



Epimicrobiome Shifts With Bleaching Disease Progression in the Brown Seaweed *Saccharina japonica*

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Recent bacterial induced bleaching disease events of the commercially farmed brown seaweed *Saccharina japonica* has resulted in significant reduction in healthy sporeling supply. However, to date the host associated epimicrobial community shifts with the disease progression have not been characterized. We investigated the dynamic shifts in the composition of the epibacterial communities of *S. japonica* with disease progression using 16S rRNA gene amplicon sequencing. We found that the alpha diversity was significantly higher in the control group than in the infected group over disease progression ($P < 0.01$). There was a significant shift in the composition and predicted functions of the epibacterial communities in both control and infected groups. Indicator species, belonging to *Stenotrophomonas*, *Pseudomonas* and *Sphingomonas* were significantly higher in the control group than infected group, suggesting that these taxa are associated with healthy *S. japonica*. In contrast, *Vibrio*, *Pseudoalteromonas* and *Colwellia* were keystone taxa in the infected group at 24 h, and thus maybe considered to be the secondary opportunistic pathogens. Our study describes the changes of epibacterial communities associated with the progression of bleaching disease in *S. japonica*. This new information not only extends our baseline knowledge of the *S. japonica* epimicrobiome, but also paves the way for developing measures to mitigate disease outbreaks for the sustainable aquaculture of *S. japonica*.

Keywords: bleaching disease, epimicrobiome shifts, pathogenic bacteria, 16S rRNA gene amplicon sequencing, *Saccharina japonica*

INTRODUCTION

Macroalgae (seaweeds) associated bacteria are critical for the normal development and health of their host (Barott et al., 2011; Egan et al., 2013; Singh and Reddy, 2014; Saha and Weinberger, 2019). For example, they are thought to provide vital nutrients for the healthy growth of the host (Croft et al., 2006) and produce antimicrobial compounds for fouling defence (Penesyanyan et al., 2009; Egan et al., 2013) as well as produce chemical signals that can induce the release and settlement of algal spores (Joint et al., 2007; Weinberger et al., 2007). However, seaweed associated microbes are not

always beneficial and as in other systems [e.g. corals (Damjanovic et al., 2019), humans (Hajishengallis and Lamont, 2016) and shrimp (Lu et al., 2020)], disruption of the hosts natural microbiota has been correlated with the occurrence of various disease in seaweeds (Egan and Gardiner, 2016; Kumar et al., 2016; Longford et al., 2019; Egan et al., 2020; Li et al., 2022).

Microbiome shifts have been found in bleaching diseased red seaweed *Delisea pulchra*. Healthy and diseased *D. pulchra* harbor distinct microbial communities (Campbell et al., 2011; Case et al., 2011; Fernandes et al., 2011), with the abundance of known and putative pathogens (e.g. *Aquimarina* sp. (healthy: 27%, diseased: 55%), *Phaeobacter gallaeciensis*) increased double roughly during the disease progression (Zozaya-Valdes et al., 2015; Kumar et al., 2016; Zozaya-Valdes et al., 2017). Further, it has been suggested that beneficial bacteria can impede this bacterial shift and thus reduce bleaching rates (Longford et al., 2019; Li et al., 2021). Similar correlation of microbiome change and bleaching disease was also found in the habitat forming kelp *Ecklonia radiata* (Marzinelli et al., 2015). These studies indicate that bleaching disease in seaweeds is generally accompanied by host-associated microbiome shifts.

Microbial communities are often characterized by keystone taxa, which drive community composition and function irrespective of their abundance (Banerjee et al., 2018). Microbial co-occurrence network analysis is a powerful analytical tool for identifying the keystone taxa (i.e. highly connected taxa within the community) and has been used for human (Shetty et al., 2017; Banerjee et al., 2018), plant (Agler et al., 2016; Weiss et al., 2016) and shrimp (Dai et al., 2020) studies. For example keystone taxa found in the gut of diseased shrimp have been identified as candidate pathogens across multiple studies (Banerjee et al., 2018; Dai et al., 2018; Dai et al., 2020). Similar approaches have also been applied to identify keystone taxa associated with the gut microbiota of healthy shrimp with the potential to be developed into novel probiotics (Dai et al., 2018; Xiong, 2018). However, to date, whether there exist keystone taxa and how they change with the disease progression in seaweeds has not been investigated.

Saccharina japonica is one of the most important commercially farmed seaweeds worldwide. Like land crops, *S. japonica* has continued to be under threat from various diseases at both nursery and field cultivation stages since the successful implement of scale-cultivation in 1958 in China (Gachon et al., 2010; Wang et al., 2014). In some severe cases, diseases can cause a 20 - 50% reduction in yield volume (Gachon et al., 2010). In addition to previously characterized diseases (such as rotten hole disease (Wang et al., 2008; Zhang R. et al., 2020) and green rotten disease (Li et al., 2020), a new bleaching disease has recently been described to occur during the late nursery stage of *S. japonica* (Zhang et al., 2022a). Previous studies have observed differences in the microbial communities associated with healthy and diseased individuals (Li et al., 2020; Zhang R. et al., 2020). Moreover, laboratory assays have shown that this disease is in part due to an infection with the bacterium *Pseudoalteromonas piscicida* X-8 (PpX-8) (Zhang et al., 2022a). However, nothing is known about the interaction of the pathogen with the natural

epimicrobiome of *S. japonica* or if and how the host epimicrobiome changes during an infection. Given the importance of the microbiome in maintaining the health status in a range of host systems, the main objectives of this study were to use 16S rRNA gene amplicon sequencing to (a) assess the dynamic changes in the *S. japonica* epimicrobiome during disease progression after infection with PpX-8 and (b) identify potential health associated bacteria and putatively pathogenic bacteria by analysis of indicator species and keystone taxa associated with farmed *S. japonica*. Our results will serve as a basis for developing disease diagnosis and therapies for the nursery farms of *S. japonica*.

MATERIALS AND METHODS

Sample Collection

Healthy juvenile sporophytes (10 - 15 cm in length) of *Saccharina japonica* were freshly collected from Weihai Changqing Ocean Science & Technology Co., Ltd, located in Rongcheng, Shandong province, China (37°19' 41" N, 122°17' 21" E), on 24th December 2020. Samples were shipped in sterile sealed bag with ice packs within 4 h to the laboratory. After the samples arrived at the laboratory, juvenile sporophytes were immediately washed with sterile seawater and then were cultured in the incubator (10 ± 1°C, 80 μE m⁻² s⁻¹, light: dark= 12 h: 12 h).

Infection Assay and Epibacterial Collection

The pathogen *Pseudoalteromonas piscicida* X-8 (PpX-8) (Zhang et al., 2022a) was routinely cultured on the Zobell 2216E marine agar plates at 25°C. For infection assays, a single colony was inoculated into 100 mL Zobell 2216E medium and incubated shaking for 8 h at 25°C. Thereafter 1mL was subcultured into 100 mL of fresh medium until the optical density (OD₆₀₀) reached 0.5 (bacterial density: 1.0 × 10⁸ CFU/mL). PpX-8 cultures were centrifuged at 2000 g for 15 min and the cell pellet was rinsed at least twice with sterilized seawater and were resuspended in an equal volume of sterilized seawater.

Forty eight healthy juvenile sporophytes were washed with sterilized seawater at least three times. Six small tissue pieces (3.0 × 3.0 cm) were cut from each sporophyte with a sterile surgical blade resulting in a total of 288 tissue pieces. Each independent biological replicate consisted of six tissue pieces randomly assigned into each well of a 6 - well cell culture plates (USA). There were 8 replicates (each replicate included six tissue pieces) for both control and infected groups at each time point, resulting in a total of 48 replicate samples (i.e. 8 biological replicates × 2 treatments × 3 time points, **Figure S1**). Individual wells were then inoculated with either 1 mL of the PpX-8 suspension (described above) or 1 mL of sterile seawater as a control (C). The plates were incubated at 10 ± 1°C, 80 μE m⁻² s⁻¹ (light: dark= 12 h:12 h) for 4 h, 14 h and 24 h, respectively. Tissue pieces were rinsed with sterilized seawater at least three times to remove PpX-8 before collecting epiphytic bacteria. Epiphytic bacteria were sampled by swabbing tissue surface with sterile cotton swab. Three sampling time points (4 h, 14 h and 24 h) were designed

for both control (C) and PpX-8 infected groups (X-8). The collected epiphytic bacteria were kept frozen at -80°C until DNA extraction. Microscopic images were taken at each sampling time to record the disease symptoms under microscope (Nikon, Japan).

DNA Extraction, Amplification, and Sequencing of the 16S rRNA Gene Amplicon Sequencing

DNA of epiphytic bacteria was extracted using the HiPure Soil DNA Kits (Magen, Guangzhou, China) according to manufacturer's protocols. The V3-V4 hypervariable regions of the bacterial 16S rRNA gene were amplified with the primers 341F (5' -CCTACGGGNGGCWGCAG-3') and 806R (5' -GGACTACHVGGGTATCTAAT-3'). PCR reactions were performed in 50 μL mixture contained 100 ng template DNA, 10 \times Buffer KOD (5 μL), dNTPs (5 μL , 2 mM), primers 341F and 806R (1.5 μL of each, 10 μM), KOD DNA Polymerase (Toyobo, Japan). PCR cycling conditions were as follows: 94°C for 2 minutes, 30 cycles of 98°C for 10 seconds, 62°C for 30 seconds, 68°C for 30 seconds and final extension for 5 minutes at 68°C . Amplicons were extracted from 2% agarose gels using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer's instructions and quantified using ABI StepOnePlus Real-Time PCR System (Life Technologies, Foster City, USA). Addition of sequencing adapters, the qualified amplicons was then sequenced on the Illumina Novaseq6000 platform to generate 2×250 bp paired-end reads by Guangzhou Genedenovo Biotechnology (Guangzhou, China).

Analyses of Illumina Sequencing Data

The raw reads were first filtered to remove low-quality reads (containing more than 10% of unknown nucleotides) using FASTP. The paired end clean reads were merged as raw tags using FLSAH 1.2.11 with a minimum overlap of 10 bps and mismatch error rates were less than 2%. After raw tags filtering, the clean tags were clustered into operational taxonomic units (OTUs) of $\geq 97\%$ similarity using UPARSE (Edgar and Robert, 2013). The tag sequence with highest abundance was selected as representative sequence within each cluster. The representative OTU sequences were classified into organisms by a naive Bayesian model using RDP classifier 2.2 (Wang et al., 2007) based on SILVA database (v132) (Pruesse et al., 2007) with the confidence threshold values ranging from 0.8 to 1. On the basis of this classification, sequences of chloroplast, mitochondria and archaea origin were removed from the dataset. The raw reads were deposited into the NCBI Sequence Read Archive (SRA) database (Accession Number PRJNA775653).

Indicator Species Analysis

To identify the bacterial taxa that are indicative of the epibacterial communities associated with either control and infected groups, calculation of IndVal and associated tests was performed using the labdsv package (version2.0-1) in R project

(Roberts, 2016). The indicator species were selected at genus level with $p < 0.01$ and IndVal > 0.95 (Lu et al., 2020).

Association Network Analysis

Interspecies interactions in epimicrobial communities were assessed by the association network analysis. The association network was acquired using Pearson correlation coefficient from the OTUs abundance. The co-occurrence OTU pairs was selected if the Pearson correlation coefficient $|r| > 0.7$ and statistically significant at $P < 0.05$ using R psych package (Banerjee et al., 2018). Each network (three networks for control and three networks for the infected group) was constructed using all 8 replicates. For each group, putative keystone OTUs were defined according to previous studies (Banerjee et al., 2018; Gao et al., 2021) as those with degree centrality higher than 11 and closeness centrality greater than 0.6.

Shift of the Predicted Function of Epibacterial Communities Over Disease Progression

The KEGG (Kyoto Encyclopedia of Genes and Genomes) Pathway was performed using Tax4Fun software to predict the function of epibacterial communities based on the OTU abundance (Abhauer et al., 2015). Cluster analysis was conducted on the abundance of multiple KEGG functions, and the sample functional distribution was visually displayed with the heatmap (Pheatmap Package in R Project Version 1.0.12).

Statistical Analysis

The stacked bar plot of the community composition was visualized with ggplot2 package 2.2.1. Chao1 and Shannon index were calculated in QIIME and the alpha diversity comparison between groups was calculated by Welch's t-test. Canonical correspondence analysis (CCA) and permutational multivariate analysis of variance (PERMANOVA) were performed to evaluate the differences among sampling time points and infected treatment in the epibacteria based on Bray-Curtis metric using Vegan package 2.5.3.

RESULTS

Distribution of Epiphytic Bacterial Communities of Control and Infected Juvenile Sporophytes

Morphological images of both healthy juvenile sporophytes and tissue pieces of *S. japonica* were showed in **Figures 1A, B**. No bleaching symptoms were observed in the control group at 4 h (**Figure 1C**), 14 h (**Figure 1D**) and 24 h (**Figure 1E**). There was no significantly different in tissue color between control (**Figure 1C**) and PpX-8 infected tissues (**Figure 1F**) at 4 h. While, compared to the corresponding control at 14 h and 24 h, PpX-8 infected group showed moderate bleaching symptoms at 14 h (**Figure 1G**) and high levels of bleaching were observed at 24 h (**Figure 1H**).

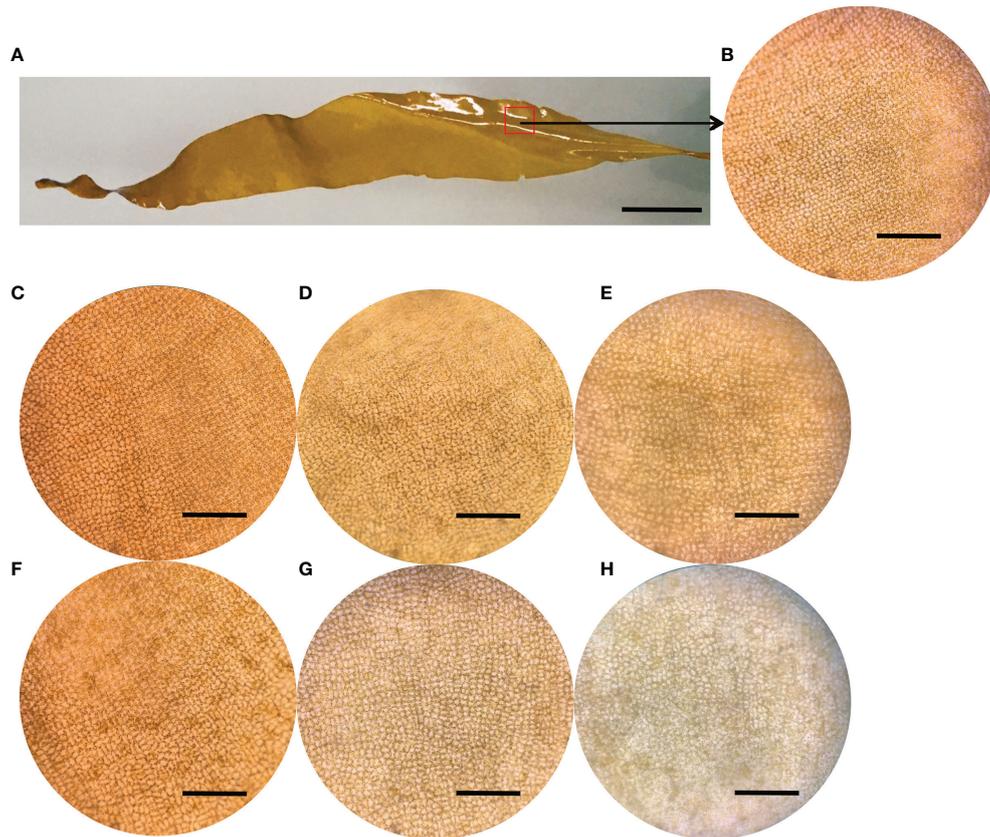


FIGURE 1 | The microscopic images of healthy juvenile sporophytes of *S. japonica* and the morphological images of tissue pieces in both control and PpX-8 infected groups. Control and infected tissue pieces were cultured under condition at $10 \pm 1^\circ\text{C}$ with the light intensity of $80 \mu\text{E m}^{-2} \text{s}^{-1}$ and the photoperiod of 12 h: 12 h (L: D). **(A)** healthy juvenile sporophytes; **(B)**, tissue of healthy juvenile sporophytes; **(C–E)** control group at 4, 14 and 24 h, respectively; **(F)** infected group at 4 h; **(G)** infected group at 14 h with moderate bleaching symptom; **(H)** infected group at 24 h with high level of bleaching. Bars, a: 1.2 cm; b-h: 400 μm .

A total of 6,088,736 reads, with an average of $126,849 \pm 5574$ (mean \pm standard deviation) reads per sample, were obtained from the 48 samples. Overall, 11,397 OTUs were assigned across all the samples based on the SILVA database at the cut-off level of 97%. Control group harbored a higher number of OTUs than the PpX-8 infected group. Rarefaction analysis indicated that the sequencing effort reached saturation all samples (see **Figure S2**) and this further supported Good's coverage estimates of greater than 98% for all samples. The obtained OTUs were classified into 30 phyla, 66 classes, 131 orders, 203 families, 330 genera.

Regarding alpha diversity, Shannon indices were significantly higher in the control group than in the infected group at 4 h, 14 h and 24 h (*t*-test, $p < 0.001$). Species richness estimates (Chao 1) were significantly higher in the control group than in infected group at 14 h and 24 h (*t*-test, $p < 0.01$). In addition, diversity (Shannon index) gradually increased from 4 h to 24 h, whereas the richness (Chao 1) decreased gradually across the time courses in the infected group (**Table 1**).

The most dominant phylum was Proteobacteria in both control (87.14%) and infected groups (99.70%). Other major phyla including Verrucomicrobia (4.79%), Planctomycetes

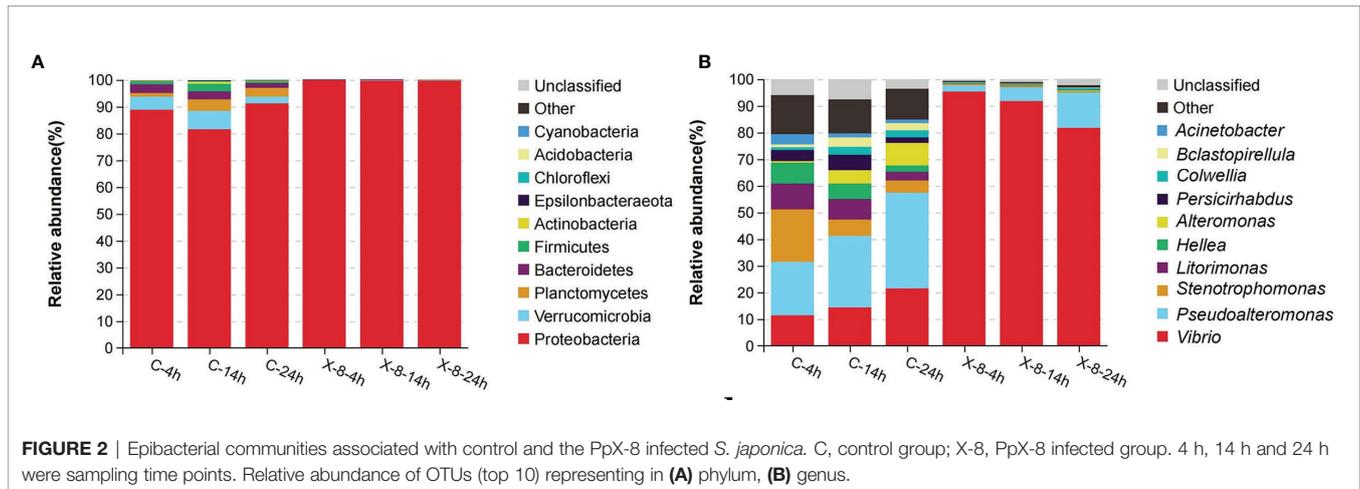
(2.94%) and Bacteroidetes (2.72%) in control group (**Figure 2A**). *Pseudoalteromonas* and *Vibrio* were the most abundant genera in both control and infected groups. In particular, the relative abundance of *Pseudoalteromonas* increased gradually in both control (ranging from 19.94% to 35.88%) and infected groups (ranging from 2.45% to 13.22%) over the time. This included sequences belonging to

TABLE 1 | Alpha diversity of the epibacterial communities associated with control and the infected groups at each time point.

Sampling time	Shannon		<i>P</i>	Chao1		<i>P</i>
	Control	X-8		Control	X-8	
4 h	3.99 \pm 0.39	0.92 \pm 0.09	< 0.001	295.86 \pm 66.85	289.10 \pm 56.53	0.830
	4.50 \pm 0.52	1.20 \pm 0.09		434.24 \pm 94.71	274.72 \pm 70.24	
24 h	4.09 \pm 0.93	1.89 \pm 0.32	< 0.001	329.29 \pm 85.96	212.98 \pm 35.70	0.006

X-8, PpX-8infected group.

Bold values indicate significant difference.



Pseudoalteromonas strain PpX-8, which represented 0.8% and 0.2% of the community in the control and infected groups, respectively. In contrast, the relative abundance of *Vibrio* showed the high but slightly decreasing overtime (from 95.34% to 81.68%) in the infected group (Figure 2B).

The Shifts of Epibacterial Community Structure Associated With the Juvenile Sporophytes of *S. japonica* Infected by PpX-8

A canonical correspondence analysis (CCA) biplot showed that there was no distinct separation over time in control and infected group, whereas infected treatment significantly affected the structures of the epibacterial communities (Figure 3). Further,

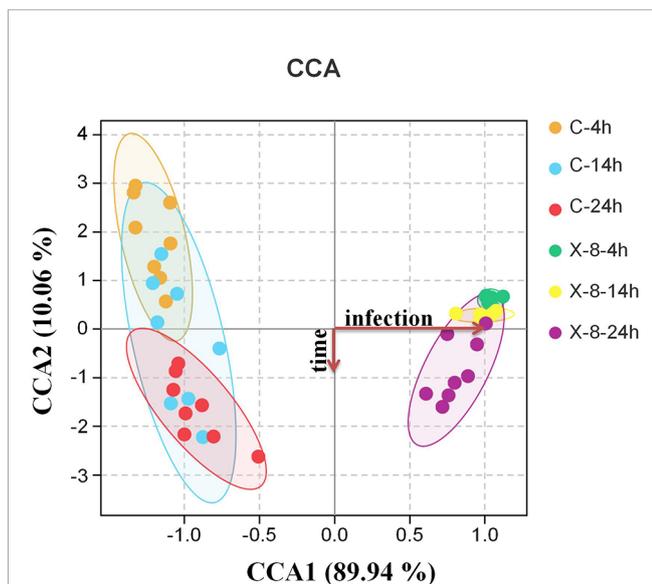


FIGURE 3 | Canonical correspondence analysis (CCA) biplot of epibacterial communities associated with control and infected groups at each time point based on Bray-Curtis distance. C, control group; X-8, PpX-8 infected group. 4 h, 14 h and 24 h were sampling time points.

a PERMANOVA revealed that sampling time, infected treatment and their interaction contributed 0.2328, 0.6164 and 0.716 variation in the epibacterial communities of *S. japonica*, respectively (Table 2). By pairwise comparisons, the distance of epibacterial community structure between control group and infected group was decreased gradually over disease progression (Table 3).

Indicator analysis showed indicator species at each time point to reflect the impact of PpX-8 on epibacterial dynamics during the disease progression (Figure 4). With respected to infected group, *Stenotrophomonas*, *Persicirhabdus*, *Acinetobacter*, *Pseudomonas* and *Sphingomonas* were indicator species at 4 h, 14 h and 24 h at control group. Specially, the relative abundance of *Stenotrophomonas*, *Pseudomonas* and *Sphingomonas* was significantly higher in control group (*t*-test, $p < 0.05$) than those in infected group (the average relative abundance was between 0.03% and 0.3%). These three genera are thought to be positively associated with healthy *S. japonica* (Figure S3). While, for the PpX-8 infected group, *Vibrio* was the indicator species at 4 h, 14 h and 24 h.

Association Network of Epibacterial Communities

To determine the effects of PpX-8 infection on the interaction between epiphytic bacterial members, we performed an association network analysis and found distinct networks according to sample type (Figure 5). The number of edges in the networks increased over time in both control and infected groups (Figure 5), with less total number of edges in the infected group. Whereas, the number of negatively correlated edges in the infected group (28-91) was all more than those in the control

TABLE 2 | The effects of sampling time and PpX-8 on variation in community composition using permutational multivariate analysis of variance (PERMANOVA) with adonis based on Bray-Curtis.

	Df	SumsOfSqs	MeanSqs	F value	R ²	P value
Infection	2	0.1403	0.0701	16.8747	0.6164	0.001
Time	2	0.793	0.3965	3.1861	0.2328	0.008
Infection: Time	5	6.8078	1.3616	21.1745	0.716	0.001

TABLE 3 | Pairwise comparison using permutational multivariate analysis of variance (PERMANOVA) with adonis based on Bray–Curtis.

	F value	R ²	P value	Significant
CK-4h-vs-X-8-4h	42.0407	0.7502	0.001	**
CK-14h-vs-X-8-14h	28.4562	0.6702	0.001	**
CK-24h-vs-X-8-24h	25.6919	0.6473	0.001	**

C, control group; X-8, PpX-8 infected group. 4 h, 14 h and 24 h were sampling time points. **extremely significant.

group (12–71) at each sampling time points. The average clustering coefficient increased with time, suggesting that the more complexity and higher connectivity of network (Table S1).

Putative keystone taxa were identified based on the highest degree and highest closeness centrality scores (Banerjee et al., 2018; Dai et al., 2019; Gao et al., 2021). For each network, OTUs with degree > 11, closeness centrality > 0.6 were selected as the putative keystone taxa. Firstly, there were a total of 24 and 25 keystone taxa in the control and in the infected networks, respectively (Table S2). There were some changes in putative keystone taxa with time in both control and infected groups, suggesting that PpX-8 infection greatly affected the structure of epibacterial community. The putative keystone taxa in the control group included 11 genera, which mainly belonged to family Alteromonadaceae and accounted for 62.5%. In contrast, predicted keystone taxa of the infected group contained 10 genera, among which belonged to family Alteromonadaceae and Hyphomonadaceae, each accounting for 28% respectively, while, Pseudoalteromonadaceae accounted for 24%. Interestingly, *Vibrio*, *Colwellia* and *Pseudoalteromonas* were the common putative keystone taxa at 24 h in both control and infected groups (Table S2), they were also indicator species for the infected group at 24 h (Figure 4). In addition, *Hellea* and *Litorimonas* became keystone taxa at 14 h and 24 h in the infected group, whereas were not found in the control group

(Table S2). Therefore, these two taxa may play a role during the progression of bleaching disease.

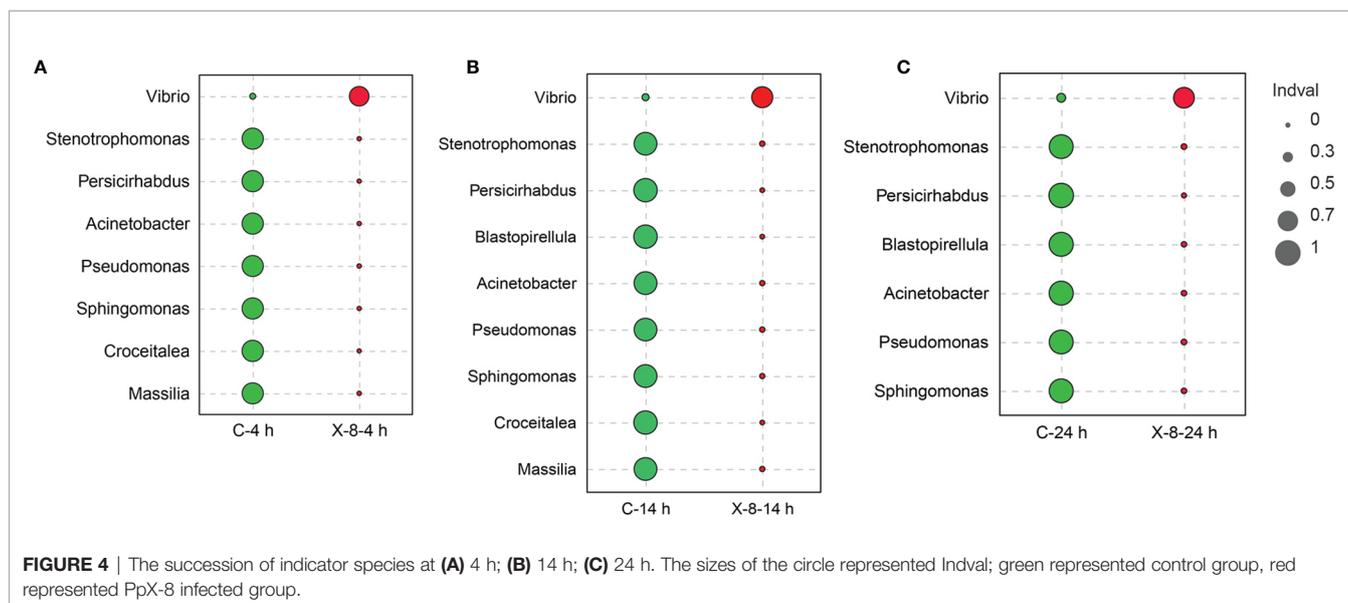
To assess the importance of these predicted keystone taxa, we performed a co-occurrence network analysis in which these were removed. The results demonstrate that the absence of keystone taxa leads to fragmentation of the network (Figure S4), which indicated that these keystone taxa were important for maintaining the balance of bacterial communities of *S. japonica*.

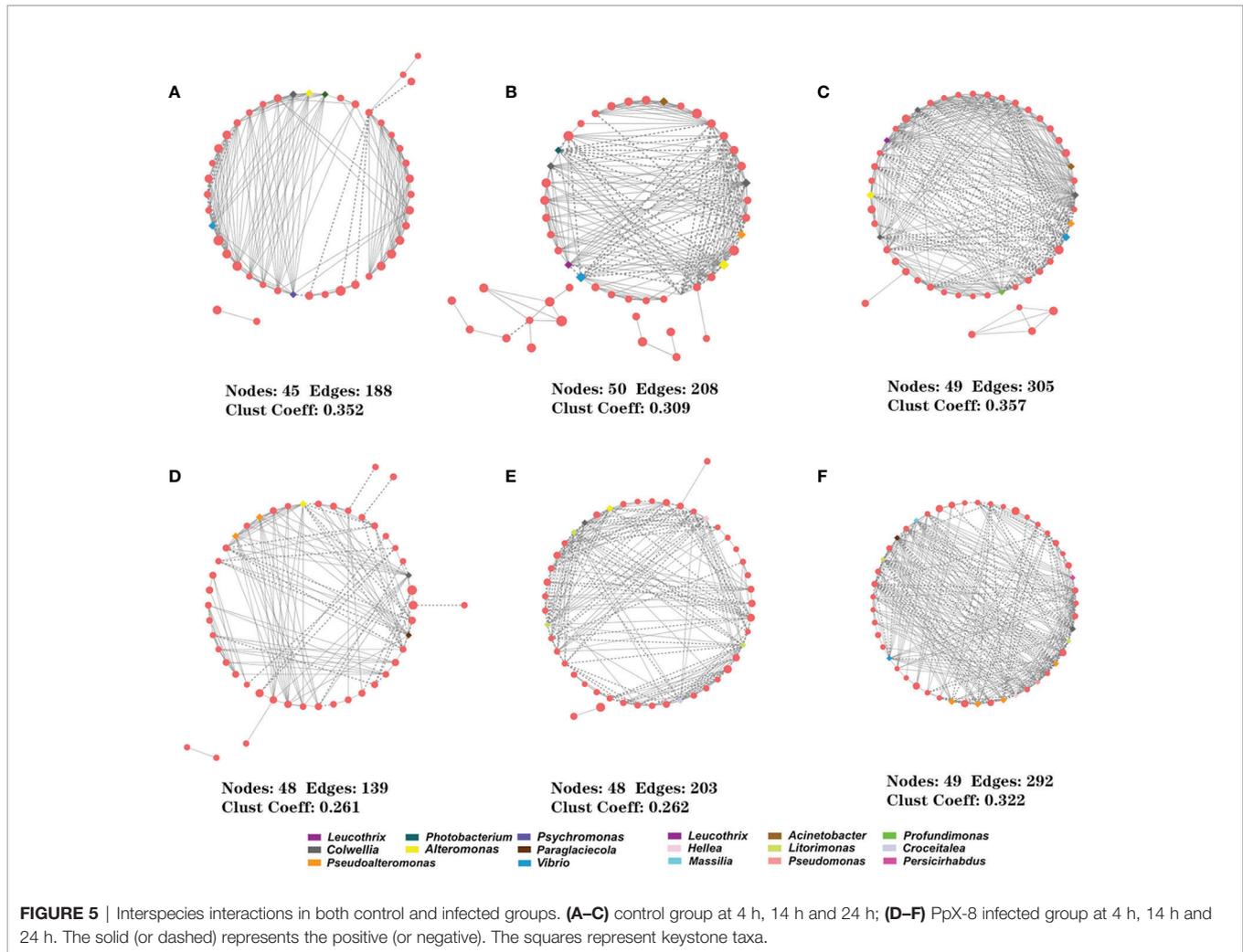
The Predicted Gene Functions of Epibacterial Communities After PpX-8 Infection

The functional changes of epibacterial communities in both control and infected groups were predicted using Tax4Fun in Figure 6. Among them, the abundance of 9 pathways in infected group was significantly higher than in control group (*t*-test, *p* < 0.05), including metabolism (carbohydrate metabolism, lipid metabolism, glycan biosynthesis and metabolism, metabolism of terpenoids and polyketides), environmental information processing (membrane transport, signal transduction and molecules and interaction), infectious diseases and cell communication. Twelve pathways were significantly enriched in the control group (*t*-test, *p* < 0.05), and included metabolism (energy metabolism, amino acid metabolism, metabolism of cofactors and vitamins and biosynthesis of other secondary metabolites), genetic information processing (translation, replication and repair, folding, sorting and degradation, transcription), cellular processes (cell motility, growth and death) and environmental adaption (Figure 6).

DISCUSSION

In this paper, we assessed the diversity of epibacterial communities during bleaching disease progression in farmed



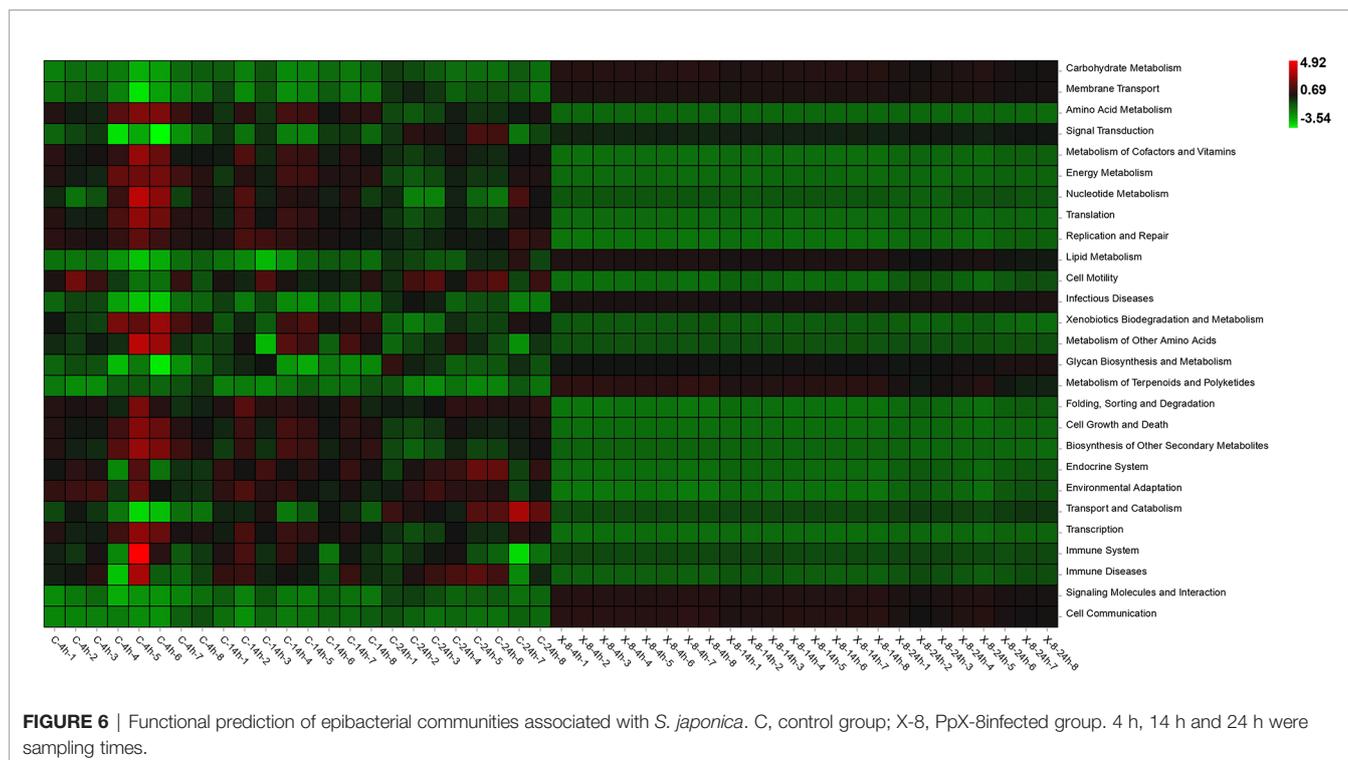


S. japonica after inoculation with the pathogenic bacterium - PpX-8. We observed a significant temporal shift in epibacterial communities specific to both the control and infected *S. japonica* groups. In addition, the healthy associated microbes in the control group - *Stenotrophomonas*, *Pseudomonas* and *Sphingomonas* and potentially opportunistic pathogens in the infected group - *Vibrio*, *Pseudoalteromonas* and *Colwellia* all suggest that the bleaching disease of *S. japonica* may be linked to an imbalance of the epibacterial communities. The imbalance could cause the bleaching disease symptoms and provide the opportunity for one or multiple secondary colonizers to exacerbate disease symptoms. To our knowledge, this is the first characterization of the epibacterial shifts during the bleaching disease progression in farmed seaweeds.

Usually, diverse microbial communities function as highly stable and resilient for the health of the host (Rungrasamee et al., 2016; Longford et al., 2019) and can provide colonization resistance against the invading pathogens and perturbation (Loreau et al., 2001; Girvan et al., 2005; De Schryver and Vadstein, 2014). Compared to the control group, we found that the richness and diversity of epibacterial communities was

significantly decreased after treatment with the algal pathogen PpX-8. This decrease in diversity was likely due to the sharp increase in the relative abundance of the most two dominant genera *Vibrio* and *Pseudoalteromonas* in the infected group (**Figure 2B**). Similar decreases in bacterial diversity after infection with a pathogen and/or in diseased hosts compared to healthy counterparts have been seen in both seaweeds (Zozaya-Valdes et al., 2015; Kumar et al., 2016; Longford et al., 2019), corals (Damjanovic et al., 2019) and shrimp (Huang et al., 2016; Rungrasamee et al., 2016; Xiong, 2018; Dai et al., 2019) and maybe an early indicator of poor host health.

We identified specific bacterial taxa that were indicative of both control and infected epibacterial communities. These indicator species included taxa belonging to *Stenotrophomonas*, *Pseudomonas* and *Sphingomonas*, which were in high abundance and decreased over the time in the control group. Therefore, these three genera are thought to be associated with healthy *S. japonica*. *Stenotrophomonas* is ubiquitously present in the environment, plants and soil etc. (Ryan et al., 2009) and engages in beneficial interactions with plants (Vilchis-Carmona et al., 2021). For example, *Stenotrophomonas* can produce factors



which promote plant growth, and secrete antibacterial compounds protecting plants from damage (Ryan et al., 2009; Elufisan et al., 2020). *Pseudomonas*, especially *Pseudomonas putida*, can protect plants against phytopathogens because of their type VI secretion systems (T6SS) (Bernal et al., 2017). *Sphingomonas* are environmentally friendly bacteria and can degrade various hazardous organic compounds (Zhang et al., 2021) and enhance the growth of microalgal strain *Oocystis* sp. KNUA044 (Na et al., 2021). In addition, *Sphingomonas* are often found in soils and have been identified as beneficial microbes in crops, such as rice and tomato, degrading toxic substances and directly reducing pathogen population (Chen et al., 2021; Cheng et al., 2021; Choi et al., 2021; Feng et al., 2021). Based on the beneficial functions of the above three taxa, we speculate that these taxa may play a similarly beneficial role for maintaining the health of farmed *S. japonica*. Moreover, the results in this study are in part similar to our previous studies, in which Xanthomonadaceae (*Stenotrophomonas*) were enriched in healthy *S. japonica* (Zhang et al., 2020; Han et al., 2021). However, the specific beneficial effects of these three taxa on *S. japonica* need to be further elucidated.

Compared to the control group, the shifts in microbiome composition occurred as early as at 4 h after PpX-8 infection in the infected group. Both *Vibrio* and *Pseudoalteromonas* became the most dominant two genera and their relative abundance reached more than 90% (Figure 2B). *Vibrio* has been identified as the pathogenic bacteria for shrimp white feces syndrome (Lu et al., 2020), coral bleaching (Ben-Haim and Rosenberg, 2002; Rosenberg and Falkovitz, 2004), mariculture fish (Zhang X-H. et al., 2020), rotten hole disease of *Laminaria japonica*

(synonymous of *Saccharina japonica*) (Wang et al., 2008) and yellow spot disease of *Pyropia* (Yang et al., 2020; Zhu et al., 2021). Therefore, it seemed that *Vibrio* was one of the secondary opportunistic pathogens in this study.

Pseudoalteromonas and *Colwellia* were keystone species at 24 h in the infected group. Moreover, the relative abundance of *Pseudoalteromonas* and *Colwellia* increased during the infection period. Previous studies have shown that *Pseudoalteromonas* and *Colwellia* seaweed isolates are capable of agar degradation (Sánchez Hinojosa et al., 2018), with some *Pseudoalteromonas* producing cold-adapted hydrolytic enzymes and exhibiting high agar-hydrolyzing activity, even at low temperatures (Hollants et al., 2013; Tropeano et al., 2013; Ivanova et al., 2014; Borchert et al., 2017; Sánchez Hinojosa et al., 2018). Other *Pseudoalteromonas* isolates have been identified as causative agents of disease of *Laminaria japonica* (Sawabe et al., 1998) and *S. japonica* (Zhang et al., 2022). Similarly, isolates of *Colwellia* have the ability to digest agar and other algal phycocolloids (Martin et al., 2015; Xu et al., 2017; Christiansen et al., 2018). Higher abundance of *Colwellia* in the infected group is also consistent with that of bleaching diseased *D. pulchra* (Egan et al., 2013). Given the high relative abundance of these *Vibrio*, *Pseudoalteromonas* and *Colwellia* in the infected group, together with previous reports of virulence traits associated with related strains, it is possible that these taxa represent secondary opportunistic pathogens which may act to hasten disease development in *S. japonica*. In addition, the *Litorimonas* and *Hellea* were keystone taxa in infected group at 14 h and 24 h. The genus *Litorimonas*, created by Jung et al. (2011), was considered as opportunistic pathogen of green rotten disease in *S. japonica*

because of their relative abundance in diseased samples increased 7.5 times (Li et al., 2020). *Hellea* was generally found in seaweed, and the relative abundance in diseased *S. japonica* and *D. pulchra* was significantly higher than healthy ones (Zozaya-Valdes et al., 2017; Zhang et al., 2020). Thus, we speculated that *Litorimonas* and *Hellea* may play an important role in the progression of disease. However, further work including isolation and virulence characterization of these abundant strains will be required to determine their importance in the progression of bleaching disease in farmed *S. japonica*. Compared with the control group, predicted functions of epibacteria in infected group changed significantly as early as 4 h after PpX-8 infection. During the course of 24 h infection, immune system of the epibacterial community in the control group was more abundant than that in the infected group. Immune system has been found to be associated with the gut microbiome of healthy shrimp (Lu et al., 2020) and may prevent the invasion of opportunistic pathogens. Therefore, we speculate that KEGG pathways of immune system of epimicrobiome in control group may also maintain the health of *S. japonica*. While, KEGG pathways of signaling molecules interaction and cell communication were significantly abundant in the infected group. These pathways have been found to be closely related to disease, particularly cell communication can increase the virulence of opportunistic bacteria (Chadha et al., 2021) and were reported to be present in algal pathogens of Roseobacters (Wagner-Dobler et al., 2005; Case et al., 2011). The potential enrichment of these pathways within the epibacterial communities of the infected group may facilitate the progression of bleaching disease after Pp-X-8 infection. In addition, infectious diseases were significantly more abundant in the infected group. The KEGG pathway of infectious diseases has been reported in white feces syndrome shrimp (Lu et al., 2020). An increase in this pathway may contribute to promote invasiveness of other opportunistic pathogens. Moreover, the enriched pathways associated with carbohydrate metabolism in the infected group may be due to the potentially secondary opportunistic pathogen (such as *Pseudoalteromonas* and *Colwellia*) with activity of digesting agar and other algal phycocolloids.

CONCLUSIONS

Considering the frequency and severity of the disease outbreaks at the nursery stage in farmed *S. japonica*, it is important and urgent to understand the mechanisms of disease pathogenesis and resistance. Our results add to the growing body of evidence that pathogenic bacteria-induced epibacterial shifts during the

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disease progression. Bacterial taxa associated with healthy seaweed and the secondary opportunistic pathogens identified in this study enrich our baseline knowledge of the biology of farmed seaweeds and pave the way for the application of beneficial microbiota to protect against disease in seaweed farming industries.

DATA AVAILABILITY STATEMENT

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/**Supplementary Material**.

AUTHOR CONTRIBUTIONS

FL and GW conceived and designed the experiments and wrote the manuscript. FL finished the experiments. SE participated sequencing analysis and English writing. YZ helped the analysis of sequencing data. LC and LX provided the samples of *Saccharina japonica*. QY involved in designing the experiments. All authors contributed to the article and approved the submitted version.

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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.2022.865224/full#supplementary-material>

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