assimilated nitrogen.[37] Hence, nitrogen assimilation seems to play an indispensable role in the nitrogen metabolism of consortium Z31. A notable level of extracellular organic nitrogen was presented in the nitrogen balance, which may have been released from lysis of the

Table 1. Nitrogen balance of consortium Z31 after 3 days of aerobic incubation in DM

	Final nitrogen					
Initial TN	NO_3^-–N	NO_2^-–N	NH_4^+–N	Extracellular organic N[a]	Intracellular N[b]	Gaseous N[c]
86.36 ± 0.97	0 ± 0	0.88 ± 0.52	0 ± 0	19.68 ± 1.53	27.86 ± 1.88	38.24 ± 0.65

All data was presented as mg·L^{-1}. Means ± SD for triplicate treatments.
[a]Extracellular organic N = final TN in media – NO_3^-–N – NO_2^-–N – NH_4^+–N.
[b]Intracellular N = final TN – final TN in media.
[c]Gaseous N = initial TN – final TN.

dead cells in view of the significant biomass decline after 40 h, and/or derived from the excretion of organic nitrogenous matter via nitrate assimilation and thence a variety of metabolism related to amino acids.[4,38]

Community composition and dynamics of consortium Z31 during nitrate removal

At all 5 sampling time points, a total of 456 ASVs were obtained, of which 24 were classified as bacteria and 432 as fungi. Figure 2 depicts the community composition and dynamics of consortium Z31 at the genus level. The most abundant bacterial genera were *Vibrio* (72.12%), *Marinobacter* (26.24%) and *Ruegeria* (1.06%). The major fungal ASVs belonged to the genera *Fusarium* (57.00%), *Exophiala* (9.57%), *Gibberella* (5.72%), Nectriaceae *unclassified* (4.84%), *Saitozyma* (2.92%), *Apiotrichum* (2.90%), *Mortierella* (1.84%), *Meyerozyma* (1.83%), *Chaetomium* (1.79%) and *Talaromyces* (1.29%). In the highly diverse fungal community of consortium Z31, the genus *Fusarium* had the highest relative abundance at all time points. On the contrary, there were only a few bacterial genera, and these were clearly dominated by *Vibrio* and *Marinobacter*. The fungal community with a much higher diversity than their bacterial counterpart can be assumed to play an important role in microbial interactions, which was also supported by network analysis (see section node-level topological features and network patterns).

Our results, to a certain extent, were abiotically controlled by the culture conditions. Generally, pH and salinity are two important environmental stressors for nitrogen removal processes. On one side, many bacteria capable of nitrogen removal adapted well to neutral and alkaline environments, but their performances were inhibited at slightly acidic pH.[39,40] Conversely, fungi may perform nitrogen removal better than bacteria in acidic environments.[41] On the other side, for the majority of microorganisms, the optimal salinity to utilize nitrogen is lower than 1%, since a high salinity will cause a dramatic increase in cell osmotic pressure, and a subsequent alteration in cellular metabolism and inhibition of microbial enzymatic activities.[40,42] The pH and salinity used in our experiments (7.0 and 3%, respectively) were similar to seawater of the sampling site from which consortium Z31 (7.38 and 3.5%, respectively) has been obtained. The relatively neutral pH condition might slightly favor bacteria over fungi, whereas the salinity was excluding microorganisms with a poor salt tolerance. Hence, it is not surprising that a number of microorganisms with a certain salt tolerance potential were found, e.g., *Vibrio*, *Marinobacter*, *Ruegeria*, *Fusarium* and *Exophiala*. Yet, applied pH conditions seemed not to play any obvious role in shaping microbial community composition, given the rich diversity of fungi. In addition, the carbon source, as the electron donor, can also play a significant role in cell growth and denitrification. The sodium acetate in the DM is widely used as a carbon source for achieving an enhancement of nitrogen removal from wastewater.[43] It is suitable for many denitrifier cultures, but may not represent the optimal carbon source for denitrification. For example, for some members of *Marinobacter* and *Fusarium* sodium succinate and fructose represent the optimal carbon sources, respectively.[12,17] Thus, the used carbon source might impact the higher relative abundance of *Vibrio* than *Marinobacter* in our results, as sodium acetate was found to be the optimal carbon source for nitrogen removal in *Vibrio*.[44] Optimization of the carbon source potentially further enhances aerobic denitrification performance of selected microbes, where strategies of carbon source combination also deserve to be taken into account in the future, as it may allow for lower carbon source costs and a more satisfying effluent pH during application.[45]

During the phase of high denitrification activity between 32 h and 48 h, relative abundance of core genera *Vibrio*, *Fusarium*, *Exophiala* and *Gibberella* increased. This suggests that aerobic denitrification performance of consortium Z31 may greatly depend on these few genera. However, relative abundance of *Marinobacter*, *Saitozyma*, *Apiotrichum*, *Mortierella* and *Chaetomium* declined after 32 h, which indicated a poorer competition in nitrogen uptake during increased aerobic denitrification. Thus, these genera may

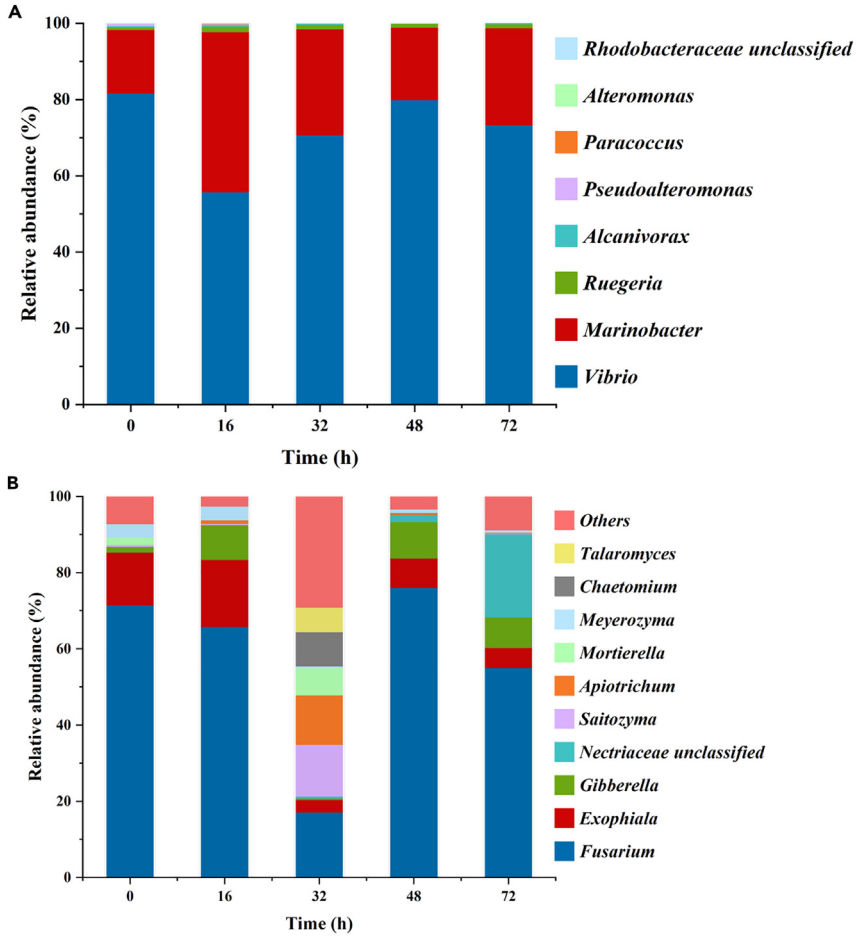

Figure 2. Composition and dynamics of consortium Z31 at genus level

(A and B) The results were based on 16S rRNA gene sequences for (A) bacterial community and ITS rRNA gene sequences for (B) fungal community.

exert a negative impact on possible nitrogen losses. The fungal genus *Fusarium* and its teleomorph *Gibberella* have been widely studied in terms of their capacities of anaerobic denitrification.[46,47] Given that *F. solani* has been proven to be an aerobic denitrifier,[12] thus, our new findings of a potential aerobic denitrification by *Fusarium* are not surprising. In contrast, *Exophiala* has not been reported to utilize inorganic nitrogen to conduct denitrification, which suggests that *Exophiala* may only indirectly promote aerobic denitrification. Moreover, the bacterial genera *Vibrio* and *Marinobacter* prevailed in many marine environments together,[48,49] and many species of both genera have been shown to perform aerobic denitrification, e.g., *Vibrio* sp.,[50] *Vibrio diabolicu*,[44] and *Marinobacter hydrocarbonoclasticus*.[51] Nevertheless, in our study, *Marinobacter* with a continuous decline of relative abundance between 16 h and 48 h, did not seem to significantly contribute to high aerobic denitrification activity. Aside from the potential effects of the present carbon source, the salinity of 3% in the DM medium, slightly lower than that at the original sampling site of the consortium Z31 (3.5%), was probably not sufficient for *Marinobacter* to perform any effective denitrification, taking the salt-dependent denitrifying activity of *Marinobacter* spp. into account.[52]

Node-level topological features and network patterns

We visualized the overall network pattern, co-occurrence and co-exclusion to better understand bacterial-fungal interactions based on Spearman's rank correlations (Table S2). All nodes with discrete modularity classes were resolved into different network modules, and each module presented distinct constituent features. For all nodes and their pairwise links (Figure 3), the overall network was dominated by module I and module IV. Module I primarily consisted of fungi, whereby *Fusarium*, *Exophiala* and *Meyerozyma* had positive links between each other and negative links with other fungi. Module IV contained bacterial-fungal

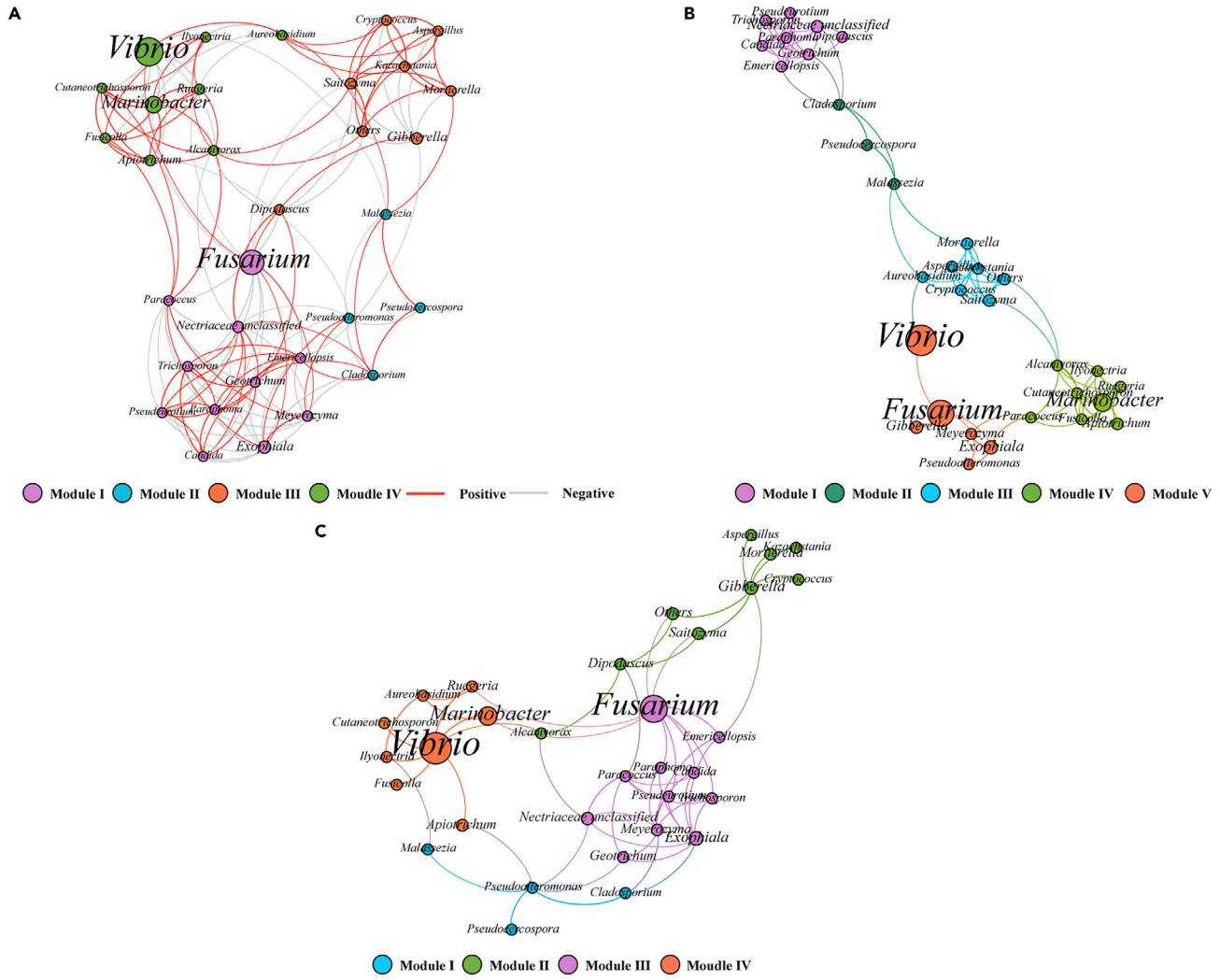

Figure 3. Network patterns of consortium Z31 revealing the interactions between microbial genera during the aerobic nitrate removal process

(A–C) (A) overall, (B) co-occurrence, and (C) co-exclusion networks. The pairwise links with a high Spearman correlation score (R) above 0.57 were presented. The size of nodes represents the relative abundance of genera. See also Figure S1, Tables S2 and S3.

links between core bacterial genera and rare fungal genera. *Marinobacter* occupied the center of module IV and had positive links with the majority of other fungal genera, whereas *Vibrio* and *Aureobasidium* showed negative links with the others. We further measured the importance of nodes in the network by using the betweenness centrality and closeness centrality as proxies of the node location (Table S3). High betweenness centrality values indicate core locations and stronger abilities of the nodes in forming connections among different groups, and high closeness centrality values indicate close relationships of the nodes with many other nodes and hence these nodes can be important influencers of the network.[53,54] In the network including all nodes and their pairwise links, *Fusarium* was the keystone taxon with the highest betweenness centrality of 81.31 and closeness centrality of 0.61. Several rare genera, i.e., *Alcanivorax*, *Malassezia*, *Aureobasidium*, *Paracoccus* and *Pseudoalteromonas* with the next highest betweenness centralities of 25.73–38.80, also played crucial roles for linking between different modules. Some major genera, i.e., *Meyerozyma*, *Exophiala*, *Ruegeria*, *Saitozyma*, *Marinobacter* and *Vibrio* with the next highest closeness centralities of 0.54–0.55, possessed closer relationships with many genera and more intensively interacted in consortium Z31.

In the co-occurrence network (Figure 3), all nodes were resolved into five modules in a nearly linear way. The adjacent modules were linked through 1–2 nodes with higher betweenness centralities of 56.35–211.00 but

lower closeness centralities of 0.28–0.34, when compared to other network patterns of consortium Z31, such as *Alcanivorax*, *Aureobasidium*, *Cladosporium*, *Malassezia*, *Vibrio* and *Paracocccus*. This result suggests weak relationships among the modules and a clear boundary to distinguish different modules, and therefore the nodes of the same module seem to have similar functions and niches according to network theory.[55] The genera in modules I and II had relative abundances <1% and positive Spearman's rank correlations with TN (Figure S1). The relative abundance of genera in modules III and IV had no common features of positive or negative correlations with TN, though there were some abundant genera, such as *Saitozyma*, *Marinobacter* and *Ruegeria*. These results suggest that modules I, II, III and IV did not relate to, but may even hinder aerobic denitrification. In contrast, module V was notably composed of the majority of the most abundant genera, including *Vibrio*, *Fusarium*, *Gibberella*, *Meyerozyma*, *Exophiala* and *Pseudoalteromonas*, which generally had negative Spearman's rank correlations with TN. Consequently, these genera positively interacted and acted as potential aerobic denitrifying bacteria-fungi groups, which hold the potential for a more effective nitrate removal in artificial consortia designs.

In the co-exclusion network (Figure 3), all nodes were resolved into four modules. The nodes and edges in modules I, II and IV were constructed in an approximately spiral-shaped distribution, and revolved around individual hubs with high betweenness centrality, including the genera *Vibrio*, *Gibberella* and *Pseudoalteromonas* with 98.56, 116.83 and 75.93, respectively. There were also several hubs with high betweenness centrality located in the junctions between module III and modules I, II and IV, such as the genera *Fusarium*, *Emericellopsis*, *Meyerozyma*, *Exophiala* and Nectriaceae *unclassified* with 127.50, 71.84, 45.35, 45.35 and 44.55, respectively. These hubs in the junctions also had high closeness centralities of 0.38–0.43. In contrast, most other nodes surrounding the hubs exhibited lower betweenness centralities below 45 and closeness centralities below 0.38. Their connections between each other were mostly fewer than their connections with the hubs, meaning that they were in peripheral locations to exert less influences in the co-exclusion network. Notably, six of the hubs with the highest betweenness centrality in the co-exclusion network belonged to co-occurrence module V. This interesting phenomenon suggests that the potential aerobic denitrifying group antagonized the majority of other microorganisms in our consortium Z31. Combined with the aforementioned results of co-occurrence and Spearman's rank correlations with TN, the majority of other microorganisms could be interpreted as potential nutrient competitors of the denitrifiers. The antagonistic interactions with denitrifiers may play an important role for total denitrification activity.[56] Firstly, lower nutrient uptake of denitrifiers slowed down the increase in cell biomass and extended their lag time. Furthermore, the denitrification rate before substrate exhaustion may decline with the lower biomass level of denitrifiers. In addition, the absolute amount of electron donors and electron acceptors available for denitrifiers is reduced, which could alleviate the denitrification activity as reported.[30] The proportion of electron flux to aerobic denitrification may also drop for the adaptation of denitrifiers in consortium Z31 to the reduction of electron donors and electron acceptors, given the branched respiratory chains of denitrifiers and the advantage for oxygen as electron acceptor over nitrate in energy production.[57,58]

Network-level topological features

The topological parameters of network patterns allowed to measure graph properties of the network structures (Table 2) and to compare connection features of nodes within different network relationships. Diameter and average path length yield the maximal and average number of nodes, respectively, when two nodes are directly or indirectly connected with each other. The modularity score shows if the network community consists of smaller groups of highly associated nodes that are poorly associated with others. The average clustering coefficient indicates the clustering level at which nodes associated with the same nodes tend to form connections between each other. Thereby, the co-occurrence network had the maximal diameter of 8, average path length of 3.74, modularity of 0.64 and average clustering coefficient of 0.68. In contrast, the lower counterparts of the co-exclusion network were 6, 2.96, 0.46 and 0, respectively. Those of the overall network were 3, 1.98, 0.49 and 0.66, respectively. Our results suggest that the co-occurrence connections of microorganisms in consortium Z31 were wider and less direct in the entire community. They were more focused on a small number of genera to resolve into modules that may indicate different functional groups. Moreover, in general, a network pattern with a modularity score of >0.4 was considered to be structured in a modular way.[59] Thus, the resolution into modules in the overall, co-occurrence and co-exclusion networks was plausible in the present work.

Use of consortium Z31 for aerobic denitrification

The performance stability is a desirable property of microbial consortia-based nitrogen removal methods, because changes in microbial community structure throughout the incubation may impact the nitrogen

Table 2. Topological properties of overall, co-occurrence and co-exclusion network patterns

	Average degree	Max component	Diameter	Average path length	Modularity	Number of triangles	Average clustering coefficient
Overall	8.875	32	3	1.98	0.486	259	0.662
Co-occurrence	5.125	32	8	3.744	0.644	90	0.682
Co-exclusion	3.75	32	6	2.96	0.456	0	0

removal performance. The performance stability of the sub-cultures was assessed by repeating the 48 h-cultivations five times. After 10 days of incubation, a steady nitrogen removal performance was observed (Figure 4). When the initial TN was set to about 80 mg L^{-1}, the residual TN of the five incubation rounds were 43.49 mg L^{-1}, 47.33 mg L^{-1}, 34.84 mg L^{-1}, 41.14 mg L^{-1} and 46.84 mg L^{-1}, respectively. As the number of sub-culturing rounds increased, the TN removal efficiency fluctuated from 41.88% to 58.59%, and finally settled around 44.07%. With the TN removal efficiency >40% throughout the whole cultivation, the sub-cultures of natural consortium Z31 showed an excellent aerobic denitrification performance and repeatability, and thus can be reused for efficient aerobic denitrification applications.

Limitations of the study

Our network analysis was based on comparison between relative abundances of microorganisms across sampling time points, during nitrate removal process mediated by consortium Z31. This includes primer bias and a dependence on relative rather than absolute abundance. We studied the biotic interactions, but not considered the interactions between microorganisms and physicochemical parameters, which may contribute to time course variations in microbial community structure. This also includes the impact of defined culture conditions on microbial aerobic denitrification.

STAR★METHODS

Detailed methods are provided in the online version of this paper and include the following:

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  - Data and code availability
- EXPERIMENTAL MODEL AND SUBJECT DETAILS
- METHOD DETAILS
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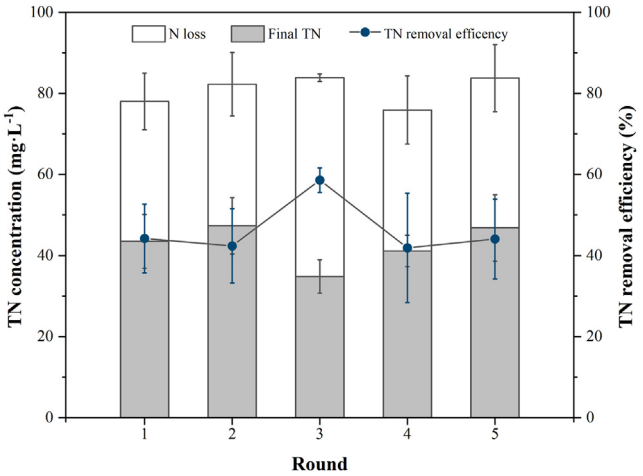

Figure 4. Stability of aerobic denitrification performance during sub-culturing of consortium Z31 for five rounds of sub-cultures

Means ± SD (error bars) for triplicate treatments.

- Isolation and screening of microbial consortia
- Determination of time-course variations of microbial biomass, inorganic nitrogen and total nitrogen
- Nitrogen balance for nitrogen removal pathways
- Illumina amplicon sequencing of 16S rRNA and ITS genes

- QUANTIFICATION AND STATISTICAL ANALYSIS
  - Network analysis

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.isci.2023.106824.

ACKNOWLEDGMENTS

This study was supported by the Natural Science Foundation of Xiamen, China (3502Z20227242), the Natural Science Foundation of Fujian Province of China (2022J02011), Xiamen Ocean Research and Development Institute Project (KFY202201), the National Natural Science Foundation of China (41776170 and 91951102), Public Welfare Project of Fujian Science and Technology Department (2015R1003-12 and 2020R1013001), Fujian Province Marine and Fishery Structure Adjustment Special Project (2020HYJG05 and 2021HYJG09), and the German Science Foundation (GR 1540/30-1 and 33-1). We sincerely appreciate sampling and experimental assistance of Haisheng Chen at the Fishery Technology Promotion Station of Dongshan, Zhangzhou, China.

AUTHOR CONTRIBUTIONS

Conceptualization, X.Z., W.X. and Z.L.; Formal Analysis, X.Z., Y.G. and K.Z.; Investigation, X.Z., S.J., Y.L., M.L., Z.W. and J.H.; Data Curation, Y.L.; Writing - Original Draft, X.Z. and W.X.; Writing - Review & Editing, S.W., K.Z., H-P.G. and Z.L.; Supervision: Z.L.

DECLARATION OF INTERESTS

The authors declare no competing interests.

Received: March 24, 2023
Revised: May 1, 2023
Accepted: May 3, 2023
Published: May 6, 2023

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STAR★METHODS

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological samples		
Consortium Z31	This paper	N/A
Chemicals, peptides, and recombinant proteins		
Denitrification medium	This paper	N/A
Trace element solution	Duan et al., 2015	N/A
FastDNA SPIN Kit for soil	MP Biomedicals, USA	116560200-CF
Deposited data		
16S rRNA and ITS rRNA gene sequence data	This paper	PRJNA945202
Oligonucleotides		
338F	Majorbio Bio-Pharm Technology, China	5′-CCTACGGGAGGCAGCAG-3′
806R	Majorbio Bio-Pharm Technology, China	5′-GGACTACHVGGGTWTCTAAT-3′
ITS1F	Majorbio Bio-Pharm Technology, China	5′- CTTGGTCATTTAGAGGAAGTAA-3′
ITS1R	Majorbio Bio-Pharm Technology, China	5′- GCTGCGTTCTTCATCGATGC-3′
Software and algorithms		
MicrobeCommunities (MATLAB package)	Connor et al., 2017	https://github.com/nkinboulder/MicrobeCommunities
Gephi 0.9.2	Gephi	RRID: SCR_004293; https://gephi.org/

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources should be directed to and will be fulfilled by the lead contact, Zhuhua Luo (luozhuhua@tio.org.cn).

Materials availability

This study did not generate new unique reagents. All chemicals were obtained from commercial resources and used as received.

Data and code availability

- All sequencing data have been deposited at NCBI Sequencing Read Archive and are publicly available as of the date of publication. The accession number is listed in the key resources table. Other experimental and analytical data reported in this paper is available from the lead contact upon request.
- This paper does not report original code.
- Any additional information required to reanalyze the data reported in this paper is available from the lead contact upon request.

EXPERIMENTAL MODEL AND SUBJECT DETAILS

This study did not use experimental models typical in life sciences.

METHOD DETAILS

Culture conditions

The consortia obtained in this study were cultured in denitrification media (DM). The DM contained 0.425 g $\cdot L^{-1}$ of $NaNO_3$ (5 mM nitrogen), 1.913 g$\cdot L^{-1}$ of sodium acetate (C/N ratio = 8), 1 g$\cdot L^{-1}$ of K_2HPO_4, 0.50 g$\cdot L^{-1}$ of KH_2PO_4, 0.05 g$\cdot L^{-1}$ of $MgSO_4 \cdot 7H_2O$, 2 g$\cdot L^{-1}$ of KCl, 30 g$\cdot L^{-1}$ of NaCl and 3 mL of trace-element

solution per liter. The pH was adjusted to 7.0. The preparation of trace-element solution is given by Duan et al.[44] The solid DM was prepared in microbial consortia isolation by addition of 20 $g \cdot L^{-1}$ of agar per liter. For the other experiments, a typical liquid culture was performed in Erlenmeyer flasks sealed with a gas-permeable film. The inoculation ratio (seed culture/DM) was set as 1:20. The flasks were incubated for 48 h or 72 h after inoculation, with constant shaking at a speed of 120 rpm at 28 °C.

Isolation and screening of microbial consortia

We collected surface water samples for consortia isolation at eight sampling sites in maricultural areas of the Fujian and Guangdong provinces, Southern China. The detailed geochemical data of the sampling sites was shown in Table S1. Samples were taken at a water depth ranging from 0 to 1 meter, filled into 50 mL sterile vials, and immediately transported to the lab in cooling containers maintaining *in situ* water temperatures.

In isolation and preliminary screening, we spread 100 μL of water samples onto DM plates. After a week of incubation at 25°C, colonies were investigated and harvested by washing them off the DM plates with 5 mL of 10 mM phosphate buffer saline (pH = 7.4). The colonies from the same plate constituted one consortium. Eight natural consortia were isolated after the first screening. Their colony suspensions were inoculated into 250 mL Erlenmeyer flasks containing 100 mL of liquid DM and then incubated at 28°C on a shaker for 72 h to provide seed cultures.

In secondary screening, seed cultures were inoculated into fresh liquid DM and incubated. We determined the loss proportion of total nitrogen in each culture as a proxy of aerobic denitrification capacity after incubation. Six of the preliminary screened consortia showed a TN loss of > 5% in cultures and were verified to be capable of aerobic denitrification. Among them, consortium Z31 obtained from sampling site #31 in Lufeng exhibited the highest aerobic denitrification performance and hence was selected for further research in this study.

Determination of time-course variations of microbial biomass, inorganic nitrogen and total nitrogen

The culture of consortium Z31 were sampled from each replicate at time intervals of 8 h. Subsamples of 10 mL were centrifuged at 10000 × g for 10 min to harvest microbial biomass for genomic DNA extraction, and the supernatant was used to determine the left-over inorganic nitrogen concentration (NO- 3-N, NO- 2-N and NH+ 4-N). Two-mL subsamples (no centrifugation) were used to measure optical density (OD) at 600 nm as a proxy of biomass and to determine TN containing biomass nitrogen for aerobic denitrification performance.

We used Spark® Cyto (Tecan, Switzerland) to measure optical density. The determinations of NO- 3-N, NO- 2-N, NH+ 4-N and TN concentration were in accordance to Chinese national standard methods.[60] Nitrite was determined as OD_{543} using the N-(1-naphthyl) ethylenediamine dihydrochloride spectrophotometric method,[61] while nitrate and ammonium were turned into nitrite for coupling determination by zinc-cadmium reduction and sodium hypobromite oxidation, respectively.[62,63] For determination of TN, all nitrogen-containing compounds were first transformed into nitrate by alkaline potassium persulfate oxidation,[64] and the corrected nitrate was subsequently determined as OD_{220} minus double OD_{275} using the UV spectrophotometric method.

Nitrogen balance for nitrogen removal pathways

The analysis involved the balance of initial TN, final nitrogen and gaseous nitrogen (nitrogen loss). The concentrations of initial and final TN, nitrate, nitrite and ammonium in final nitrogen were directly measured. The other forms of nitrogen in the nitrogen balance were not directly determined, and we evaluated their contents as follows: The extracellular organic nitrogen was calculated as final TN in the supernatant minus the sum of nitrate, nitrite and ammonium; the nitrogen assimilated into biomass (intracellular nitrogen) was calculated as final TN in the cell suspension minus that in the supernatant; the gaseous nitrogen derived from aerobic denitrification was calculated as initial TN minus final TN.

We subjected the aliquots of culture separately to centrifugation at 10000 × g for 10 min and no treatment. By this way, the supernatant was used to determine final TN in the ambient media, and the cell suspension was used to determine final TN in the cell suspension.

Illumina amplicon sequencing of 16S rRNA and ITS genes

The amplicons (i.e., the16S rRNA genes of bacteria and the inter transcribed spacer (ITS) rRNA genes of fungi) were sequenced by Illumina MiSeq. The total genomic DNA of cultures was extracted using the MP FastDNA SPIN Kit for soil (MP Biomedicals, USA) following the manufacturer's manual. DNA purity and concentrations were analyzed with a NanoDrop spectrophotometer. We performed 16S rRNA gene and ITS rRNA gene amplification by using the ABI GeneAmp® 9700. The V3-V4 region of 16S rRNA genes and the ITS1-ITS2 region of ITS rRNA genes were amplified using primer pairs 338F and 806R as well as ITS1F and ITS2R, respectively. PCR was carried out with 3 min at 95°C, 27 and 35 cycles (for 16S rRNA and ITS rRNA genes, respectively) at 95°C for 30 s, 55°C for 30 s and 72°C for 45 s, and a final extension step of 10 min at 72°C. Amplicons were subjected to Illumina MiSeq sequencing in accordance to standard protocols by Majorbio Bio-Pharm Technology Co. Ltd. (Shanghai, China). Amplicon sequence variants (ASVs) were inferred by DADA2, and the taxonomy of each sequence was analyzed by Naive Bayes algorithm with the Silva 138 dataset for 16S rRNA genes and the Unite 8.0 dataset for ITS rRNA genes.

QUANTIFICATION AND STATISTICAL ANALYSIS

Network analysis

To control for sample contamination and potential sequencing errors, ASVs with fewer than five sequences across all samples were discarded.[65] The network analysis was based on Spearman's correlation coefficient, which was performed at the genus level according to Huang et al.[66] The matrices of relative abundance and TN concentration at different sampling time points were used to compute the matrices of Spearman's correlation coefficients. The computation was performed with a MATLAB script "BarberanData_v9_wrap_036-thresh_TrueData" in MicrobeCommunities package.[65] A high correlation score (R value) of 0.57 was applied to the matrix of Spearman's correlation coefficients between the relative abundance values, and pairwise correlations with lower scores were omitted in the matrix. Permutation test allowed for testing the null hypothesis, i.e., the absence of any correlation against the alternative that there is a nonzero correlation, according to the corr () function in MATLAB. Details of the statistical results are given in the Table S2. Subsequently, this matrix was used to determine and visualize the network patterns of overall, co-occurrence and co-exclusion using Gephi 0.9.2. The ForceAtlas2 was set as the layout algorithm for visualization. Topological parameters were also computed using Gephi 0.9.2, which included the betweenness centrality and closeness centrality of nodes, the average degree, max component number, diameter, average path length, modularity, number of triangles, and average clustering coefficient.