



Inhibitory Effect of Isolated Bacteria from the Phycosphere of *Levanderina fissa* on the Growth of Different Microalgae

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Levanderina fissa (formerly *Gyrodinium instriatum*) frequently causes blooms in the Pearl River Estuary and has few advantages in interspecific competition with other bloom-forming algal species. Phycosphere bacteria, which closely interact with algal cells, may play an ecologically functional role in the population dynamics and bloom occurrence. To test this hypothesis, we isolated and identified cultivable bacteria coexisting in different growth stages of *L. fissa* by the gradient dilution method and investigated the characteristics of the bacterial interactions with three diatom species (*Chaetoceros curvisetus*, *Skeletonema dohrnii*, and *Phaeodactylum tricorutum*) and three dinoflagellate species (*Scrippsiella acuminata*, *Karenia mikimotoi*, and the host algae) after screening for functional bacteria. One of the isolated bacterial strains, Lf7, which was phylogenetically identified as an *Alteromonas* species, showed significant inhibitory effects on different algal species except its host. Moreover, all algal species, especially their hosts, showed significant stimulatory effects on bacterial Lf7 growth. These results indicate that the phycosphere bacterium Lf7 may play some ecological roles in the competition between its host alga *L. fissa* and other phytoplankton. The study also highlights the complicated interactions between phycosphere bacteria and host algae.

Keywords: algicidal bacteria, phycosphere, harmful algal bloom, growth, algae-bacteria interactions

1 INTRODUCTION

The phycosphere is the planktonic analogue of the rhizosphere in plants (Seymour et al., 2017). Phytoplankton–bacteria relationships in this microenvironment can span mutualism, antagonism, parasitism, and competition, indicating a multifarious and highly sophisticated ecological function of phycosphere bacteria (Cole, 1982; Findlay and Patil, 1984; Amin et al., 2012; Seymour et al., 2017; Höger et al., 2021). Competitive or antagonistic relationships of phycosphere bacteria and phytoplankton have been mostly extensively studied, including their competition for inorganic nutrients (Bratbak and Tingstad, 1985), algicidal abilities of bacteria (Mayali and Azam, 2004; Umetsu et al., 2019), and defense mechanisms of phytoplankton (Teplitski et al., 2004; Rajamani et al., 2011; Stien et al., 2016). Their mutualism associations were recently revealed and proposed to be more prevalent than antagonism (Xie et al., 2013; Buchan et al., 2014; Calatrava et al., 2018).

Some studies have described the phycosphere bacteria of certain algae as friends with benefits (Amin et al., 2009; Kim et al., 2014; Johansson et al., 2019). For example, several clades of *Marinobacter* ubiquitously found in close association with dinoflagellates and coccolithophores produce an unusually lower affinity dicitrate siderophore, vibrioferrin, which increased algal iron uptake of a representative dinoflagellate partner, *Scrippsiella trochoidea*, >20-fold (Amin et al., 2009). When *Rhizobium* sp., the most prevalent and dominant bacterium isolated from *Chlorella vulgaris*, was cocultured with green algae, it increased the algal cell count by approximately 72% (Kim et al., 2014). Several phycosphere bacterial strains (*Arenibacter algicola* strain SMS7, *Marinobacter salarius* strain SMR5, *Sphingorhabdus flavimaris* strain SMR4y, *Sulfitobacter pseudonitzschiae* strain SMR1, *Yoonia vestfoldensis* strain SMR4r and *Roseovarius mucosus* strain SMR3) stimulate the growth of the diatom partner *Skeletonema marinoi* under different environmental conditions, for example, low iron concentrations and high and low temperatures (Johansson et al., 2019). Symbiotic bacteria may also provide the host with vitamins and organic acids, including amino acids and siderophores, and are essential to the growth of their host (Croft et al., 2005; Amin et al., 2009; Sandhya and Vijayan, 2019). Moreover, these kinds of phycosphere bacteria are well adapted to a narrow range of compounds secreted by their algal host, forming an exclusively specific association with the host algae (Bell, 1984; Schäfer et al., 2002; Sapp et al., 2007; Sison-Mangus et al., 2014; Palacios et al., 2022). This may not be the only way phycosphere bacteria benefit their algal hosts. Our laboratory isolated several phycosphere bacteria that showed inhibitory activity against algal species other than their host (Wang et al., 2021).

Levanderina fissa (formerly *Gyrodinium instriatum*) has frequently caused blooms in the Pearl River Estuary in the past two decades, for example, blooms in Shenzhen Bay in 1998, 2003, and 2007 (Wang et al., 2001; Zhu et al., 2004; SOA, 2008); Lingding Bay in 2002 (Wang et al., 2003); and Zhuhai coastal waters in 2009 (Wang et al., 2011). However, blooms caused by this species elsewhere were rare and were observed only in the Gulf of Guayaquil in Ecuador (Jiménez, 1993); Hakozaki Fishing Port of Japan (Nagasoe et al., 2006; Nagasoe et al., 2010); and Bahía de Acapulco, Mexico (Gárate-Lizárraga et al., 2013). Our previous study showed that *L. fissa* had few advantages in interspecific competition with three other algal bloom-forming species, namely, *Skeletonema dohrnii* (Bacillariophyceae), *Prorocentrum micans* (Dinophyceae), and *Chattonella marina* (Raphidophyceae), using pure, sterilized cultures (Wang et al., 2021). Thus, we suspect that phycospheric bacteria may play an ecological function role by promoting the growth or competition of *L. fissa*. To test this hypothesis, we isolated and identified cultivable bacteria coexisting in different growth stages of *L. fissa* by the gradient dilution method and obtained an isolate with an inhibitory effect on algal growth by screening experiments with *C. curvisetus*. We also cocultured the isolate Lf7 at different inoculation doses with three diatom species, *Chaetoceros curvisetus*, *Skeletonema dohrnii*, and *Phaeodactylum tricorutum*, as well as three dinoflagellate species, *Scrippsiella acuminata*, *Karenia mikimotoi*, and *L. fissa*, to determine their effects on these commonly dominant phytoplankton species in the Pearl River Estuary. Our research will provide insight into the

ecological function of phycosphere bacteria on algal growth, interspecies competition, and bloom formation.

2 MATERIALS AND METHODS

2.1 Isolation of Bacterial Strains from the Phycosphere of *Levanderina fissa*

2.1.1 Microalgal Culture

Levanderina fissa (GenBank accession no. ITS: KF435124) was isolated in October 2009 during a bloom in the Pearl River Estuary, South China Sea. The cultures were maintained in autoclaved (121°C, 20 min) f/2 medium (Guillard, 1975). Algal cells at the exponential phase were incubated in 250 mL Erlenmeyer flasks containing 100 mL of sterilized f/2 medium. The medium was made with artificial sea salt (Red Coral Sea, nutrient-free formula) with a salinity of 30-31 and pH of 7.9 ± 0.1. Microalgal cultures were maintained in an incubator at 20 ± 1°C and illuminated with 100 μmol photon/m²·s cool-white fluorescent illumination with a dark:light cycle of 12:12 h.

2.1.2 Isolation of Cultivable Bacteria in the Phycosphere

Samples were aseptically collected at five growth phases of *L. fissa*, that is, the lag growth phase, the logarithmic growth phase, the stationary phase, and the early and late decline phases. One milliliter of each culture was suspended in 9 mL of Zobell 2216E marine medium (Su et al., 2007) to a dilution of 10⁻¹. Zobell 2216E marine medium was prepared using artificial seawater. These suspensions were further diluted in the same way to dilutions of 10⁻²-10⁻⁶. A 0.1 mL aliquot of the dilutions of 10⁻³-10⁻⁶ was spread onto Zobell 2216E agar plates, and the plates were incubated at 28°C for 5-7 days. Visually distinct bacterial colonies were subcultured on fresh Zobell 2216E agar plates and incubated for 5-7 days. The step was repeated three to four times to obtain purified bacterial colonies.

There were 32 bacterial isolates collected from cultures of *L. fissa*. The bacterial isolates were then identified based on sequencing of the V3 region of the 16S rRNA gene. Twelve different phylotypes were obtained after removing the duplicate rRNA gene sequences. The isolated bacteria were labelled Lf1 to Lf12. The sequences were submitted to the International Nucleotide Sequence Database Collaboration at NCBI (GenBank accession nos. KF444158-KF444169). The bacterial phylotypes belonged to Alphaproteobacteria, Gammaproteobacteria, Bacteroidetes, and Actinobacteria (Table 1 and Figure 1)

2.2 Effects of Bacteria on the Growth of Different Microalgal Taxa

2.2.1 Antibacterial Treatment of Microalgal Cultures

The clonal strains of the six phytoplankton species, including three diatom species, *C. curvisetus* (GenBank accession no.: MW793400), *S. dohrnii* (GenBank accession no.: MW795694), and *P. tricorutum* (GenBank accession no.: MW793395), and three dinoflagellate species, *K. mikimotoi* (GenBank accession

TABLE 1 | The identities of bacteria isolated from phycosphere of *Levanderina fissa* and their phylogenetically most related sequences from EzTaxon database based on partial 16S rRNA gene sequences.

Strain code	GenBank accession no.	Phylum	Class	Family	Genus	Closest relatives	GenBank accession no.	Similarity (%)
Lf1	KF444165	Alphaproteobacteria	Rhodobacteriales	Rhodobacteraceae	<i>Pseudoceanicola</i>	<i>Pseudoceanicola antarctica</i>	OBEA01000013	99
Lf2	KF444164		Rhizobiales	Stappiaceae	<i>Roseibium</i>	<i>Roseibium marinum</i>	AY628423	99
Lf3	KF444162		Rhodobacteriales	Rhodobacteraceae	<i>Ruegeria</i>	<i>Ruegeria pomeroyi</i>	CP000031	97
Lf4	KF444163					<i>Ruegeria atlantica</i>	CYPU01000053	99
Lf5	KF444159		Rhodospirillales	Rhodospirillaceae	<i>Rhodospirillum</i>	<i>Rhodospirillum rubrum</i>	CP000230	90
Lf6	KF444160		Sphingomonadales	Erythrobacteraceae	<i>Erythrobacter</i>	<i>Erythrobacter litoralis</i>	JMIX01000006	99
Lf7	KF444158	Gammaproteobacteria	Alteromonadales	Alteromonadaceae	<i>Alteromonas</i>	<i>Alteromonas macleodii</i>	CF003841	99
Lf8	KF444161				<i>Marinobacter</i>	<i>Marinobacter salsuginis</i>	EF028328	100
Lf9	KF444169		Pseudomonadales	Moraxellaceae	<i>Acinetobacter</i>	<i>Acinetobacter courvalinii</i>	KT997472	98
Lf10	KF444168	Actinobacteria	Actinomycetales	Micrococcaceae	<i>Arthrobacter</i>	<i>Arthrobacter crystallopoietes</i>	FNKH01000002	99
Lf11	KF444167	Bacteroidetes	Flavobacteriales	Flavobacteriaceae	<i>Winogradskyella</i>	<i>Winogradskyella pariferorum</i>	AY848823	100
Lf12	KF444166				<i>Robignitalea</i>	<i>Robignitalea biformata</i>	CP001712	92

no.: MW793400), *S. acuminata* (GenBank accession no.: MW793402), and *L. fissa*, were isolated between 2005 and 2009 from southern Chinese coastal waters and maintained in *f/2* media (for diatoms) or in *f/2* media without silicon (for dinoflagellates).

The stock cultures were treated with a mixture of antibiotics to destroy the external bacteria before the experiments. First, 10 µg/mL penicillin (final concentration) was added to 100 mL of *C. curvisetus* culture in the exponential phase and incubated for 24 h. Then, 10 mL of the penicillin-treated culture was inoculated into 100 mL of fresh *f/2* medium with 10 µg/mL streptomycin sulfate and incubated for another 24 h, followed by treatment with 10 µg/mL kanamycin sulfate. The antibacterial treatment cultures were maintained in sterilized *f/2* medium for the experiments.

2.2.2 Bacterial Isolation and Counting

Bacterial isolates were maintained in a Zobell 2216 marine agar plate after removing the repeated isolates by cross-comparison with the results of the rRNA gene sequences. Before the experiment, bacterial colonies were transferred to liquid Zobell 2216 medium, cultivated on a 28°C, 200 r/min shaking table for 8-10 h, and then gradually acclimated to 20°C within 2 h.

The bacterial concentration was determined by measuring the colony-forming unit (CFU) number on Zobell 2216E agar medium. Each distinct bacterial colony was counted in the plate of suitable colonies (30-300), and the concentration of each bacterium (CFU/mL) was obtained according to the dilution factor.

2.2.3 Screening of the Bacterial Strains

The effects of the 12 bacterial isolates on the growth of different microalgal taxa were screened by *C. curvisetus*. Liquid bacterial cultures of the 12 isolates in exponential phase were added to the exponential *C. curvisetus* cultures. The inoculation rate was 0.1% (v/v), and the initial bacterial concentration was 7.5×10^7 CFU/mL in the cocultures. Cultures in *f/2* medium (*f/2*) and with the same volume of liquid Zobell 2216E medium (BC) were used as the negative controls. Algal cultures were cultivated under the same conditions as outlined in Section 2.1.1. All experiments were carried out in sterile 24-well tissue culture plates. The experiment was conducted for 5 days covering the whole exponential phase and reaching the stationary phase for *C. curvisetus*, and cell numbers were counted every day. The algal cell numbers were counted daily during the incubation period. The cell counts were performed in a cell counting chamber by placing 0.05-0.1 mL of culture into the chamber, fixing with a drop of Lugol's fixative, and observing under an inverted microscope (Leica DMIRB) at a magnification of 200×. Each sample was counted more than three times until the differences in cell counts were less than 10%. When *C. curvisetus* always showed negative growth with lower cell densities than initial cell densities, the maximum cell density was recorded as 0. All experiments were conducted in triplicate.

2.2.4 Effects of Bacterial Strain Lf7 on the Growth of Microalgal Taxa

The results of the screening test showed that the bacterial strain Lf7 had the strongest inhibitory effect on the growth of *C. curvisetus*. Therefore, Lf7 was selected as the bacterial strain to study its effects

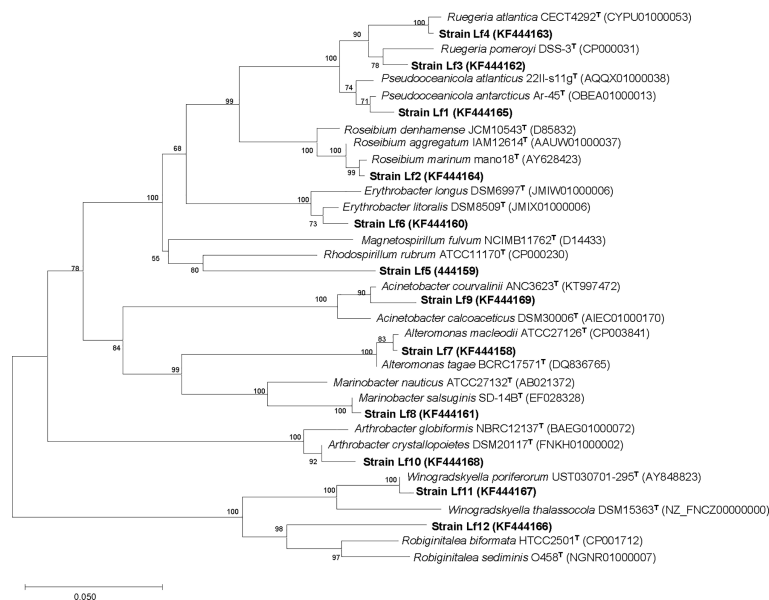


FIGURE 1 | Neighbor-joining tree, showing the phylogenetic position of bacterial strains isolated from phycosphere of *Levanderina fissa* and type strains of most phylogenetically related taxa (type species included) based on partial 16S rRNA gene sequences. GenBank accession numbers are given. Bootstrap analysis was made with 1000 resamplings; percentages of support are shown at nodes, Bar, 5% sequence divergence.

on the growth of the six different phytoplankton taxa. Four bacterial concentrations were set in the experiment, which were 7.5×10^7 , 7.5×10^6 , 7.5×10^5 , and 7.5×10^4 CFU/mL, with an inoculation rate of 0.1% (v/v). The experiment was conducted for 6 days to cover the whole exponential phase and reach the stationary phase for the algae, and algal cell numbers and bacterial concentrations were measured every day. The initial cell densities of different microalgal taxa were set differently according to their cell sizes to obtain similar initial biomasses. The size of the microalgal taxa is described in **Supplementary Table S1**. The experiment was conducted for 6 days to cover the whole exponential phase and reach the stationary phase for the algae according to our former lab experience, and algal cell numbers and bacterial concentrations were measured every day.

This additional experimental design is the same as that in Section 2.2.3.

2.3 Data and Statistical Analysis

2.3.1 Phylogenetic Analysis

Analysis of the 16S rRNA gene sequence was performed using the software package MEGA, version 3.1 (Kumar et al., 2004). The model of Jukes and Cantor (1969) was used to compute the evolutionary distance, based on which a phylogenetic tree was constructed using the neighbor-joining method (Saitou and Nei, 1987), with a bootstrap analysis derived from 1000 replications. Sequence similarity was calculated using pairwise alignment obtained from the EzTaxon database (Chun et al., 2007).

2.3.2 Inhibitory Effect

The effects of bacterial strains on the growth of algal cells are expressed as the relative maximum cell number (%), which is

defined as the maximum/final cell number in each experimental regime compared to the maximum/final cell number obtained in the *f/2* medium (*f/2*). When cell numbers in the test group were always lower than the initial density, indicating that no active growth occurred, neither the maximum cell number (cells/mL) nor the relative maximum cell number (%) was calculated.

2.3.3 Maximum Specific Growth Rate

The specific growth rate (μ , divisions/d) of algal cells was calculated every day during the experimental period using the following equation:

$$\mu (\text{divisions/d}) = (\ln N_2 - \ln N_1) / (t_2 - t_1) \quad (1)$$

where N_2 and N_1 are cell density values at times t_2 and t_1 , respectively. The maximum specific growth rate (μ_{\max}) is defined as the maximum value of μ during the experiment.

2.3.4 Statistics

The mean and standard deviation (SD) were calculated for each treatment from three independent replicate cultures. The means and standard deviations of all data were calculated and graphed. Repeated measures ANOVA was used to compare growth curves of different treatments. One-way ANOVA with *post hoc* (Tukey test) analysis was conducted for multiple comparisons of the maximum specific growth rate, relative maximum cell density, and maximum bacterial concentration. Statistical analyses were performed using SPSS 25.0, and differences were considered significant when $p < 0.05$. Detailed statistical information is shown in the **Supplementary Tables**.

3 RESULTS

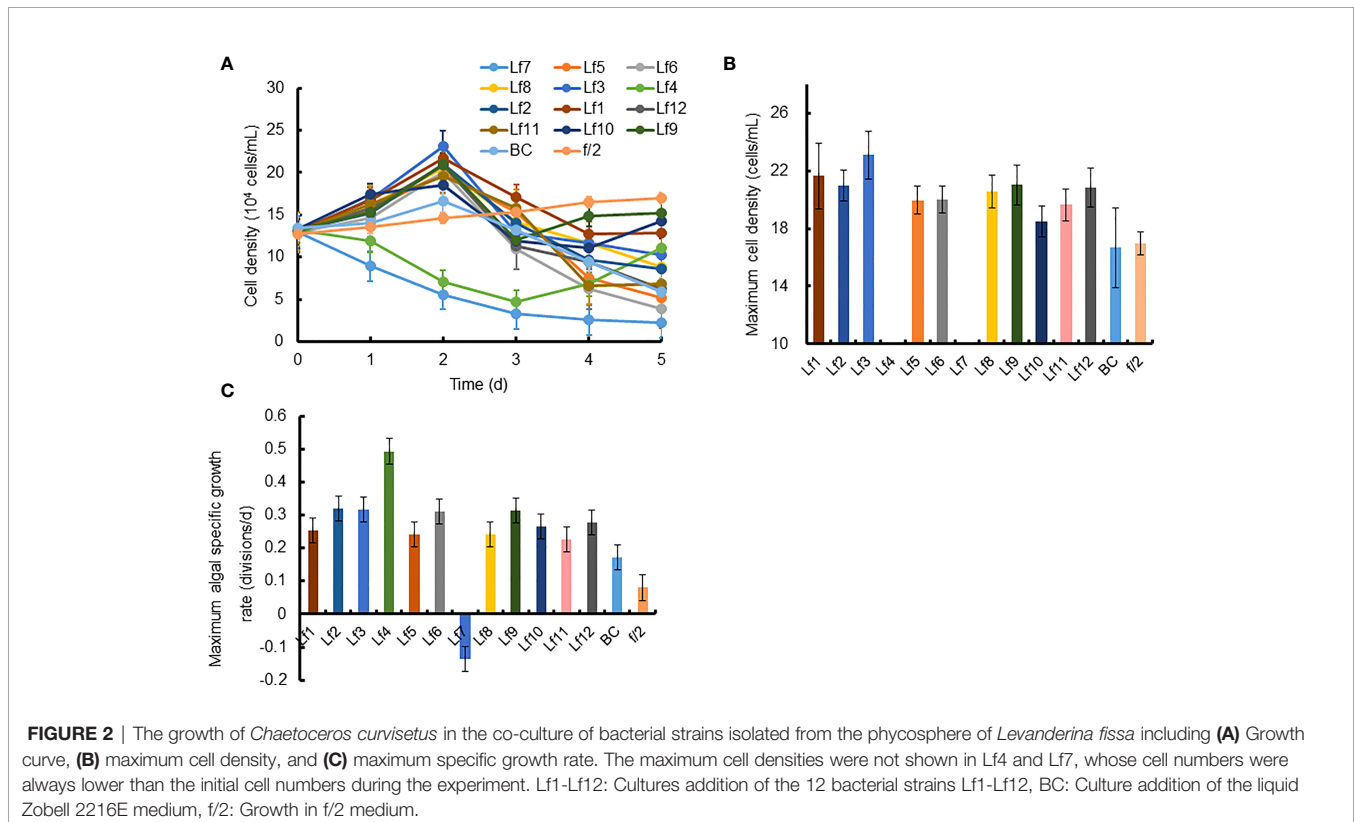
3.1 Bacteria Isolation from the Phycosphere of *Levanderina fissa*

The growth of *C. curvisetus* in the coculture of the 12 bacterial strains isolated from the phycosphere of *L. fissa* is shown in **Figure 2**. The cell density increased gradually in the f/2 medium and reached a maximum of 1.71×10^5 cells/mL at Day 5. The cell density in the BC control, which was supplemented with bacterial medium, increased and peaked at Day 2, with a maximum of 1.67×10^5 cells/mL. As shown in **Figure 2A**, most bacteria had no significant inhibition of the growth of *C. curvisetus*, and only two strains (Lf4 and Lf7) showed significant inhibitory effects ($p < 0.01$). The cell density of *C. curvisetus* decreased rapidly after the addition of the bacterial strain Lf7 and dropped to 2.2×10^4 cells/mL at Day 5, which was only 16.9% of the inoculation density. The growth of algal cells was significantly inhibited in the first 3 days after the addition of the bacterial strain Lf 4 ($p < 0.01$), and then the cell density increased at Day 4 and Day 5; however, it was still lower than the inoculation density. The cell densities of *C. curvisetus* in cocultures with Lf7 and Lf4 were always lower than the inoculation densities. Moreover, the maximum cell densities in the cocultures with the other 10 bacterial strains were significantly higher than those of the f/2 and BC media ($p < 0.05$, **Figure 2B**). Moreover, only *C. curvisetus* inoculated with strain Lf7 showed a negative maximum specific growth rate (**Figure 2C**). The results of the screening experiment suggested

that the bacterial strain Lf7 had the most obvious growth inhibition on *C. curvisetus*. Therefore, Lf7, identified as the Gammaproteobacteria *Alteromonas* species based on a partial sequence of the 16S rRNA gene (**Table 1** and **Figure 1**), was selected for further experiments.

3.2 Effects of Bacterial Strain Lf7 on the Growth of *Chaetoceros curvisetus*

The growth curves of *C. curvisetus* in different experimental treatments were similar, except for the highest bacterial inoculation group (7.5×10^7 CFU/mL). The curves initiated at a 1–2-day lag period, then a 2–3-day rapid growth period, followed by a slow increase or a slight decrease in cell density (**Figure 3A**). Significant growth inhibition occurred only in culture with 7.5×10^7 CFU/mL bacterium Lf7, in which cell numbers decreased steadily and were always lower than the incubation number (**Figure 3A**). As shown in **Figure 4A**, the maximum cell density in the BC culture was 95.7% compared to that in the f/2 medium, while the relative maximum cell densities were over 100% (106–133%) in cultures with bacterial addition at concentrations of 7.5×10^4 – 7.5×10^6 CFU/mL. The algal cells showed no effective growth after adding bacteria at a concentration of 7.5×10^7 CFU/mL, and the maximum cell density was the inoculation density. The maximum specific growth rate of *C. curvisetus* showed a negative value when inoculated with 7.5×10^7 CFU/mL bacterium Lf7, indicating negative growth throughout the whole experimental period (**Figure 4B**).



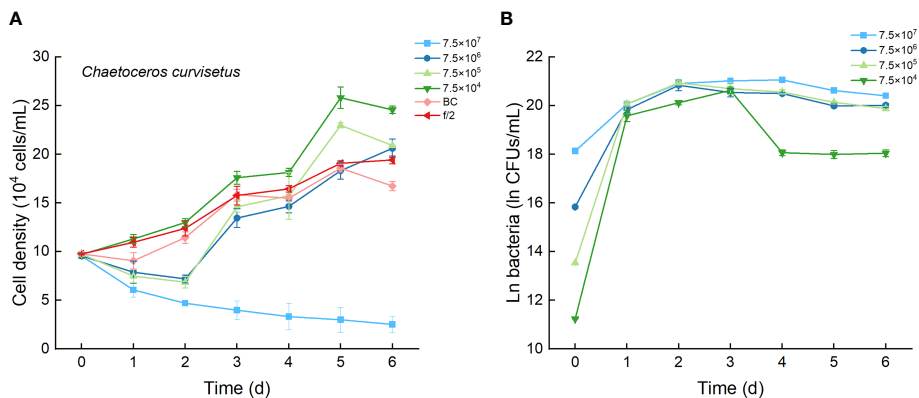


FIGURE 3 | The growth of *Chaetoceros curvisetus* in the co-cultures with different inoculum dose of bacterial strain Lf7 (7.5×10^4 - 7.5×10^7 CFUs/mL). **(A)** Growth curves of *C. curvisetus* in co-cultures with Lf7, **(B)** Lf7 abundances in the algal cultures.

In the coculture system, bacteria grew well and showed maximum abundances at 2 to 4 days (Figure 3B). The bacterial concentration in the group with the highest bacterial inoculum dose maintained the longest growing period (4 days). In all groups with different bacterial inoculum doses, the maximum bacterial concentration showed no significant difference, ranging from 8.7×10^8 - 1.42×10^9 CFU/mL (Figure 4C).

3.3 Effects of the Bacterial Strain Lf7 on the Growth of *Skeletonema dohrnii*

The cell densities of *S. dohrnii* with f/2 medium were significantly higher than those with bacterial medium. The cell densities of *S. dohrnii* inoculated with strain Lf7 were significantly lower than those inoculated with either f/2 medium or bacterial medium (Figure 5A). The density dropped to 15-24% of the initial

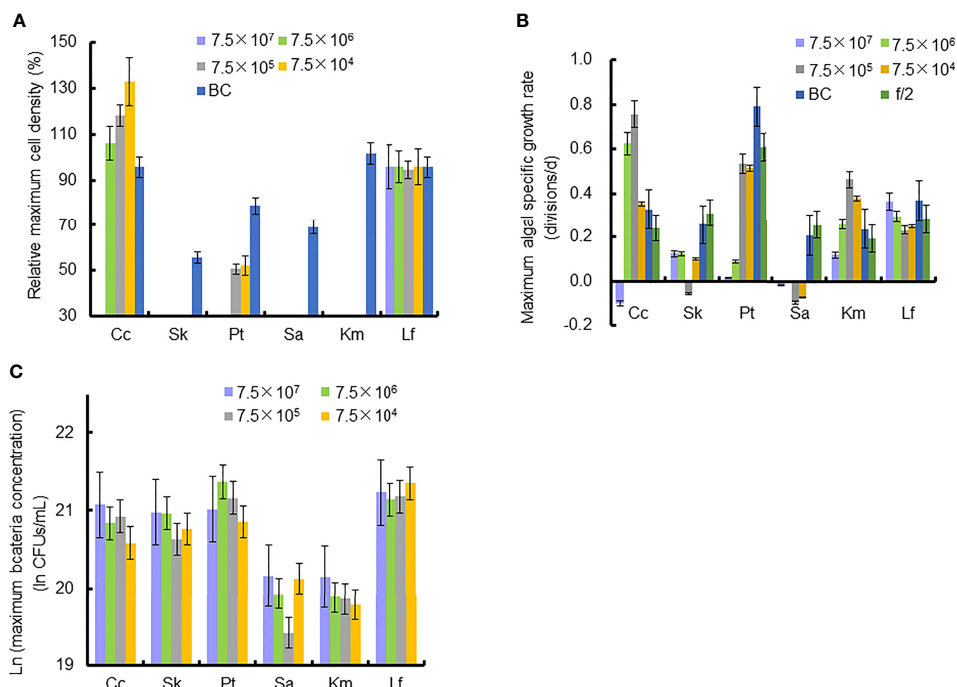


FIGURE 4 | Relative maximum algal cell density, maximum algal specific growth rate, and bacteria concentration in co-cultures of six microalgal taxa with the bacterial strain Lf7 during the six days incubation. **(A)** Relative maximum algal cell density, **(B)** maximum algal specific growth rate, **(C)** maximum Lf7 concentration in the algal cultures. Cc, co-culture of *Chaetoceros curvisetus* with Lf7; SK, co-culture of *Skeletonema dohrnii* with Lf7; Pt, co-culture of *Phaeodactylum tricornutum* with Lf7; Sa, co-culture of *Scirppsiella acuminata* with Lf7; Km, co-culture of *Karenia mikimotoi* with Lf7; Lf, co-culture of *Levanderina fissa* with Lf7.

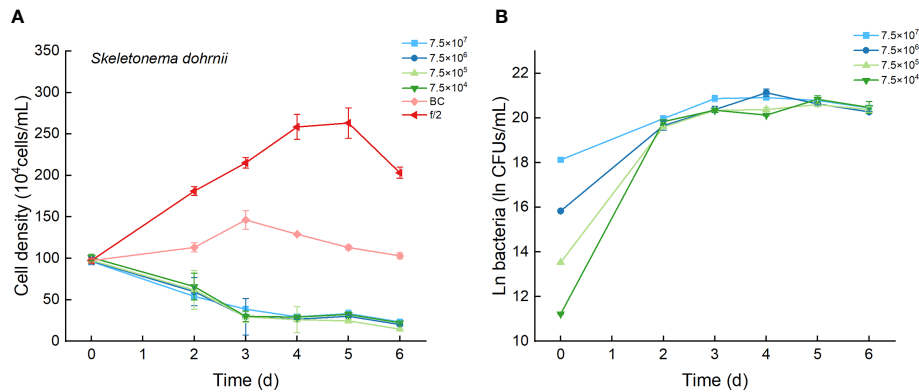


FIGURE 5 | The growth of *Skeletonema dohrnii* in the co-cultures with different concentrations of bacterial strain Lf7 (7.5×10^4 – 7.5×10^7 CFUs/mL). **(A)** Growth curves of *S. dohrnii* in co-cultures with Lf7, **(B)** Lf7 concentration in the algal cultures.

inoculation densities, indicating a strong inhibitory effect of the bacterial strain Lf7 on the growth of the algae *S. dohrnii*. The maximum specific growth rate of *S. dohrnii* showed a negative value when inoculated with 7.5×10^5 CFU/mL bacterium Lf7, indicating negative growth throughout the whole experimental period (Figure 4B).

The bacterial concentration increased significantly to 9.1×10^8 – 1.29×10^9 CFU/mL, showing no significant differences between groups with different bacterial inoculum doses (Figure 5B).

3.4 Effects of Bacterial Strain Lf7 on the Growth of *Phaeodactylum tricornutum*

As the control, *P. tricornutum* with f/2 experienced a lag phase within two days, reached maximum cell densities on the fourth day, and maintained a high cell density from Day 5 to Day 6 (Figure 6A). At a high inoculation dose of bacteria (7.5×10^7 and 7.5×10^6 CFU/mL), the

growth of *P. tricornutum* was strongly inhibited, with a decline of 90% cell density within 3 days. Afterward, the cell density of this algae decreases slowly and remains low in such a coculture condition. Whether at a low inoculation dose of bacteria (7.5×10^4 and 7.5×10^5 CFU/mL) or under additions of bacterial medium, the growth of *P. tricornutum* showed a similar growth pattern: cell densities decreased within 2 days and gradually increased to maximum values on Day 4. The cell densities of *P. tricornutum* with high inoculation doses of strain Lf7 were significantly lower than those with either low inoculation doses of strain Lf7 or bacterial medium. The maximum specific growth rate of *P. tricornutum* showed a value close to zero when inoculated with 7.5×10^4 CFU/mL bacterium Lf7, indicating almost no growth throughout the whole experimental period (Figure 4B).

When cocultured with *P. tricornutum*, CFUs of strain Lf7 increased significantly (Figure 6B). The highest bacterial concentration occurred on Day 2 when the growth of this algae was strongly suppressed. The bacterial concentration increased to 1.14 – 1.9×10^9 CFU/mL by the end of

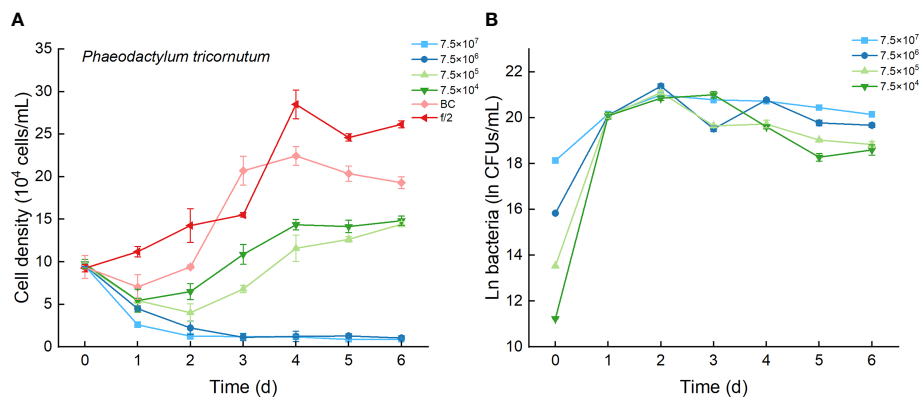


FIGURE 6 | The growth of *Phaeodactylum tricornutum* in the co-cultures with different concentrations of bacterial strain Lf7 (7.5×10^4 – 7.5×10^7 CFUs/mL). **(A)** Growth curves of *P. tricornutum* in co-cultures with Lf7, **(B)** Lf7 concentration in the algal cultures.

the experiment, showing no significant differences between groups with different bacterial inoculum doses ($p > 0.05$).

3.5 Effects of Bacterial Strain Lf7 on the Growth of *Scrippsiella acuminata*

As the control, the cell densities of *S. acuminata* with additions of f/2 medium and bacterial medium showed no significant differences. The cell densities of *S. acuminata* at all inoculation doses of Lf7 declined dramatically (Figure 7A), indicating a strong inhibitory effect of Lf7 on *S. acuminata*. Moreover, the concentration of strain Lf7 increased under the coculture conditions, with maximum values within 2.7 - 5.7×10^8 CFU/mL from Day 3 to Day 4 (Figure 7B). The maximum specific growth rate of *S. acuminata* showed a negative value at all inoculation doses of the bacterium Lf7, indicating negative growth throughout the whole experimental period for all the coculture treatments (Figure 4B). Among the three diatoms used in the coculture experiments, Lf7 showed the lowest maximum concentration when cocultured with *S. acuminata* (Figure 4C).

3.6 Effects of the Bacterial Strain Lf7 on the Growth of *Karenia mikimotoi*

As the control, the cell densities of *K. mikimotoi* with additions of f/2 medium and bacterial medium showed no significant differences in their growth curves ($p > 0.1$, Figure 8A). Only the maximum specific growth rate of *K. mikimotoi* cocultured with 7.5×10^6 CFU/mL bacterium Lf7 was significantly lower than that of the control (*P. tricornutum* cultured with f/2 or BC) (all $p < 0.05$, Figure 4B). However, the cell densities of *K. mikimotoi* at all inoculation doses of Lf7 declined dramatically on the first day and were maintained at a low density, reaching 42-68% of the inoculum dose. Lf7 showed inhibitory effects on the growth of *K. mikimotoi* under the addition of all bacterial concentrations (Figure 8A).

The concentration of strain Lf7 under coculture conditions with *K. mikimotoi* was also similar to that with *S. acuminata* and significantly lower than that with other diatoms, reaching maximum values within 2.7 - 5.7×10^8 CFU/mL ($p < 0.01$, Figures 4C, 8B).

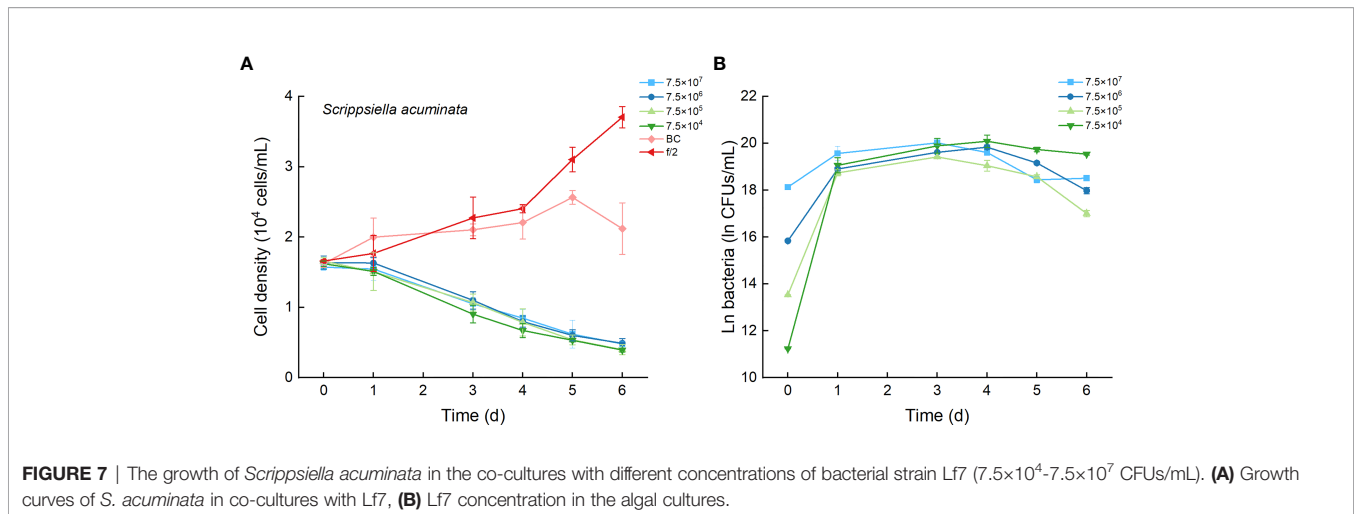


FIGURE 7 | The growth of *Scrippsiella acuminata* in the co-cultures with different concentrations of bacterial strain Lf7 (7.5×10^4 - 7.5×10^7 CFUs/mL). (A) Growth curves of *S. acuminata* in co-cultures with Lf7, (B) Lf7 concentration in the algal cultures.

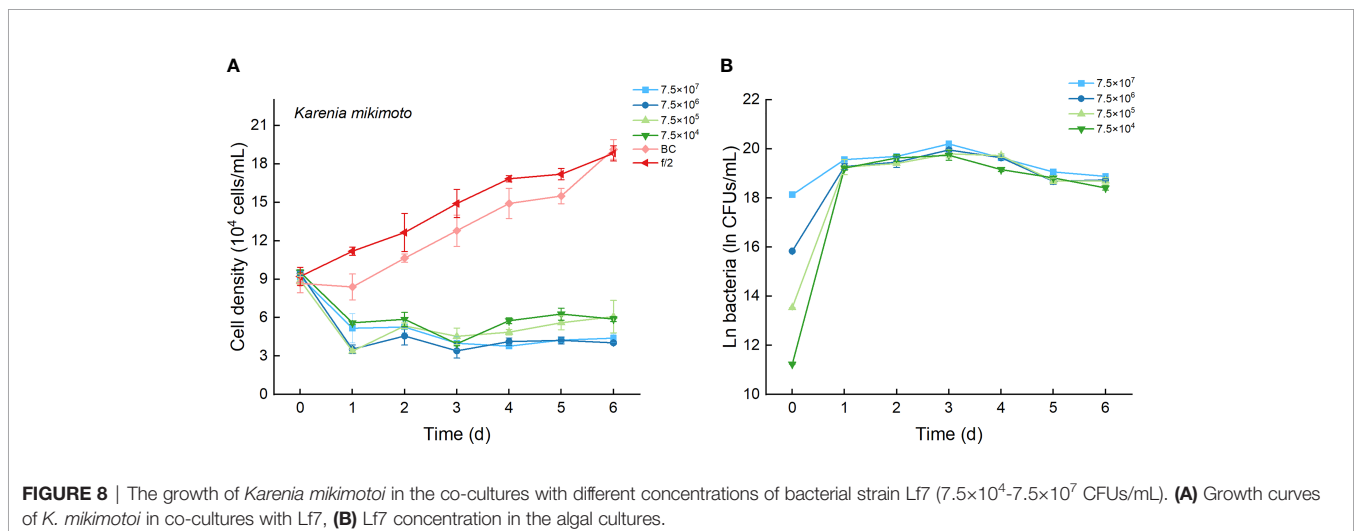


FIGURE 8 | The growth of *Karenia mikimotoi* in the co-cultures with different concentrations of bacterial strain Lf7 (7.5×10^4 - 7.5×10^7 CFUs/mL). (A) Growth curves of *K. mikimotoi* in co-cultures with Lf7, (B) Lf7 concentration in the algal cultures.

3.7 Effects of Bacterial Strain Lf7 on the Growth of *Levanderina fissa*

Regardless of treatments (whether with different inoculation doses of bacteria or under additions of different media), the cell densities of *L. fissa* increased to a maximum value at Day 5 (Figure 9A). Although the daily maximum specific growth rate occurred differently between several of the two treatments ($p < 0.05$, Figure 4B), the growth curves showed no significant differences ($p > 0.05$), indicating that the growth of *L. fissa* was not inhibited by Lf7. However, the concentration of strain Lf7 in coculture conditions with *L. fissa* reached maximum values within $1.52\text{--}1.87 \times 10^9$ CFU/mL, with the highest average values among those of strain Lf7 in coculture conditions with all algal species in this experiment (Figures 4C, 9B).

4 DISCUSSION

The study isolated and identified a total of 12 strains of cultivable bacteria coexisting in different growth stages of *L. fissa* by the gradient dilution method. Only strain Lf7, identified phylogenetically as an *Alteromonas* species using a partial 16S rRNA gene sequence, showed a significant inhibitory effect on different algal species except its host, and its growth was promoted by coculture with the host alga *L. fissa*.

Alteromonas species are frequently reported as phycosphere bacteria with two opposite interacting actions with their host (Meyer et al., 2017; Cao et al., 2021). These bacteria can directly cause the lysis of their host algae by producing β -glucosidase and chitinase (Imai et al., 1995; Wang et al., 2010; Lin et al., 2013; Umetsu et al., 2019). Shi et al. (2018) isolated an algicidal bacterium (FDHY-03) from a bloom of *Prorocentrum donghaiense* and found the characteristics of its action against *P. donghaiense* by digesting cell wall polysaccharides from the megacytic growth zone. 16S rRNA gene sequence analysis placed this strain in the genus *Alteromonas*. The beneficial effects of *Alteromonas* species on their hosts have also been reported (Ferrier et al., 2002; Sandhya and Vijayan, 2019). By examining how the entire transcriptome of

Isochrysis galbana changed when it was cocultured with *A. macleodii* isolated from its phycosphere, Cao et al. (2021) revealed transcriptome changes, including notable increases in transcripts related to photosynthesis, carbon fixation, oxidative phosphorylation, ribosomal proteins, biosynthetic enzymes, and transport processes, as well as the depletion of transcripts encoding DNA repair enzymes, superoxide dismutase (SOD), and other stress-response proteins. Taken together with the lab-enhanced growth of *I. galbana* by coculturing with *A. macleodii*, the presence of *A. macleodii* enhanced photosynthesis and biosynthesis of *I. galbana* and protected it from stress, especially oxidative stress.

Our isolated Lf7 showed neither direct beneficial nor inhibitory effects on its host, as discussed above. However, the strain showed different inhibitory effects on the growth of five other tested algal species, including *K. mikimotoi*, *S. acuminata*, *P. tricornutum*, *C. curvisetus*, and *S. dohrnii*. All five algal species occurred in the Pearl River Estuary. Except for the model diatom *Phaeodactylum tricornutum*, the other four were reported as bloom species elsewhere (Shikata et al., 2008; Begum et al., 2015; Tse and Lo, 2017; Li et al., 2019). *L. fissa* blooms are frequent in the Pearl River Estuary, while the phenomenon is not common worldwide (Jiménez, 1993; Nagasoe et al., 2006; Nagasoe et al., 2010; Gárate-Lizárraga et al., 2013). *L. fissa* showed few advantages in interspecific competition with three other algal bloom-forming species, *Skeletonema dohrnii* (Bacillariophyceae), *Prorocentrum micans* (Dinophyceae), and *Chattonella marina* (Raphidophyceae), using pure, sterilized cultures in previous research (Wang et al., 2021). Thus, the reason *L. fissa* outcompeted other algae in the Pearl River Estuary and other limited places might be a key factor explaining the frequent blooms. Wang et al. (2017) proposed the assumption that the ability to dissolve organic phosphorus utilization of *L. fissa* offers this species a competitive advantage in the phytoplankton community. In this study, our results hint at another possible explanation that bacteria associated with *L. fissa* might have some ecological roles in its competition with other phytoplankton.

Mutualism associations often refer to a serious chemical exchange between phytoplankton and phycosphere bacteria; for example, diatoms secrete the amino acid tryptophan, which is

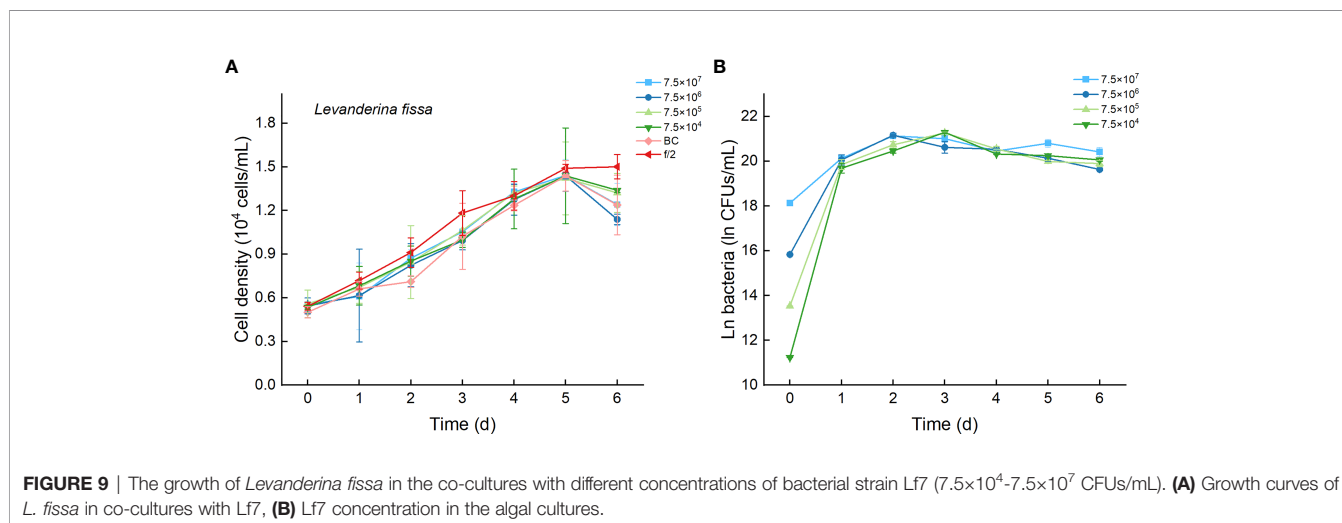


FIGURE 9 | The growth of *Levanderina fissa* in the co-cultures with different concentrations of bacterial strain Lf7 (7.5×10^4 – 7.5×10^7 CFUs/mL). **(A)** Growth curves of *L. fissa* in co-cultures with Lf7, **(B)** Lf7 concentration in the algal cultures.

converted by the bacterium into the hormone indole-3-acetic acid (IAA) (increasing bacterial growth simultaneously), which is then transferred from the bacterium back to the diatom to promote its cell division (Amin et al., 2015). Both bacteria and phytoplankton benefited from direct mutualism associations. *In our study, the growth of that bacterium benefited most from the original host L. fissa* among all tested algae. It is a common case that phycosphere bacteria are more adapted to a narrow range of compounds secreted by their algal host (Bell, 1984; Schäfer et al., 2002; Sapp et al., 2007; Sison-Mangus et al., 2014). Even though strain Lf7 showed no promoting effect on the growth of *L. fissa*, the inhibitory effect of strain Lf7 on other algae may lead to a competing advantage of its host. This may indicate complicated interactions between phycosphere bacteria and their host algae, which needs further lab studies.

In conclusion, by isolating and identifying cultivable bacteria coexisting in different growth stages of Levanderina fissa and subsequently monitoring the growth status of bacteria and microalgae in cocultural conditions, we obtained the bacterial strain Lf7 (taxonomically belonging to Alteromonas species), which showed inhibitory effects on the growth of five different microalgae (K. mikimotoi, S. acuminata, P. tricornutum, C. curvisetus, and S. dohrnii). We suspect that phycosphere bacteria Lf7 might have some ecological roles in the competition between its host algae L. fissa and other phytoplankton.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding authors.

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AUTHOR CONTRIBUTIONS

In this paper, ZW and RHvconceived and designed the experiments; CX and XJ contributed to data acquisition and analysis; YT contributed to drafting the manuscript. All authors approved the final submitted manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: <https://www.frontiersin.org/articles/10.3389/fmars.2022.908813/full#supplementary-material>

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