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Microbial community coexisting with harmful alga *Karenia mikimotoi* and microbial control of algal bloom in laboratory*

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Abstract Algicidal bacteria have been frequently isolated from algal blooming areas. However, knowledge regarding the microbial communities coexisting with microalgae and their potential application in preventing harmful algal blooms (HABs) is limited. In this study, we investigated the composition of the microbial community coexisting with harmful alga *Karenia mikimotoi* and its responses to algal control via nutrient stimulation or by adding algicidal strain in microcosms. The microorganisms inhabiting the *K. mikimotoi* culture consisted of 24 identified phyla, including dominant Proteobacteria (relative abundance 76.24%±7.28%) and Bacteroidetes (22.67%±8.32%). Rhodobacteraceae, *Phaeodactylibacter*, and *Maritimibacter* predominated during the algal cultivation. Both the added nutrient and fermentation broth of algicidal strain *Pseudoalteromonas* QF1 caused a massive death of *K. mikimotoi* and substantial changes in the coexisting microbial community, in which Rhodobacteraceae and *Phaeodactylibacter* significantly decreased, while *Halomonas* and *Alteromonas* increased. Core operational taxonomic units (OTUs) analysis indicated that 13 OTUs belonging to Rhodobacteraceae, *Maritimibacter*, *Marivita*, *Nisaea*, *Phaeodactylibacter*, *Citreicella*, *Halomonas*, *Alteromonas*, *Marinobacter*, *Muricauda*, and *Pseudoalteromonas* dominated the changes of the microbial communities observed in the *K. mikimotoi* culture with or without treatments. Collectively, this study indicated that microbial community inhabiting *K. mikimotoi* culture includes potential algicidal bacteria, and improves our knowledge about microbial community succession during biocontrol of *K. mikimotoi* via nutrient stimulation or by adding isolated algicidal strains.

Keyword: *Karenia mikimotoi*; microbial community; nutrient stimulation; algicidal bacteria; *Pseudoalteromonas*

1 INTRODUCTION

Karenia mikimotoi is a dinoflagellate species known to cause harmful algal blooms (HABs) (Aoki et al., 2017). Blooms induced by *K. mikimotoi* have been reported frequently in past decades worldwide, such as the coast of China (Sakamoto et al., 2021), Imari Bay (Aoki et al., 2017), the east Johor Straits of Singapore (Kok and Leong, 2019), the French Atlantic Shelf (Sourisseau et al., 2016), the waters off western Ireland (O'Boyle et al., 2016), the north-west European continental shelf (Gillibrand et al., 2016), the Kachemak Bay Alaska (Vandersea et al., 2020), and the Arabian Sea (Kumar et al., 2018). The large-

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scale HABs have adverse impacts on aquatic ecosystem, can cause mass mortality of benthic and pelagic organisms by the competition of nutrient salt, the dramatic reduction of underwater light, and the secreted toxic substances. A number of researches reported that the type of HABs can cause mortality of marine organisms, such as oysters (Mizuno et al., 2015), zooplankton, *Penaeus vannamei* and *Scophthalmus maximus* (Li et al., 2017), and rotifers (Li et al., 2018). Thus, the control of *K. mikimotoi* has recently attracted more attention.

Physical and chemical methods have been developed and applied to control HABs (Nagai et al., 2016; Park et al., 2017). Algaecides, such as copper-based products and natural clays, had been used to control HABs. However, copper-based algaecides may cause environmental issues, such as toxicity to aquatic organisms (Closson and Paul, 2014), and are banned internationally. Due to the low flocculation efficiency and high field consumption, it is difficult to achieve large-scale application of natural clay. Recently, eco-friendly controlling methods, such as using ultrasonic technology, modified clays, allelochemicals secreted by macroalgae, algaecides produced by bacteria, and nutrient competition between organisms, are receiving increasing attention. Among them, algicidal bacteria have strong applied potential in control of HABs because of the accessibility and excellent biocompatibility.

To date, a large number of algicidal bacteria have been isolated from seawater (Zheng et al., 2018), reservoirs (Shimizu et al., 2017), lake sediments (Su et al., 2016), mangroves (Yu et al., 2018), and soils (Cai et al., 2019). The reported algicidal microorganisms and algicidal mechanisms were collated in Supplementary Table S1 (Zheng et al., 2019). These microorganisms were mainly affiliated with Bacteroidetes, α-Proteobacteria, β-Proteobacteria, γ-Proteobacteria, Actinomycetes, and Firmicutes. The algicidal bacteria inhibit algae by influencing algal cell integrity, enzymatic activities, gene expression, photosynthesis, respiration, and reproduction. For example, *Myxococcus* parasitized *Phormidium* and resulted in algal cell lysis (Burnham et al., 1984), and most algicidal bacteria inhibit algae via producing algicidal substances (Zhang et al., 2020a, b).

Bacteroidetes (*Flavobaterium*, *Cytophaga*, and *Cellulophaga*), α-Proteobacteria (*Paracoccus*, Rhodobacteraceae), β-Proteobacteria (*Thalassospira*), γ-Proteobacteria (*Alteromonas*, *Idiomarina*, *Vibrio*, *Pseudoalteromonas*, *Halomonas*, and *Marinobacter*), Actinomycetes (*Kocuria*), and Firmicutes (*Bacillus*) have proven to be able to inhibit or kill *K. mikimotoi* (Imai et al., 2006; Lu et al., 2016; Zheng et al., 2018), showing potential application in controlling *K. mikimotoi*. In our previous work, an isolated *Pseudoalteromonas* showed algicidal activity against *K. mikimotoi*, and caused the accumulation of reactive oxide species (ROS) and the apoptosis of algal cells (Zheng, 2019). Previous studies also reported that *Pseudoalteromonas* could inhibit algae from Dinophyceae (*Gymnodinium catenatum*, *Cochlodinium polykrikoides*, *Akashiwo sanguinea*, and *Alexandrium tamarense*), Raphidophyceae (*Chattonella marina* and *Heterosigma akashiwo*), and diatom (*Skeletonema costatum*) (Lovejoy and Bowman, 1998; Lee et al., 2000; Oh et al., 2011; Sun et al., 2016; Lyu et al., 2019). Due to its algicidal activity, the isolated *Pseudoalteromonas* QF1 was added into the culture of *K. mikimotoi* to investigate its influences on the co-existing microbial community.

Recently, the microbiomes coexisting with algae have received extensive attention for better understanding the process and mechanism of algal bloom formation and extinction. However, the algicidal effects of the coexisting microbial communities on microalgae have rarely been studied. In this study, the microbial community coexisting with *K. mikimotoi*, their responses to nutrient addition and added algicidal strain *Pseudoalteromonas* QF1 were investigated in microcosms. The hypothesis of nutrients stimulation is: microalgae are photoautotrophic; compared with microalgae, microorganisms have shorter growth and metabolism cycles, and give priority to nutrients; the added nutrients is limited, and not enough to cause massive growth of microalgae.

2 MATERIAL AND METHOD

2.1 Material

2.1.1 *Karenia mikimotoi* and cultivation

Karenia mikimotoi was donated by Ocean University of China and was preserved in our laboratory. The culture was incubated and preserved in a sterile f/2 medium prepared with seawater (Guillard and Ryther, 1962) in 500-mL glass conical flasks capped with aseptic breathable parafilm. The used seawater was first filtered through a 0.45-μm filter membrane to eliminate microorganisms. The f/2 medium was autoclaved for 20 min at 121 °C before

use. Unless otherwise noted, all the algal cultivation was performed at 25±1 °C under a 12-h:12-h light-dark cycle with a light intensity of 3 000 lx in a light incubator. The cultures were manually shaken three times per day to prevent the algae from growing against the wall of the flask.

Axenic algae culture was obtained via repetitive filtration using a 5.0-μm mixed cellulose ester membrane filter, which allows the microorganisms existing in the *K. mikimotoi* culture to pass through the membrane, while the algal cells are trapped (Baker and Kemp, 2014). The number of cultivable bacteria remained on the axenic culture were measured based on colony forming units (CFUs) counts: plating aliquots of samples on 2216E agar plates that were incubated at 25±1 °C for 24 h. The 2216E agar medium consisted of 1-g yeast extract, 5-g peptone, 15-g agar, and 1-L seawater, with pH of 7.2, and was autoclaved for 20 min at 121 °C before use. After the aseptic processing, the bacteria in the algal culture decreased from 1.1×10^7 to 2.1×10^2 cells/mL.

The algal cells were fixed with Lugol's solution and counted under a light microscope using hemocytometer. The Lugol's solution consisted of 4-g iodine (I_2) and 6-g potassium iodide (KI) in 100 mL of distilled water.

2.1.2 *Pseudoalteromonas* QF1

Pseudoalteromonas QF1 can effectively inhibit the growth of *K. mikimotoi* through indirect way (Zheng et al., 2018). Strain QF1 was previously isolated from estuarine of the Yellow Sea, China. The strain was cultured in 200-mL sterile 2216E medium in 500-mL conical flasks at 25±1 °C.

2.2 Method

2.2.1 Algicidal experiment

Because the lack of organic carbon source in the f/2 medium, it cannot meet the growth of microorganisms. Therefore, 2216E medium was used to stimulate the growth of microorganisms inhabiting *K. mikimotoi* culture. Algicidal experiments consisting of control group (C), nutrient-stimulated group (E), and QF1 treated group (J) were conducted. The control group had no 2216E medium and QF1 fermentation broth and was used to investigate the succession of the coexisting microbial community. In the nutrient-stimulated group, 4.5 mL (3%, 2216E medium volume/algal culture volume) and 9 mL (6%) of sterile 2216E medium were respectively added to 150-mL *K. mikimotoi* culture with an initial algal cell concentration of 8.0×10^4 cells/mL to investigate the influence of the 2216E medium on *K. mikimotoi* and the coexisting microbial community. In addition, 4.5-mL and 9-mL sterile 2216E was respectively added to 150-mL axenic *K. mikimotoi* culture to form the control group of nutrients (A–E) to test the influence of the 2216E medium on the growth of *K. mikimotoi*. In the QF1 treated group, 4.5-mL (3%) and 9-mL (6%) QF1 cultures in the stationary phase (8.5×10^8 cells/mL) were respectively added to 150-mL *K. mikimotoi* culture to investigate the influence of the added algicidal strain on *K. mikimotoi* and the coexisting microbial community. Each of the experiments above was conducted in triplicate. The algal cells in each group were counted every 24 h, and the algal inhibition ratio, representing the algicidal activity, was estimated according to the following equation:

$$\text{Algicidal rate } (\%)=(1-N_t/N_c)\times100,$$

where N_c refers to the number of algal cells in the control group, and N_t refers to the number of algal cells in the treatment group.

For microbial DNA extraction, 50-mL microalgae suspension from each group was collected on the 3rd, 6th, and 9th day, respectively, and were centrifuged at 8 000 r/min at 4 °C for 5 min to collect microbial cells. The pellets from centrifugation were frozen at -80 °C before DNA extraction. Additionally, the number of cultivable microorganisms in *K. mikimotoi* cultures was measured based on counts of CFUs on 2216E plates.

2.2.2 Microbial community 16S rRNA gene sequencing and analysis

Microbial genomic DNA was extracted using glass bead grinding method combined with an AxyPreP bacterial genomic DNA kit. Universal prokaryotic primers 336f (5′-GTACTCCTACGGGAGGCAG-CA-3′) and 806r (5′-GTGGACTACHVGGGTWTC-TAAT-3′) were used to amplify the V3–V4 region of microbial 16S rRNA gene. PCR products of the same sample in triplicate were mixed to minimize bias. The 16S rRNA amplicons were examined by DNA electrophoresis on 2% agarose gel, and were recovered using AxyPrep DNA gel Recovery Kit. Amplicons were paired-end sequenced on the MiSeq platform based on PE300 (2×300 bp) in Allwegene Technology Co. Ltd., Beijing, China. Fastq data were processed using QIIME, version v.1.8.0 (Caporaso et al., 2010). After filtering low quality reads and chimeras, 16 714 effective sequences were selected at random for each

sample for community analysis to reduce sequencing deviation. Sequences were assigned to operational taxonomic units (OTUs) at a 97% sequence similarity level using the UPARSE pipeline (Edgar, 2013). Goods coverage was used to assess the sequencing depth for the samples. The representative sequence sets were aligned and given a taxonomic classification by ribosomal database project (RDP) against the SILVA Small Subunit rRNA database at an 80% confidence threshold (Cole et al., 2014). The representative sequences of OTUs with the highest abundance were selected for tree building using 'Mafft' and 'Fasttree'. The tree was visualized using R software (https://www.r-project.org/) based on the abundance and evolutionary relationship at the genus level. 'Unweighted uniFrac' only considers whether the microbial taxon appears in the community, but does not consider the abundance. However, the relative abundance of different microbial taxon can be critical for describing the community changes. Therefore, 'weighted uniFrac' that considers on abundance information during calculations was used here to illustrate the responses of the microbial community existing in *K. mikimotoi* culture to the added 2216E medium and QF1 broth. Non-metric multidimensional scaling analysis (NMDS) based on Bray-Curtis of the OTUs and heatmap based on weighted unifrac distances were performed to visualize the changes in the microbial community in the control and treated groups. To determine the significant differences in microbial β-diversity, analysis of molecular variance (AMOVA) depending on the weighted unifrac distance matrixes was analyzed using the 'anosim' function of 'ade4' package in R. Then, a Wilcoxon rank-sum test was performed to determine the microbial populations with statistically significant difference between the control and treated groups.

3 RESULT

3.1 Growth of *K. mikimotoi* in microcosms

The influences of the added 2216E medium and QF1 broth on the growth of *K. mikimotoi* were investigated in the algicidal experiments consisting of control group (C), nutrient-stimulated group (E), and QF1 treated group (J). For the control group, the algae rapidly reproduced, with algal cell density increasing from 8.4×10^4 to 55.8×10^4 cells/mL in 9 days. Adding both nutrients and QF1 fermentation broth caused mass mortality of *K. mikimotoi*, and the algal inhibition ratio reached up to 90% after 3 days of cultivation.

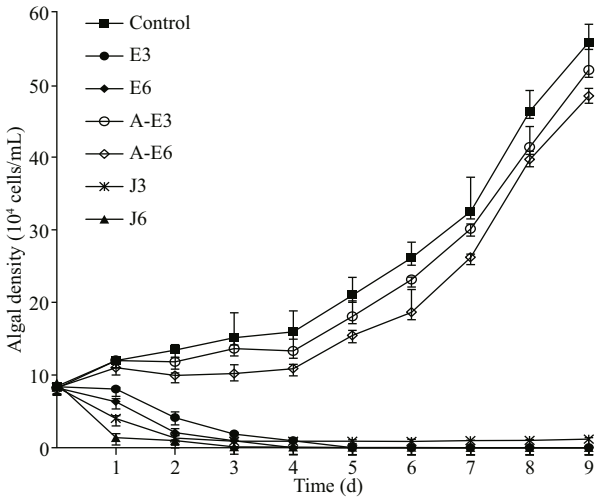

Fig.1 The growth curves of *K. mikimotoi* in microcosms

E3 and E6 represents 3% and 6% (v/v) nutrients treated groups; A-E3 and A-E6 represents 3% and 6% nutrients control groups, in which, microorganisms were removed; J3 and J6 represents 3% and 6% QF1 fermentation broth treated groups.

The same amount of sterile 2216E medium was also added to the same volume of axenic *K. mikimotoi* culture, and no inhibiting effects on the growth of *K. mikimotoi* were observed (Fig.1): the algal cell density increased from 8.4×10^4 to 52.0×10^4 cells/mL in 9 days for groups with 3% 2216E medium (A-E3), and 48.8×10^4 cells/mL for groups with 6% 2216E medium (A-E6). The results indicated that adding nutrients and QF1 broth could efficiently inhibit the growth of *K. mikimotoi* in non-sterile environment. Additionally, the microorganisms existing in *K. mikimotoi* culture would be stimulated by nutrients.

3.2 Sequencing information and the shared microbial OTUs

The sequencing coverage of the microbial communities in each sample ranged from 99.70% to 99.96%, which could reflect the whole of the microbial community in each sample. The responses of microorganisms existing in *K. mikimotoi* culture to the added 2216E medium and QF1 broth were further investigated. A total of 424 OTUs were detected in the control group (average: 85±41 OTUs/sample), nutrient-stimulated group (average: 87±43 OTUs/sample), and QF1 fermentation broth treated group (average: 82±34 OTUs/sample). Additionally, 140 shared OTUs were detected in all groups. Among them, 13 OTUs were simultaneously detected in each sample, and were distributed in Rhodobacteraceae, *Maritimibacter*,*Marivita*,*Nisaea*,*Phaeodactylibacter*, *Citreicella*, *Halomonas meridiana*, *Alteromonas*, *Marinobacter*, *Muricauda*, and *Pseudoalteromonas*

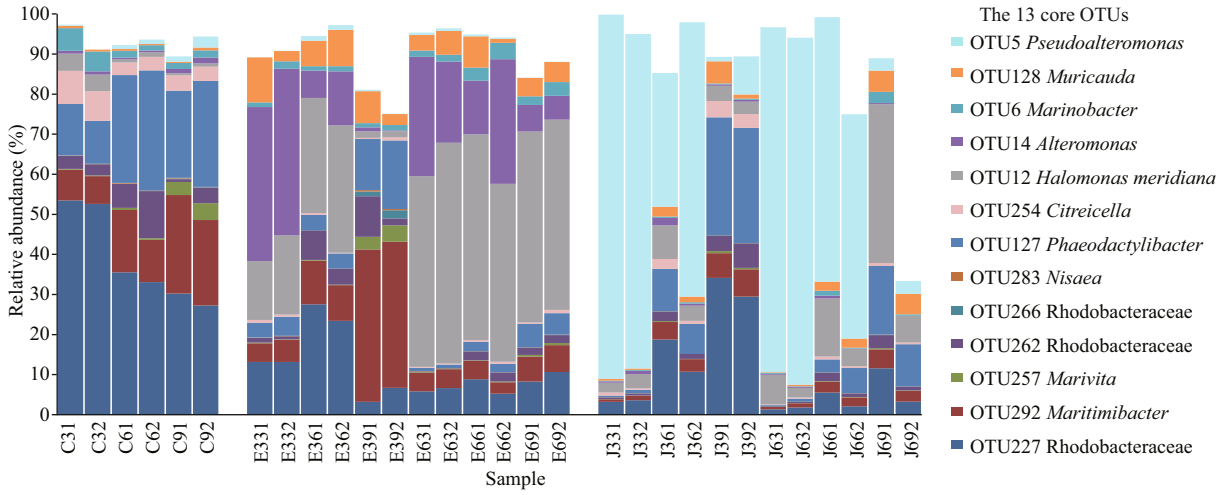

Fig.2 The relative abundances of the 13 core OTUs

There were OTUs detected in control group (letter C + number), nutrients stimulated group (letter E + number), and QF1 fermentation broth treated group (letter J + number). The first number following E and J represents the proportions of added nutrients or QF1 fermentation broth, the second number and the first number following C represents the sampling time points in algal cultivation process, the last number represents the replicates. OTUs: operational taxonomic units.

(Fig.2). The 13 OTUs accounted for 88.10%–97.04%, 75.05%–96.03%, and 8.98%–88.18% of the microbial total relative abundances in the control, nutrient-stimulated, and QF1 fermentation broth treated groups, respectively.

3.3 Microbial community coexisting with *K. mikimotoi*

The number of cultivable microorganisms in the non-sterile *K. mikimotoi* culture was approximately 1.2×10^7–1.9×10^7 cells/mL during algal cultivation. As shown in Fig.3, many microorganisms with diverse phylogenetic relationships coexist with well-grown *K. mikimotoi*. These microorganisms were affiliated with 1 unidentified phylum and 24 identified phyla. The main phyla were Proteobacteria and Bacteroidetes, accounting for 76.24%±7.28% and 22.67%±8.32% of the whole community, respectively (Fig.3). A total of 38 identified classes were detected, including the dominant Alphaproteobacteria, Sphingobacteriia, Gammaproteobacteria, and Deltaproteobacteria, which accounted for 66.29%±5.60%, 21.51%±7.99%, 8.69%±3.21%, and 1.24%±1.16% of the microbial community, respectively (Fig.4a). An unidentified genus belonging to Rhodobacteraceae, *Phaeodactylibacter*, *Maritimibacter*, *Citreicella*, *Marinobacter*, *Halomonas*, *Marivita*, *Oceanococcus*, and *Pseudoalteromonas* predominated in *K. mikimotoi* culture, and accounted for 45.87%±10.97%, 21.49%±7.99%, 14.45%±7.34%, 4.91%±2.29%, 2.84%±1.93%, 1.96%±1.75%, 1.45%±1.80%, 1.44%±2.46%, and 1.07%±0.97% of the microbial community, respectively (Fig.4b).

As shown in the NMDS graph (Fig.5a) and heatmap of the weighted unifrac dissimilarity matrix (Fig.5b), microbial communities from the 3rd, 6th, and 9th day of the *K. mikimotoi* culture were clustered together. However, a slight succession of the microbial community was observed during the cultivation of *K. mikimotoi* (Figs.4 & 5): the unidentified Rhodobacteraceae genus decreased from 57.52%±0.35% (3rd day) to 33.19%±0.47% (9th day), *Citreicella* decreased from 7.84%±0.60% (3rd day) to 3.67%±0.17% (9th day), *Halomonas* decreased from 4.20%±0.12% (3rd day) to 0.67%±0.16% (9th day), while *Phaeodactylibacter* increased from 11.80%±1.46% (3rd day) to 24.13%±3.34% (9th day), and *Maritimibacter* increased from 7.33%±0.53% (3rd day) to 22.99%±2.29% (9th day).

3.4 Responses of the microbial community to nutrient stimulation

Compared with the control group, the number of coexisting microorganisms increased to 2.5×10^8–3.9×10^8 cells/mL in the nutrient-stimulated group, and the microbial community compositions showed an obvious change (Figs.4 & 6a), forming a distinct cluster in the NMDS graph (Fig.5a), with a significant weighted unifrac dissimilarity (Fig.5b; AMOVA, Fs=12.637 7, P<0.001). The Wilcoxon rank-sum test revealed the genera with significantly different abundances between the control and nutrient-stimulated groups (Fig.6a, P<0.05). Compared with the control group, the genus that belongs to

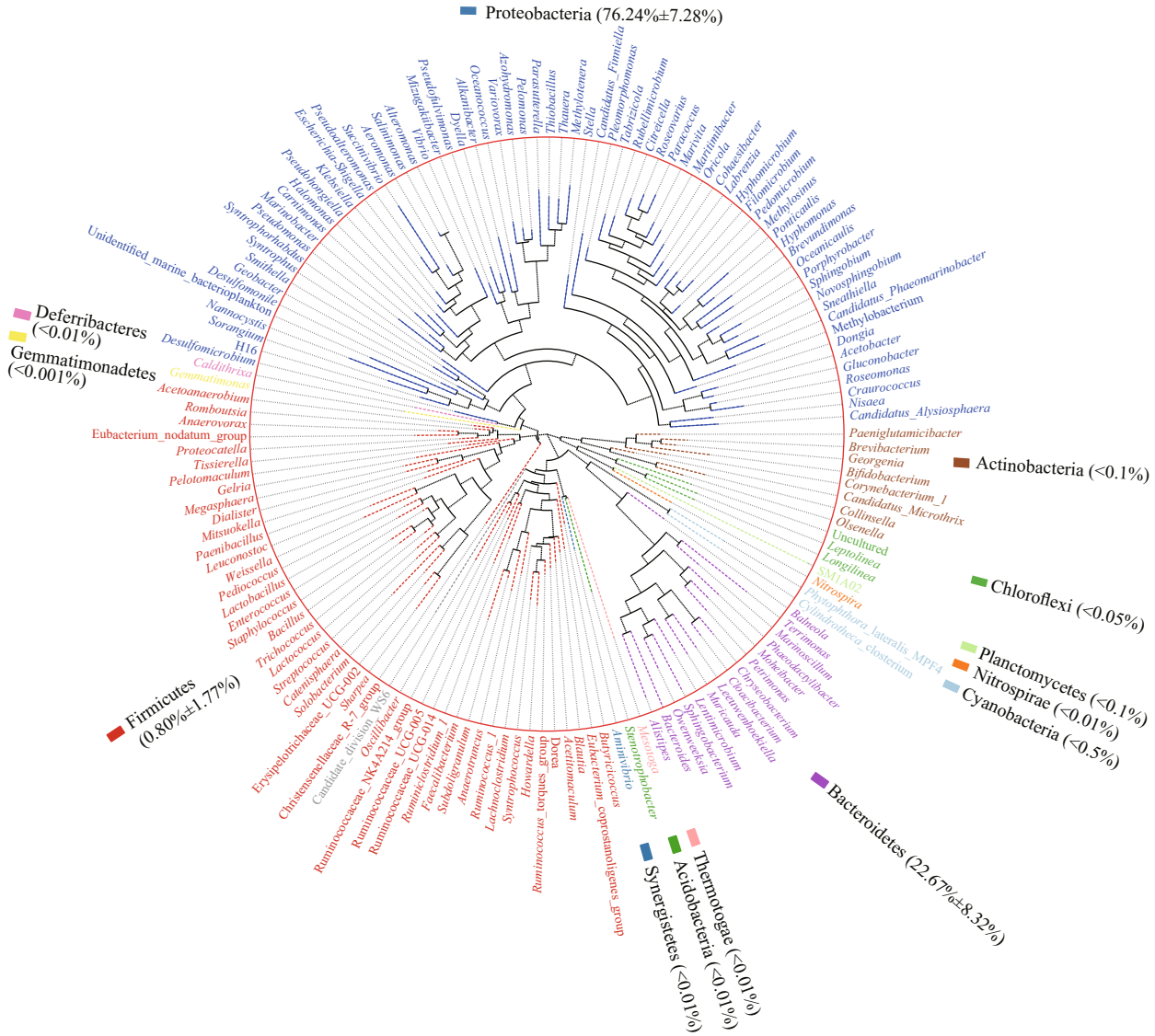

Fig.3 Phylogenetic tree and abundance at phylum level of the microorganisms coexisting with *K. mikimotoi*

Rhodobacteraceae, *Phaeodactylibacter*, and *Maritimibacter* significantly decreased, while *Halomonas* and *Alteromonas* became dominant on the 3rd and 6th days in the 3% nutrient-stimulated group. It is worth noting that *Halomonas* and *Alteromonas* decreased again on the 9th day of algal cultivation, while *Phaeodactylibacter* and *Maritimibacter* obviously increased. Accordingly, the microbial community on the 9th day in the 3% nutrient-stimulated group showed a higher similarity with those in the control group (Fig.5b), and clustered together in the NMDS plot (Fig.5a). Differing with the 3% nutrient-stimulated group, *Halomonas* and *Alteromonas* always dominated during the algal cultivation process in the 6% nutrient-stimulated group, accounting for 44.38%–55.13% and 5.97%–31.13% of the microbial community, respectively. The community in the 6% nutrient-stimulated group had a higher dissimilarity with those in the control group (Fig.5).

3.5 Responses of the microbial community to added algicidal strain QF1

QF1 fermentation broth treatment significantly changed the microbial community coexisting with *K. mikimotoi* (Figs.4 & 6b; AMOVA, Fs=10.204 9, P<0.001). The changes in the microbial community were also significantly different from those in the nutrient-stimulated group (Figs.4 & 6c; AMOVA, Fs=12.399 8, P<0.001). In the 3% QF1 treated group, the relative abundances of *Pseudoalteromonas* were 83.52%–90.83% at the 3rd day, decreasing to 33.42%–68.45% at the 6th day, with further decreases to 1.10%–9.50% at the 9th day. Conversely, the genus that belongs to Rhodobacteraceae, *Phaeodactylibacter*,

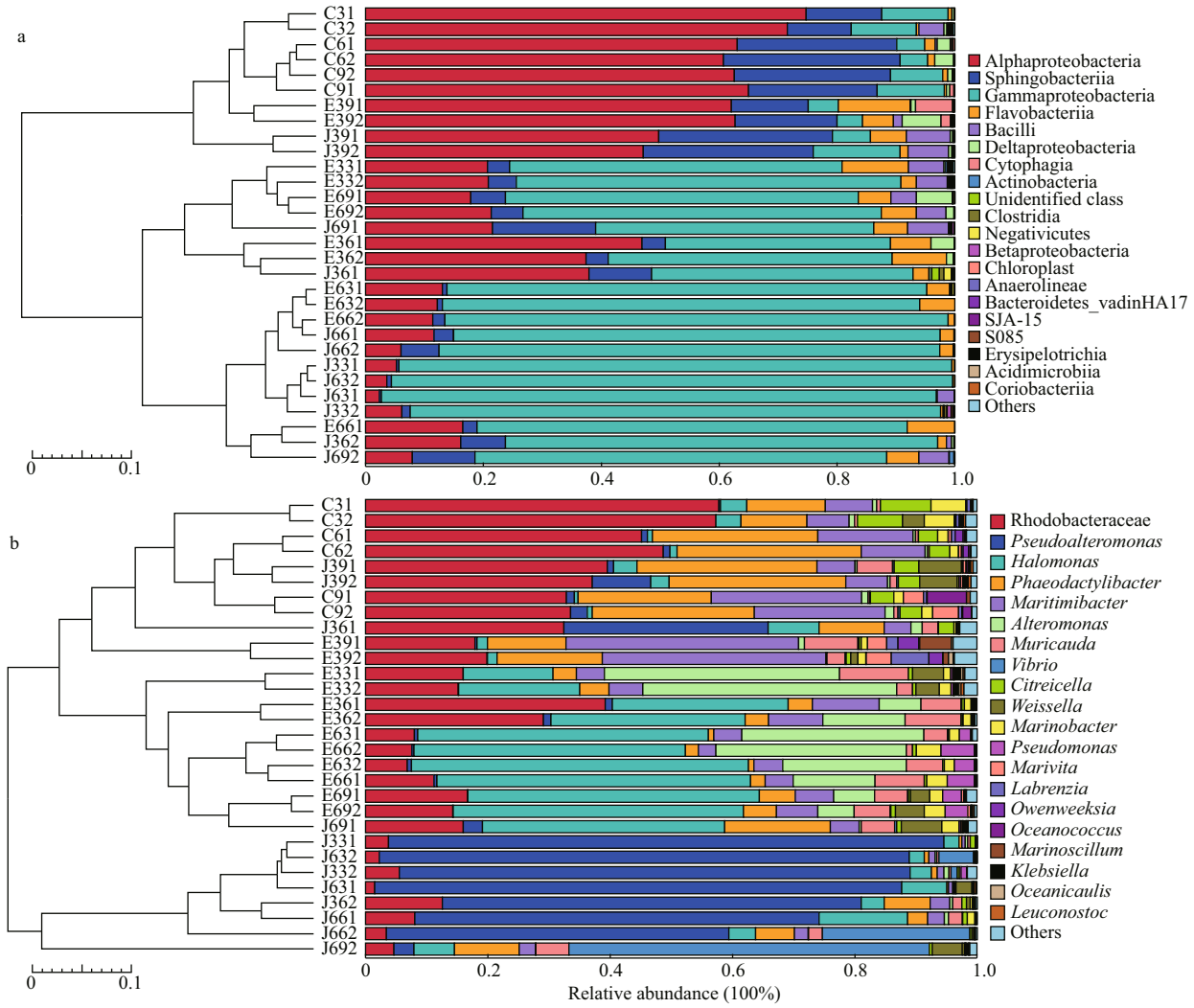

Fig.4 Composition and succession of the microbial communities at the class (a) and genus (b) level

Letter C, E, and J represents control, nutrients-stimulated, and QF1 treated groups, respectively. The first number following E and J represents the proportions of added nutrients or QF1 fermentation broth, the second number and the first number following C represents the sampling time points in algal cultivation process, the last number represents the replicates.

and *Maritimibacter* significantly increased from 3.74%–5.52%, 0.42%–0.89%, and 0.62%–1.14% on the 3rd day to 37.10%–39.49%, 28.86%–29.45%, and 6.19%–6.80% on the 9th day, respectively. The microbial community at the 9th day in the 3% QF1 treated group showed a higher similarity with those in the control group (Fig.5b), and clustered together in the NMDS plot (Fig.5a). The 6% QF1 treated group had community compositions similar to those of the 3% QF1 treated group at the 3rd and 6th day, and *Pseudoalteromonas* significantly decreased on the 9th day. Differing with 3% QF1 treated group, the unidentified Rhodobacteraceae genus and *Phaeodactylibacter* did not obviously increase on the 9th day. As a result, the microbial community in the 6% QF1 treated group at the 9th day showed higher dissimilarity with those in the control group.

4 DISCUSSION

This study detected a number of microbial populations in *K. mikimotoi* culture without nutrients and QF1 broth stimulation. The dominant populations were assigned to Rhodobacteraceae and Saprospiraceae, which were broadly observed in marine algal-associated microbial communities, such as *Ulva australis* (Burke et al., 2011) and *Gymnodinium*-diatomm bloom cycles (Shao et al., 2020). Rhodobacteraceae is frequently detected in marine environments, consisting of diverse species which can utilize various organic and inorganic compounds and play important roles in sulfur and carbon biogeochemical cycling (Pujalte et al., 2014). This type of microorganism includes *Citreicella*, *Marivita*, *Labrenzia*, *Maritimibacter*, and *Roseovarius*. Species from Saprospiraceae are also frequently isolated

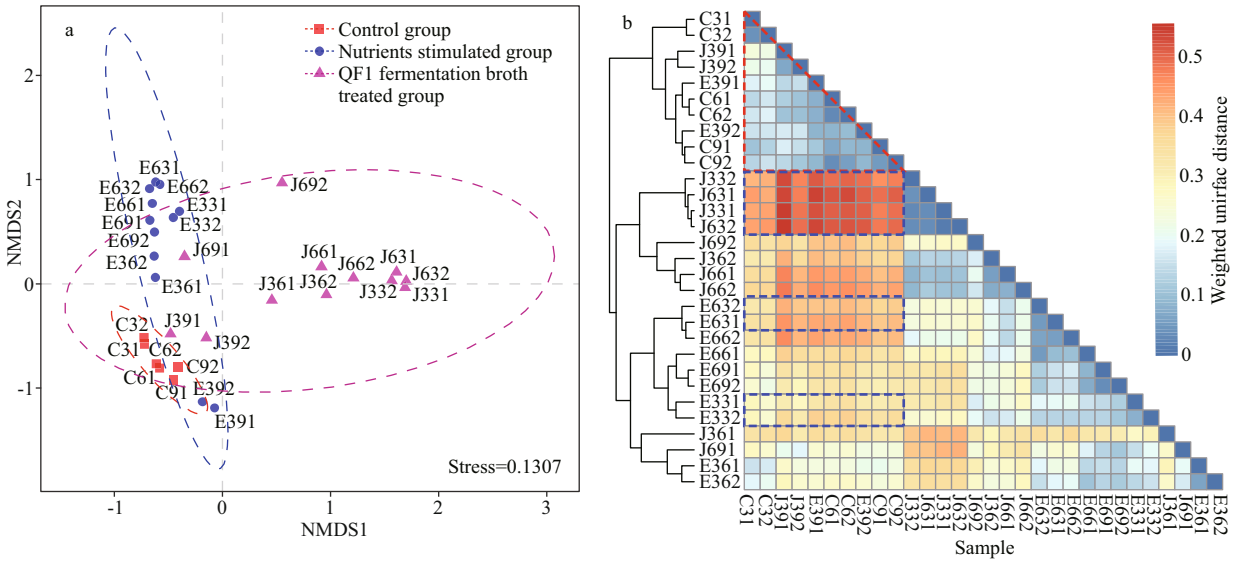

Fig.5 NMDS analysis (a) based on Bray-Curtis distance and heatmap (b) of weighted unirfac distance of the microbial communities in control and treated groups

Letter C, E, and J represents control, nutrients-stimulated, and QF1 treated groups, respectively. The first number following E and J represents the proportions of added nutrients or QF1 fermentation broth, the second number and the first number following C represents the sampling time points in algal cultivation process, the last number represents the replicates. The red triangle in (b) shows that the microbial communities on the 9th day in the 3% nutrient-stimulated group and QF1 treated group had a higher similarity with those in the control group, and the blue square shows that the microbial communities on the 3th day in the 3% and 6% nutrient-stimulated group and QF1 treated group had greater differences with those in the control group.

from marine environments, which are generally aerobic and chemoheterotrophic, and can hydrolyze complex carbon sources (Mcilroy and Nielsen, 2014). In this study, *Phaeodactylibacter*, a genus of Saprospiraceae, was detected in the *K. mikimotoi* culture. Previous researches have isolated the species from *Phaeodactylibacter* from alga *Phaeodactylum tricornutum* and *Picochlorum* sp. (Chen et al., 2014; Lei et al., 2015). These microorganisms likely benefit from the microalgae, which produce dissolved organic carbon compounds and oxygen through photosynthesis, and in turn may improve algal growth by providing nutrient, such as vitamins, iron, and ammonia (Amin et al., 2012).

Except for the dominant populations, *Halomonas*, *Alteromonas*, *Pseudoalteromonas*, and *Marinobacter* were also detected in *K. mikimotoi* culture without nutrients and QF1 broth stimulation. Previous studies have reported that these populations could inhibit the growth of some harmful microalgae. Extracts of *Halomonas* sp. HSB07 could inhibit the red-tide microalgae *Gymnodinium* (Pyrrophyta) (Liu et al., 2013). *Alteromonas* sp. A14 caused a significant decrease in *Cochlodinium polykrikoides* (Lee et al., 2008). *Pseudoalteromonas* S1 can lyse *Akashiwo sanguinea* in both direct and indirect ways (Sun et al., 2016). In our previous work, algicidal strains from *Halomonas*, *Alteromonas*, *Pseudoalteromonas*, and *Marinobacter* were also isolated from co-cultures of *K. mikimotoi* with sea water and estuarine soil of the Yellow Sea, China, and showed a high inhibition of *K. mikimotoi* (Zheng et al., 2018). Although these microbial populations increased slightly during the cultivation of *K. mikimotoi*, they never predominated in the coexisting microbial community. In addition, the number of cultivable microorganisms in the *K. mikimotoi* culture was low during algal cultivation. This could possibly explain why the growth of algal cells was not obviously inhibited, even when coexisting with these microbial populations. This phenomenon indicated that the limited nutrient in the culture of *K. mikimotoi* were not sufficient to promote the growth of the coexisting microorganisms. The limit nutrient refers to the f/2 medium, which contains 75-mg/L $NaNO_3$ and 5-mg/L NaH_2PO_4 that can satisfy the growth of microalgae. However, it cannot meet the massive growth of microorganisms because the lack of organic carbon source. As for achieving the purpose of algal inhibition in nutrient-stimulated groups, it was demonstrated later.

Nutrient addition increased microbial abundance and obviously changed the composition of the microbial community existing in the *K. mikimotoi* culture. After nutrient stimulation, the relative abundance of an unclassified genus belonging to Rhodobacteraceae and *Phaeodactylibacter* significantly decreased, while *Halomonas* and *Alteromonas* gradually became the

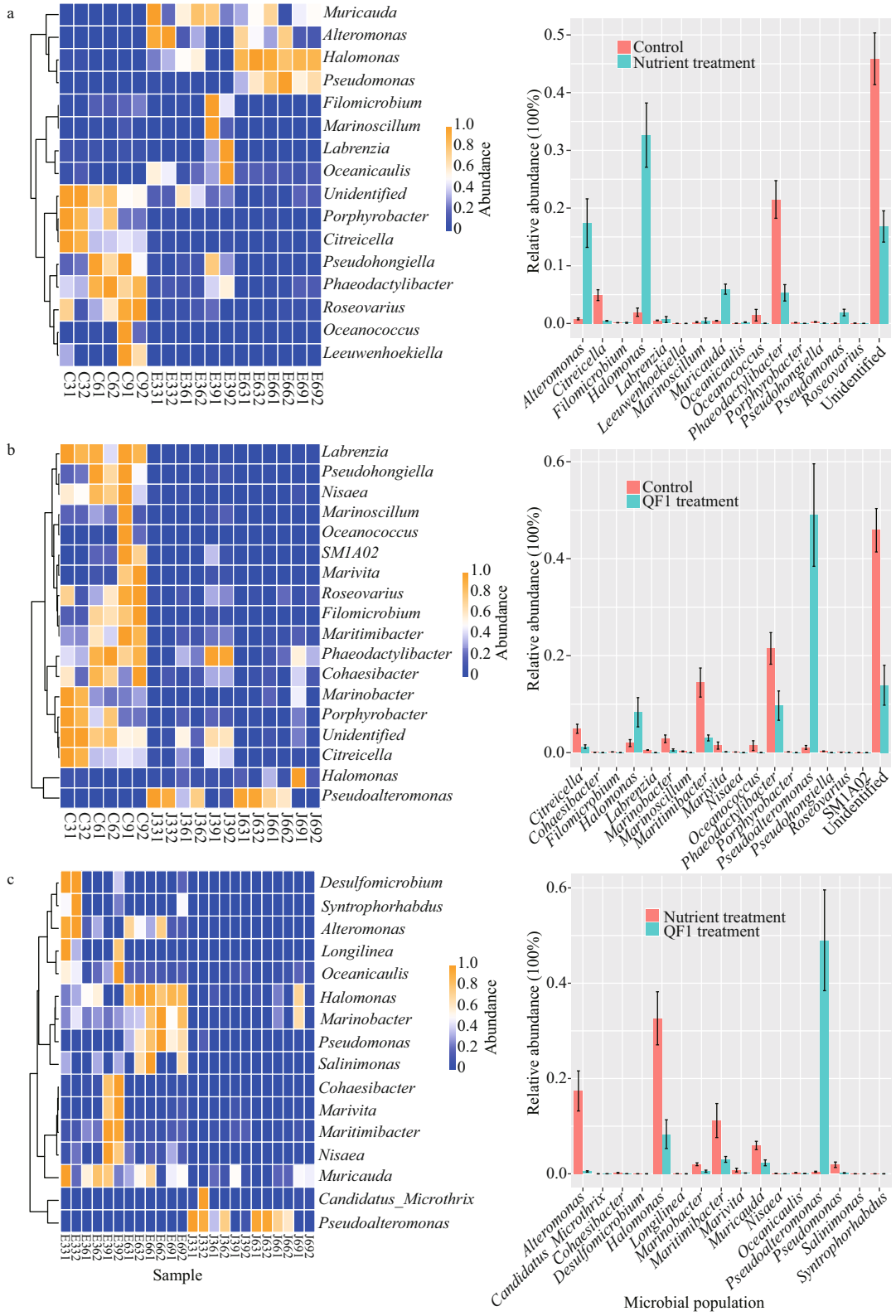

Fig.6 Heatmaps and Histograms with error bar of the microbial populations revealed by Wilcoxon rank-sum test

The microbial populations with significantly differential abundance between the control and nutrients stimulated groups; b. the control and QF1 treated groups; c. nutrients stimulated and QF1 treated groups. Letter C, E, and J represents control, nutrients-stimulated, and QF1 treated groups, respectively. The first number following E and J represents the proportions of added nutrients or QF1 fermentation broth, the second number and the first number following C represents the sampling time points in algal cultivation process, the last number represents the replicates.

dominant populations. In nutrient-stimulated groups, a high mortality of *K. mikimotoi* was observed. Furthermore, the 6% nutrient stimulation resulted in a higher mortality of *K. mikimotoi* than the 3% nutrient-stimulated groups. Algal inhibition was not observed when we added the same nutrient to the axenic *K. mikimotoi* culture, indicating that the growth of some microorganisms rather than the nutrient components inhibited algal growth in nutrient-stimulated group. Therefore, it has been concluded that nutrient stimulated the growth and metabolism of the coexisting microorganisms in the *K. mikimotoi* culture, and the latter inhibited algal growth. Almost as remarkably, the microbial communities of 3% nutrients treated groups became similar with those of the control groups at the 9th day, and the relative abundance of *Halomonas* and *Alteromonas* decreased, but always dominated in the 6% nutrient-stimulated group during the cultivation process. These results indicated the robustness of the microbial community in the *K. mikimotoi* culture to low-dose nutrient disturbance.

The QF1 fermentation broth had a high algicidal activity against the *K. mikimotoi*. Although the added QF1 fermentation broth rapidly changed the composition of the microbial community existing in *K. mikimotoi* culture in the initial phase, the relative abundance of *Pseudoalteromonas* substantially decreased in the microbial community on the 9th day. Furthermore, *Pseudoalteromonas* was detected in *K. mikimotoi* culture, yet it did not become a dominant population in the nutrient stimulation process. It seems that *Pseudoalteromonas* does not have competitive edges in the microbial community existing in *K. mikimotoi* culture. In addition, the robustness of the microbial community in the *K. mikimotoi* culture to low-dose QF1 fermentation broth disturbance was also observed: the microbial communities of QF1 treated groups became similar with those of the control groups on the 9th day. Therefore, adding fermentation broth of *Pseudoalteromonas* QF1 has potential application on controlling *K. mikimotoi*.

It should be noted that 13 OTUs were simultaneously detected in each sample, and predominated in *K. mikimotoi* culture with or without treatment. These OTUs were assigned to Rhodobacteraceae, *Maritimibacter*, *Marivita*, *Nisaea*, *Phaeodactylibacter*, *Citreicella*, *Halomonas meridiana*, *Alteromonas*, *Marinobacter*, *Muricauda*, and *Pseudoalteromonas*. Furthermore, these OTUs include the dominant populations in *K. mikimotoi* culture, such as Rhodobacteraceae, *Phaeodactylibacter*, *Maritimibacter*, and the dominant populations in nutrient-treated *K. mikimotoi* culture, such as *Halomonas meridiana* and *Alteromonas*. This phenomenon indicated that the aforesaid 13 OTUs are the principal parts of the microbial communities in the *K. mikimotoi* culture, and the changes of microbial communities observed in the *K. mikimotoi* culture with or without treatments could be represented by the changes of the 13 OTUs.

5 CONCLUSION

Having investigated the succession of the microbial community existing in *K. mikimotoi* culture, and the influences of added nutrients and exogenous algicidal strain on the growth of *K. mikimotoi*, diverse microbial populations were detected in *K. mikimotoi* culture and could be selectively stimulated by nutrients, thereby inhibiting the growth of *K. mikimotoi*. Adding algicidal strain *Pseudoalteromonas* also effectively inhibited the growth of *K. mikimotoi*. Moreover, the microbial community existing in *K. mikimotoi* culture showed robustness to low-dose nutrients and QF1 disturbance. This study provides insights for microbial control of *K. mikimotoi* via nutrient stimulation of the coexisting microorganisms or by adding fermentation broth of isolated algicidal strains. However, the amount of the added nutrients or fermentation broth of isolated algicidal strain will be studied to avoid secondary pollution to the marine environment.

6 DATA AVAILABILITY STATEMENT

The raw reads from 16S rRNA gene Illumina MiSeq sequencing have been submitted to the NCBI Sequence Read Archive (SRA) with the accession number SUB6782573. The 16S rRNA gene sequence of QF1 have been deposited in the GenBank database with the accession number MG457253.

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Electronic supplementary material

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