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Impact of Abiotic and Biotic Factors on the Growth of Betaphycus gelatinus and Diversity Analysis of Its Associated Epiphytic and Endophytic Microbiomes

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Impact of Abiotic and Biotic Factors on the Growth of *Betaphycus gelatinus* and Diversity Analysis of Its Associated Epiphytic and Endophytic Microbiomes

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Abstract *Betaphycus gelatinus* is a crucial carrageenophyte grown in tropical and subtropical coastal ecosystems. However, the environmental factors influencing its growth and the microbial community changes linked to its health status remain unclear. This study assesses the impact of selected abiotic conditions and seawater treatment on *B. gelatinus* growth using orthogonal experimental design. We characterized epiphytic, surface-sterilized tissue-associated bacterial communities, and ITS2-derived epiphytic eukaryotic communities in marine-cultured, bleached, and indoor-cultured thalli through amplicon sequencing. The growth experiments indicated significant associations between phosphate, iron, and seawater treatment and *B. gelatinus* growth, highlighting the combined effects of nutrient conditions and seawater-related factors on the richness and evenness of its microbial communities. Microbial community analysis revealed distinct host-condition-associated shifts. Marine-cultured thalli displayed higher bacterial richness but lower evenness, while bleached thalli exhibited increased bacterial evenness and distinct enrichment of bleaching-associated taxa (e.g., *Ruegeria* and *Methylophaga*). Surface-sterilized tissue-associated bacterial communities varied among host conditions, with bleached thalli showing higher richness and evenness. ITS2-derived communities demonstrated significant taxonomic shifts, notably ciliate-related taxa's dominance in bleached samples, though functional differences were limited. Function prediction suggested that bacterial communities under different host conditions varied in metabolic potential, including terpenoid- and steroid-related pathways in marine-cultured samples, and amino acid and central carbon metabolism in bleached samples. These findings reveal

that *B. gelatinus* health status is intimately linked to the restructuring of host-associated microbial communities, providing an initial microbial ecological basis for understanding bleaching-associated community shifts and developing future health monitoring and cultivation strategies for *B. gelatinus*.

Keywords: *Betaphycus gelatinus*; abiotic factors; microbial communities; amplicon sequencing

Declarations

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Competing interests

The authors declare no competing interests.

Availability of data and material

The orthogonal experimental and sequencing data during the current study are publicly available in the Zenodo repository. [https://doi.org/10.5281/zenodo.20482417]

Code availability

No custom code was developed in this study.

Authors' contributions

Guohong Peng: Sample collection and cultivation, data curation, formal analysis, visualization, writing-original draft. Zhou Chen: Sample collection and testing. Jun Wang: Sample processing and guided experiment. Zhiyuan Liu: Conceptualization, methodology, supervision, project administration, article framework and revision.

Introduction

Macroalgal ecosystems are the largest and most productive of coastal vegetated ecosystems on Earth (Pessarrodona et al., 2022), making significant contributions to coastal primary productivity, habitat formation, and carbon sequestration (Krause-Jensen and Duarte, 2016). As crucial primary producers, macroalgae provide food, shelter, and habitat for a diverse array of marine organisms, thus supporting the structure and functioning of coastal ecosystems (Best et al., 2014; Gallardo et al., 2021; Tano et al., 2016). In addition to their ecological significance, many macroalgae are also economically valuable, serving as resources for food, hydrocolloids, pharmaceuticals, and the aquaculture industry.

Betaphycus gelatinus, a carrageenan-producing red alga of the Rhodophyta division, primarily inhabits tropical and subtropical regions, including Hainan and Taiwan Island in China, as well as the Philippines, Indonesia, Vietnam, and Japan. It is an important raw material for carrageenan production in industry (Tseng, 2001) and is cultivated in the southern coastal regions of China and Vietnam (Tran et al., 2025). However, observations from field cultivation indicate that *B. gelatinus* is highly sensitive to local environmental conditions. Significant variations in survival and growth rates across similar latitudes suggest a strong influence of local seawater conditions on the cultivation performance of *B. gelatinus*. Its growth is influenced by both abiotic and biotic factors. While previous studies have primarily addressed abiotic factors such as temperature, light, culture density, and nutrient concentrations (Han et al., 2025; Lideman, 2012; Tran et al., 2025), the role of microbial factors in its growth and health status is not yet well understood.

Increasing evidence supports the concept of macroalgae as holobionts, wherein the host and its associated microbial communities jointly contribute to the health, development, stress response, and disease susceptibility of the macroalgae (Wahl et al., 2012). The nutrient-rich thallus surface serves as ecological niches for diverse bacteria and fungi, which may affect host performance through the recycling of algal exudates, nutrient supply or the provision of growth-related compounds, production of substances that promote morphogenesis, and regulation of microbial interactions on the algal surface (Egan et al., 2013; Hollants et al., 2013; Wichard, 2015). Furthermore, some epiphytic bacteria produce substances that are similar to morphogenesis-promoting agents and other bioactive

compounds, which may support normal thallus development and the establishment of early life stages (Cortez et al., 2026; Mathew et al., 2026). Although less studied than bacteria, recent evidence suggests that fungal associates also contribute to metabolite production, microbial-network stability, and stress-related interactions within the holobiont (Arcela-Castro et al., 2026; Rousseau et al., 2025).

In addition to epiphytic microorganisms, tissue-associated or endophytic bacteria may also constitute a significant but underexplored component of macroalgal microbiomes. Previous studies have demonstrated that bacterial communities isolated from internal or surface-sterilized algal tissues can differ from both epiphytic communities and surrounding seawater communities (Hollants et al., 2011; Longford et al., 2007; Lachnit et al., 2009). These communities may be influenced by host-related factors, such as sex, reproductive structures, developmental stages, and health status (Cao et al., 2025; Xiang et al., 2026; Zhao et al., 2024). Functionally, macroalgal tissue-associated bacteria are indicated to contribute to host growth, regeneration, antimicrobial activity, pollutant degradation, salt tolerance, and stress resistance (Deutsch et al., 2023; Deutsch et al., 2021; Li et al., 2024; Singh et al., 2011). These findings suggest that both epiphytic and tissue-associated microbial communities are closely linked to macroalgal performance and health.

Macroalgal microbiomes are dynamic entities influenced by various factors, including host identity, environmental conditions, and seasonal variations (Aires et al., 2016; Lachnit et al., 2011; Roth-Schulze et al., 2016). In favorable environments, the host-associated microbiota may contribute to the stability of the holobiont. Conversely, environmental stress can disrupt microbial community structures, leading to dysbiosis. This disruption may precipitate opportunistic colonization, tissue degradation, and decline in photosynthetic efficiency, thereby fostering disease in macroalgae (Murúa et al., 2023; Spagnuolo and Genovese, 2024; Ward et al., 2020; Zozaya-Valdes et al., 2015). For instance, the bacterium *Nautella* sp. R11 has been identified as a pathogen linked to the bleaching disease in the red alga *Delisea pulchra* (Fernandes et al., 2011). Similarly, the ice-ice disease observed in carrageenophytes, such as *Kappaphycus* spp. and *Eucheuma* spp., is likely caused by a combination of abiotic stressors and opportunistic bacteria such as *Vibrio*, *Bacillus*, and other associated taxa (Riyaz et al., 2019; Tahiluddin and Terzi, 2021). Further research into *Saccharina japonica* has revealed that bleaching disease

correlates with shifts in the structure of epiphytic bacterial communities and an enrichment of potential opportunistic taxa such as *Vibrio*, *Pseudoalteromonas*, and *Colwellia* (Ling et al., 2022; Ma et al., 2023). This evidence indicates that restructuring of microbial communities is a significant indicator of deteriorating macroalgal health. Moreover, recent studies on *B. gelatinus* have demonstrated that bleaching is associated not only with tissue damage and loss of pigments but also with reduced photosynthetic capability and alterations in epiphytic bacterial communities (Ma et al., 2024).

Despite these advances, the growth requirements of *B. gelatinus* remain poorly understood, and the relationship between its health status and associated microbiota has not been thoroughly investigated. Specifically, there is limited knowledge about whether healthy, bleached, and indoor-cultured *B. gelatinus* host distinct epiphytic and tissue-associated microbial communities, or whether specific microbial taxa and predicted functional profiles are associated with different health conditions. Addressing these questions may offer preliminary insights into the microbial ecology of *B. gelatinus* and help identify potential microbial indicators or strains for future cultivation management. Accordingly, this study aimed to investigate the effects of selected abiotic conditions and seawater sterilization treatments on the growth of *B. gelatinus* and to characterize its associated microbial communities under varying health conditions. Initially, we examined the effects of phosphorus, nitrogen, iron, vitamins, trace elements, light, and seawater treatments using an orthogonal experimental design. Subsequently, amplicon analysis was utilized to compare the taxonomic diversity and the predicted functional potential of epiphytic bacteria, tissue-associated bacteria, and ITS2-derived surface-associated eukaryotic taxa among healthily marine-cultured, bleached, and indoor-cultured *B. gelatinus*. By associating growth responses with shifts in host-associated microbial communities, this study established a preliminary framework for understanding the potential role of microbial communities in the cultivation and health management of *B. gelatinus*.

Material and method

Sample collection and processing

The marine red algae species, *B. gelatinus*, was collected in April 2024 from a

seaweed cultivation region in Haiwei Town, Hainan Island, China. A total of 18 thalli were collected manually from cultivation ropes at a depth of 0.5 meters. We then categorized the samples into three groups based on their growth conditions and visible health statuses: (1) marine-cultured healthy thalli, which exhibited normal pigmentation without visible signs of bleaching or tissue damage; (2) bleached thalli, which displayed naturally occurring localized bleaching symptoms within the cultivation area; and (3) indoor-cultured thalli. The latter group comprised thalli initially collected from sea cultivation and subsequently transferred to indoor tank culture conditions, where a reduction in growth performance was observed. For each group, individual thalli were collected as biological replicates. Each sample was rinsed with seawater to remove loosely associated impurities, then stored in separate sterile plastic bags, placed on ice in a portable container, and transported to the laboratory. Seedlings from healthy *B. gelatinus* were incubated in a tank filled with natural seawater at 27°C, sourced from Changjiang, Hainan Island. Seedling growth was monitored by measuring and recording their fresh weight and length periodically. The relative growth rate (RGR, expressed as % day^{-1}) of *B. gelatinus* was calculated based on changes in fresh weight according to the following equation (Glenn and Doty, 1992):

$$\text{RGR (\% day}^{-1}) = \frac{(\ln Mt - \ln M0)}{\Delta t} \times 100 \quad (1)$$

where M_0 represents the initial fresh weight of *B. gelatinus* (g), M_t denotes the fresh weight after cultivation for Δt days (g), and Δt is the duration of cultivation in days.

Healthy marine-cultured algae demonstrated a relative growth rate (RGR) of 4.10% day^{-1}. In contrast, the bleached algae exhibited a negative RGR of -2.30% day^{-1}. The indoor-cultured algae, consisting of healthy *B. gelatinus*, were subsequently cultured in indoor tanks for three months, during which they maintained a stable, healthy condition with an RGR of 0.42% day^{-1}.

Assays on nutrients factors

An orthogonal L8(2)7 test design (Table 1) was employed to preliminarily assess the impact of selected abiotic and seawater-treatment factors on the growth of *B. gelatinus*. The experiment comprised seven factors: nitrogen source, phosphorus source, iron source, vitamins, trace elements, light intensity, and seawater treatment. These factors were chosen because nitrogen and phosphorus are crucial macronutrients for algal growth, pivotal to

protein synthesis, photosynthesis, and cellular metabolism. Iron, a key micronutrient, plays a critical role in photosynthetic electron transport and enzymatic reactions. Vitamins and trace elements are known to bolster physiological activities of algae and potentially affect growth performance under culture conditions. Light intensity is a direct determinant of photosynthesis and biomass accumulation, while seawater treatment was included to preliminarily determine whether the presence or absence of naturally occurring seawater microorganisms affects the growth of *B. gelatinus*.

The nutrient factors were aligned with the composition of Ningbo No. 3 liquid medium, widely recognized for microalgal cultivation and employed in preceding studies (Xiao et al., 2022). The formulated stock solutions included $NaNO_3$ as the nitrogen source, KH_2PO_4 as the phosphorus source, $FeSO_4 \cdot 7H_2O$ as the iron source, vitamins B_1 and B_{12} as vitamin supplements, and a trace-element mixture containing $ZnSO_4 \cdot 7H_2O$, Na_2MoO_4, $MnSO_4$, $CuSO_4 \cdot 5H_2O$, and Na_2EDTA. The concentrations of these stock solutions were maintained at 10 g/L for $NaNO_3$, 10 g/L for KH_2PO_4, 2.5 g/L for $FeSO_4 \cdot 7H_2O$, 6 mg/L for vitamin B_1, 50 mg/L for vitamin B_{12}, 0.23 g/L for $ZnSO_4 \cdot 7H_2O$, 0.073 g/L for Na_2MoO_4, 0.25 g/L for $MnSO_4$, 0.1 g/L for $CuSO_4 \cdot 5H_2O$, and 21.8 g/L for Na_2EDTA.

Each factor in the orthogonal design was tested at two levels: absence and addition of 0.1 mL of stock solution per 200 mL of culture medium. This gradient was chosen as a preliminary screening level based on the formulation of Ningbo No. 3 medium and aimed to explore the growth responses of *B. gelatinus* under both nutrient-deficient and nutrient-enriched conditions, rather than establishing a comprehensive dose-response relationship for each nutrient. For the factor of light, two intensities, 5200 lx and 8200 lx, were selected to represent moderate and relatively high conditions, suitable for indoor algal cultures. As for the seawater-treatment factor, a comparison of natural unsterilized seawater and sterilized seawater was conducted to evaluate the potential influences of naturally occurring seawater-associated microorganisms and seawater treatment on algal growth. It is important to recognize that sterilization may modify some physicochemical properties of the seawater, hence this factor was treated as indicative of seawater sterilization treatment rather than purely microbial influence.

Table 1. $L8(2)^7$ orthogonal design for screening the effects of nutrient

supplementation, light intensity, and seawater treatment on the growth of *B. gelatinus*.

Treatment No.	KH_2PO_4 (mL)	$NaNO_3$ (mL)	$FeSO_4 \cdot 7H_2O$ (mL)	Vitamins (mL)	Trace element (mL)	Light (lx)	Seawater
1	0	0	0	0	0	5200	Unsterilized
2	0	0	0	0.1	0.1	8200	Sterilized
3	0	0.1	0.1	0	0	8200	Sterilized
4	0	0.1	0.1	0.1	0.1	5200	Unsterilized
5	0.1	0	0.1	0	0.1	5200	Sterilized
6	0.1	0	0.1	0.1	0	8200	Unsterilized
7	0.1	0.1	0	0	0.1	8200	Unsterilized
8	0.1	0.1	0	0.1	0	5200	Sterilized

Microbes samples treatment

This study utilized samples of *B. gelatinus* from three different host conditions for microbial community analysis: (1) marine-cultured thalli, (2) bleached thalli, and (3) indoor-cultured thalli. For each condition, three independent biological replicates were prepared. Approximately 1 g of each fresh sample was placed into a sterile 50 mL centrifuge tube, which contained 10 mL of phosphate-buffered saline (PBS, 0.1M, pH 8.0). The procedures for the separation of epiphytic and endophytic microbes are detailed below.

Epiphytic microorganisms were isolated using a modified procedure based on Aires et al. (Aires et al., 2018). Each sample underwent ultrasonic washing for 1 minute and vortexing for 3 minutes, a process repeated twice to detach surface-associated microorganisms from the algal thallus. The resulting suspension was filtered through a 0.22 μm mixed cellulose esters filter membrane, then flash-frozen in liquid nitrogen and stored

at -80°C until the extraction of DNA and subsequent 16S rRNA and ITS2 amplicon sequencing. Epiphytic microbial samples were categorized into three treatments: marine-cultured (Mep), bleached (Bep), and indoor-cultured (Iep), with three replicates for each treatment.

Following the removal of the epiphytic microorganisms, tissue-associated microbial communities were harvested from surface-sterilized thallus tissues using a protocol modified from Beckers et al. (2017). Algal tissues were rinsed with sterile distilled water for 30 seconds and then cut into small fragments of approximately 2-3 cm under sterile conditions. These fragments were treated with 70% ethanol for 2 minutes, followed by a 2.5% sodium hypochlorite solution (containing 0.1% Tween 80) for 5 minutes, then immersed again in 70% ethanol for 30 seconds. The residual sodium hypochlorite and ethanol were removed by rinsing with sterile distilled water. The surface-sterilized samples were immediately flash-frozen in liquid nitrogen and stored at -80°C. Tissue-associated microbial samples from the marine-cultured, bleached, and indoor-cultured thalli were designated as Men, Ben, and Ien, respectively. Three replicates were established for each group. All samples were transported on dry ice for sequencing.

In this study, tissue-associated microbial communities are defined operationally as the microorganisms detected from the surface-sterilized thallus tissues. Due to the absence of independent verification of the surface sterilization effectiveness through culturing or sequencing of the final rinse solution, these communities should be considered as potentially surface-sterilized tissue-associated microorganisms, rather than definitively confirmed as endophytes.

DNA extraction, PCR amplification, and high-throughput sequencing

High-throughput sequencing was conducted by BioMarker Technologies. Total genomic DNA was extracted from each epiphytic and surface-sterilized tissue-associated microbial sample employing the CTAB method. The integrity of the DNA was assessed via 1% agarose gel electrophoresis, and the DNA concentration was normalized to 1 ng/μL using sterile water prior to PCR amplification. PCR amplification targeted the V3-V4 region of the bacterial 16S rRNA gene using primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′). Additionally, the ITS2 region was amplified with primers 2024F (5′-

GCATCGATGAAGAACGCAGC-3′) and 2409R (5′-TCCTCCGCTTATTGATATGC-3′). It is crucial to note that while the ITS2 primers predominantly target fungal communities, they may also amplify other eukaryotic taxa depending on primer specificity and database annotations. Consequently, ITS2-derived results were interpreted broadly as reflecting surface-associated eukaryotic taxa where appropriate. The PCR amplification was conducted in a reaction mixture containing 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 2 μM of each primer, and approximately 10 ng of template DNA. The amplification protocol included an initial denaturation at 98°C for 1 minute, followed by 30 cycles of denaturation at 98°C for 10 seconds, annealing at 50°C for 30 seconds, and extension at 72°C for 30 seconds, with a final extension at 72°C for 5 minutes. The PCR products were evaluated using 2% agarose gel electrophoresis in 1× TAE buffer. Qualified PCR products were pooled in equimolar amounts and purified using the Universal DNA Purification Kit (TianGen, China). Sequencing libraries were prepared employing the NEBNext® Ultra DNA Library Prep Kit for Illumina as per the manufacturer's instructions, and index codes were appended to differentiate the samples. Library quality was ascertained using an Agilent 5400 system. Subsequently, paired-end sequencing was executed on an Illumina platform, generating 250-bp paired-end reads.

Bioinformatic analysis

Bioinformatic analyses were conducted in QIIME2, guided by the QIIME2 "Atacama Soil Microbiome Tutorial" and supplemented with customized scripts. Raw FASTQ files were imported into QIIME2, where they underwent demultiplexing, quality filtering, trimming, denoising, merging, and chimera filtering utilizing DADA2, resulting in the production of amplicon sequence variant (ASV) feature tables (Callahan et al., 2016). Taxonomic classification was performed using the QIIME2 feature-classifier plugin against the Greengenes 13_8 99% database for 16S rRNA gene sequences and the UNITE+INSD 99% database for ITS sequences (Bokulich et al., 2018). Any contaminating mitochondrial and chloroplast sequences were excluded using the QIIME2 feature-table plugin. Alpha diversity indices, such as observed ASVs, Chao1, and Shannon, along with Bray-Curtis-based beta diversity, were calculated using QIIME2.

Data analysis

Growth data were analyzed using DPS software and are presented as the mean ± SE (Standard Error). A one-way analysis of variance (ANOVA) was utilized to compare differences in fresh weight and relative growth rate across treatments. When significant differences were detected, post hoc multiple comparison tests were conducted as appropriate. A P value of less than 0.05 was considered statistically significant.

Microbial community analyses were conducted using ASV tables generated from the QIIME2 pipeline. Alpha diversity indices, including observed ASVs, Chao1, and the Shannon index, were calculated to evaluate microbial richness and diversity. The Chao1 index estimates species richness, while the Shannon diversity index quantifies species diversity, encompassing both richness and evenness. Beta diversity was assessed using Bray-Curtis dissimilarity and visualized through principal coordinate analysis (PCoA) and non-metric multidimensional scaling (NMDS) (Vázquez-Baeza et al., 2013). Differences in community composition among treatments should be statistically tested using PERMANOVA where applicable.

Differentially abundant taxa were identified using ANCOM, ANOVA, Kruskal-Wallis, LEfSe, and DESeq2 (Segata et al., 2011; Love et al., 2014; Mandal et al., 2015). Co-occurrence relationships among predominant taxa were assessed by calculating Spearman's rank correlation analysis, and network plots were utilized to visualize potential associations among taxa. The potential functional profiles of microbial communities were predicted using PICRUSt2 (Langille et al., 2013), and predicted functional abundances were summarized at KEGG Orthology Level 3, focusing on individual metabolic pathways. Unless specified above, the parameters used in the analysis were set to defaults.

Results

Effect of environmental conditions on b. gelatinus growth

The growth of *B. gelatinus* was significantly influenced by the combined culture conditions in the orthogonal experiment ($p < 0.05$). Among the eight treatment combinations, treatments 1 and 2 exhibited relatively higher growth rates compared to the other treatments, whereas treatment 5 displayed the lowest growth rate (Fig. 1A). Orthogonal range and ANOVA analysis indicated that the seven factors varied in their

impact on algal growth (Fig. 1B). Notably, phosphate (PO_4^{3-}) supplementation, iron concentration (Fe^{3+}), and presence of microorganisms had significantly negative effects on the growth of *B. gelatinus* ($p < 0.05$), with phosphate (PO_4^{3-}) showing the strongest inhibitory effect. Conversely, vitamin supplementation and increased light intensity appeared to enhance growth, although these effects were not statistically significant. Nitrogen and trace element supplementation had relatively minor impacts under the conditions tested.

The results demonstrate that the growth of *B. gelatinus* is sensitive to both nutrient conditions and seawater treatment. Excessive phosphate and iron supplementation seemed to inhibit algal growth, whereas natural unsterilized seawater and moderate vitamin supplementation were more conducive to cultivation. These findings highlight that environmental conditions and seawater-associated factors collectively affect the physiological performance of *B. gelatinus*. This provides an experimental foundation for further analyses of host-associated microbial communities under different host conditions. From an economic perspective, an optimal growth treatment would involve minimal levels of phosphate (PO_4^{3-}) and iron ions (Fe^{3+}), natural (unsterilized) seawater, appropriate vitamin supplementation, and light intensities exceeding 5200 lx to promote the growth of *B. gelatinus*.

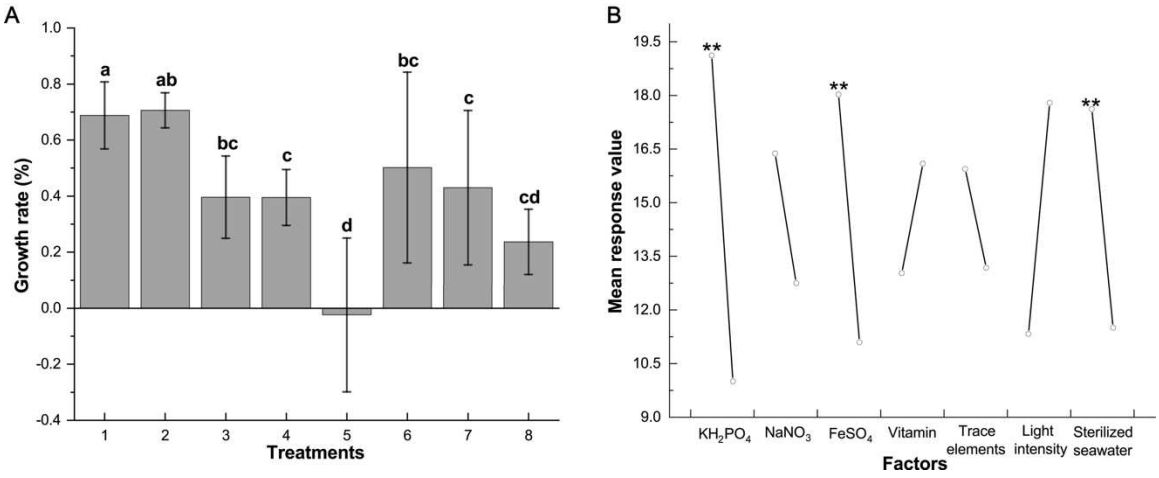


Fig. 1 Effects of the L8(2^7) orthogonal array design on the growth of *B. gelatinus*. (A) Growth rate under the eight treatment combinations. Different lowercase letters indicate significant differences. Error bars represent the standard deviation of replicates (n = 3). (B)

Main effects of the seven experimental factors on the relative growth rate of *B. gelatinus* based on orthogonal range analysis. ** indicates significant differences at $p < 0.05$

Host-condition-associated shifts in bacterial community structure

Diversity patterns of bacteria communities

A total of 1,265,760 high-quality 16S rRNA gene sequences were obtained from 18 bacterial community samples after quality filtering, with an average of 71,456 reads per sample. Across all treatments, 5,020 bacterial ASVs were identified and assigned to 32 phyla, 88 classes, 136 orders, 216 families, and 372 genera. These findings demonstrate that *B. gelatinus* harbored diverse bacterial assemblages under various host conditions.

Alpha-diversity analysis revealed distinct diversity patterns between epiphytic and tissue-associated bacterial communities across the three host conditions (Fig. 2). For epiphytic bacteria, the marine-cultured treatment exhibited the highest Chao1 richness (Fig. 2A), whereas the indoor-cultured treatment showed a significantly lower Chao1 index than the marine-cultured treatment ($p < 0.05$), indicating a reduction in epiphytic bacterial richness after transfer from sea cultivation to indoor culture. In contrast, the bleached treatment had the highest Shannon index and was significantly higher than both marine-cultured and indoor-cultured treatments ($p < 0.05$, Fig. 2B), suggesting that bleaching was associated with a marked change in epiphytic bacterial evenness and relative abundance distribution.

For tissue-associated bacteria, the bleached treatment exhibited higher Chao1 and Shannon indices than the marine-cultured and indoor-cultured treatments (Fig. 2 C–D). Although the differences in Chao1 richness among tissue-associated bacterial communities were not significant, the Shannon index of the bleached treatment was significantly higher than that of the marine-cultured treatment ($p < 0.05$), indicating increased evenness in bacteria detected from surface-sterilized tissues under bleaching-associated conditions. Compared with epiphytic communities, tissue-associated bacterial communities showed a more consistent increase in both richness and evenness in bleached thalli, whereas epiphytic communities displayed high richness in marine-cultured thalli but higher evenness in bleached thalli. Together, these results suggest that bleaching was associated with restructuring of both surface-associated and tissue-associated bacterial communities, while indoor cultivation mainly reduced the richness of epiphytic bacteria.

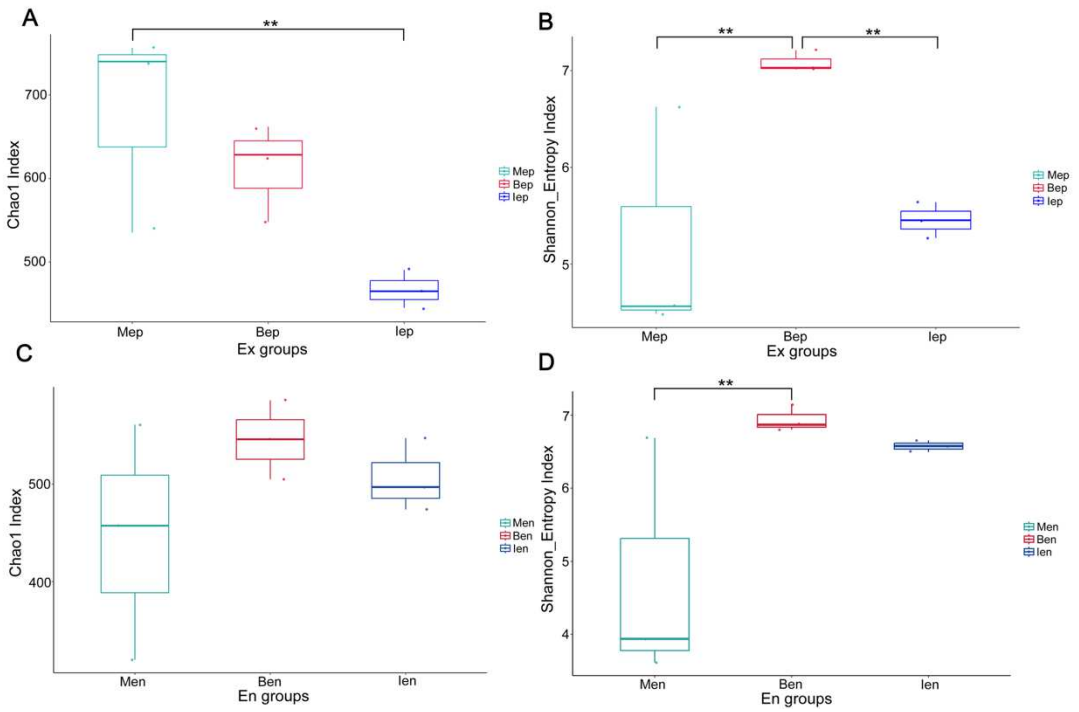


Fig. 2 Alpha diversity of epiphytic and tissue-associated bacterial communities associated with *B. gelatinus* under different host conditions. (A) Chao1 index of epiphytic bacteria. (B) Shannon index of tissue-associated bacteria. (C) Chao1 index of tissue-associated bacteria. (D) Shannon index of tissue-associated bacteria. Mep, Bep, and Iep represent epiphytic bacterial communities from marine-cultured, bleached, and indoor-cultured *B. gelatinus*, respectively. Men, Ben, and Ien represent tissue-associated bacterial communities detected from surface-sterilized tissues of marine-cultured, bleached, and indoor-cultured *B. gelatinus*, respectively. ** indicates significant differences at $p < 0.05$.

Beta-diversity analysis further delineated differences in both epiphytic and tissue-associated bacterial community composition across the three host conditions (Fig. 3A, C). For epiphytic bacteria, the PCoA based on Bray–Curtis dissimilarity explained 78.1% of the total variation, with the first and second axes accounting for 50.3% and 27.8%, respectively (Fig. 3A). Epiphytic bacterial communities from marine-cultured, bleached, and indoor-cultured thalli formed distinct clusters, indicating clear host-condition-associated differences in surface bacterial composition. For tissue-associated bacteria, the PCoA also showed obvious separation among the three groups, with the first and second axes explaining 45.2% and 33.2% of the variation, respectively (Fig. 3C). Compared with epiphytic communities, tissue-associated communities showed a particularly clear

separation between bleached and indoor-cultured samples, while the marine-cultured samples were more dispersed, suggesting that bacterial communities detected from surface-sterilized tissues also changed markedly under bleaching and indoor-cultivation conditions.

Venn analysis further revealed differences in shared and unique ASVs between epiphytic and tissue-associated bacterial communities (Fig. 3B, D). For epiphytic bacteria, only 108 ASVs were shared among all three host conditions, while marine-cultured samples contained the largest number of unique ASVs (1,276), followed by bleached samples (896) and indoor-cultured samples (687) (Fig. 3B). Marine-cultured and bleached epiphytic communities shared more ASVs with each other than either did with indoor-cultured samples, suggesting that indoor cultivation was associated with a stronger shift in surface bacterial composition. For tissue-associated bacteria, 240 ASVs were shared among the three host conditions, which was higher than that observed for epiphytic bacteria (Fig. 3D). Marine-cultured tissue-associated samples also contained the largest number of unique ASVs (753), followed by bleached samples (634) and indoor-cultured samples (627). Compared with epiphytic communities, tissue-associated bacterial communities showed a larger core ASV overlap among host conditions, indicating that bacteria detected from surface-sterilized tissues may contain a more conserved component across host states. Overall, both epiphytic and tissue-associated bacterial communities showed clear host-condition-associated restructuring, but epiphytic communities appeared more variable among conditions, whereas tissue-associated communities retained a larger shared ASV pool.

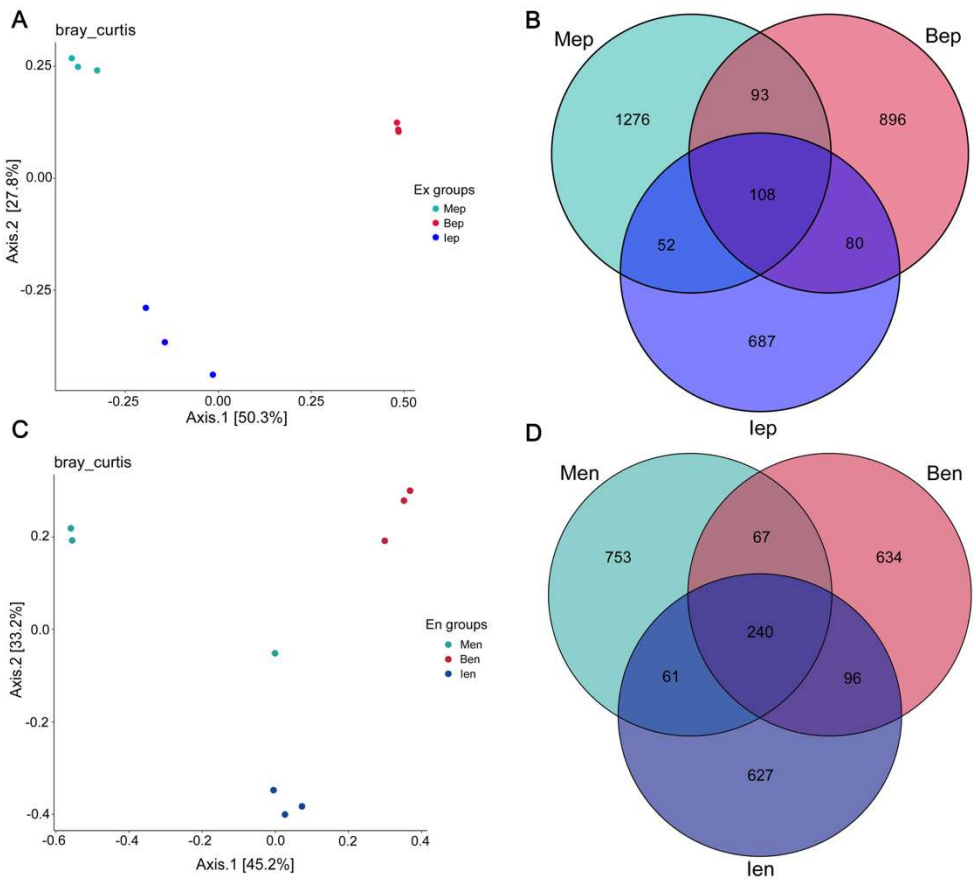


Fig. 3 Beta diversity and shared ASVs of epiphytic and tissue-associated bacterial communities associated with *B. gelatinus* under different host conditions. (A) Principal coordinate analysis (PCoA) in epiphytic bacterial community composition among different groups. (B) Venn diagram of shared ASVs among epiphytic bacterial communities from different groups. (C) PCoA in tissue-associated bacterial community composition among different groups. (D) Venn diagram of shared ASVs among tissue-associated bacterial communities from different groups. Mep, epiphytic bacteria of marine-cultured *B. gelatinus*. Bep, epiphytic bacteria of bleached *B. gelatinus*. Iep, epiphytic bacteria of indoor-cultured *B. gelatinus*. Men, endophytic bacteria of marine-cultured *B. gelatinus*. Ben, endophytic bacteria of bleached *B. gelatinus*. Ien, endophytic bacteria of indoor-cultured *B. gelatinus*.

Taxonomic composition of epiphytic and tissue-associated bacteria

Bacterial community composition varied markedly among host conditions at both the phylum and genus levels in epiphytic and tissue-associated bacterial communities (Fig. 4).

Across all epiphytic bacterial samples, the communities were primarily dominated by *Proteobacteria*, followed by *Bacteroidetes*, *Planctomycetes*, *Actinobacteria*, *Firmicutes*, and *Verrucomicrobia* (Fig. 4A). *Proteobacteria* accounted for the majority of sequences in all treatments and became particularly dominant in indoor-cultured samples. Although the dominant phyla were broadly similar among treatments, their relative abundances varied substantially with host condition.

At the genus level, epiphytic bacterial communities exhibited pronounced host-condition-associated differences (Fig. 4B). Marine-cultured thalli were mainly dominated by *Cupriavidus* (25.89%) and *Ralstonia* (21.48%), whereas bleached thalli were characterized by the enrichment of *Ruegeria* (15.34%) and *Methylophaga* (12.63%). Indoor-cultured thalli displayed a more heterogeneous community composition, with *Cupriavidus* (10.88%), *Methylophaga* (9.94%), *Alteromonas* (9.34%), and *Ralstonia* (6.88%) representing the dominant genera. *Cupriavidus* and *Ralstonia* were primarily associated with marine-cultured and indoor-cultured thalli, whereas *Ruegeria* was predominantly enriched in bleached samples. *Alteromonas* occurred across all three host conditions, suggesting that it represents a common component of the epiphytic bacterial community associated with *B. gelatinus*.

Similar patterns were observed in tissue-associated bacterial communities, although their composition differed from those of epiphytic bacteria (Fig. 4C–D). *Proteobacteria* remained the dominant phylum in all tissue-associated samples, accounting for more than 80% of the total sequences, while Bacteroidetes, *Actinobacteria*, *Firmicutes*, *Planctomycetes*, and *Cyanobacteria* were present at lower abundances (Fig. 4C). Compared with epiphytic communities, tissue-associated bacterial communities exhibited a more stable phylum-level composition among host conditions.

At the genus level, tissue-associated bacterial communities showed substantial restructuring among treatments (Fig. 4D). Marine-cultured thalli were dominated by Unclassified taxa and *Ruegeria*, whereas bleached thalli showed a marked enrichment of *Methylophaga* and *Pseudoalteromonas*. Indoor-cultured thalli were characterized by higher relative abundances of *Ruegeria*, *Methylophaga*, and *Alteromonas*. Several genera, including *Ruegeria*, *Methylophaga*, *Pseudoalteromonas*, and *Alteromonas*, were detected in both epiphytic and tissue-associated communities, suggesting that these taxa may

represent important components of the *B. gelatinus*-associated microbiome across different host-associated niches. However, their relative abundances varied considerably among host conditions, indicating that bleaching and indoor cultivation were accompanied by substantial bacterial community restructuring in both surface-associated and tissue-associated habitats. Overall, epiphytic bacterial communities exhibited stronger host-condition-dependent variation than tissue-associated communities, particularly at the genus level. In contrast, tissue-associated communities retained a more conserved phylum-level structure while undergoing shifts in dominant genera. These results suggest that host condition influences both surface-associated and tissue-associated bacterial assemblages, although the magnitude and pattern of change differ between the two microbial fractions.

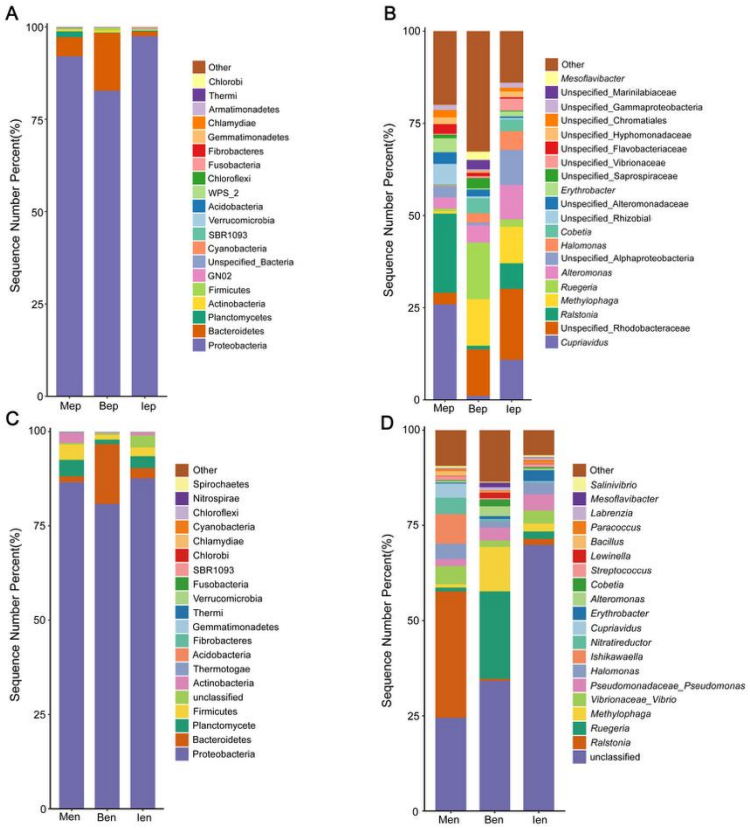


Fig. 4 Taxonomic composition of epiphytic and tissue-associated bacterial communities associated with *B. gelatinus* under different host conditions. (A) Community structure of the epiphytic bacteria in the phylum level. (B) Community structure of the epiphytic bacteria in the gene level. (C) Community structure of the tissue-associated bacteria in the phylum level. (D) Community structure of the tissue-associated bacteria in the gene level. Mep, Bep, and Iep represent epiphytic bacterial communities from marine-cultured,

bleached, and indoor-cultured *B. gelatinus*, respectively. Men, Ben, and Ien represent tissue-associated bacterial communities detected from surface-sterilized tissues of marine-cultured, bleached, and indoor-cultured *B. gelatinus*, respectively.

Shared bacterial asvs between epiphytic and tissue-associated communities

Comparative analyses of epiphytic and tissue-associated bacterial communities revealed that several dominant taxa were common to both microbial fractions, though their relative abundances varied among host conditions (Fig. 5). Marine-cultured samples showed the highest overlap between epiphytic and tissue-associated communities, whereas bleached and indoor-cultured samples exhibited more distinct bacterial assemblages. This overlap suggested that certain bacterial taxa consistently inhabit various host-associated niches within *B. gelatinus*, and changes in host condition significantly influence the relative structure and composition of these communities. Both epiphytic and tissue-associated bacterial communities underwent conspicuous host-condition-associated alterations. Specifically, bleached and indoor-cultured thalli demonstrated unique bacterial diversity patterns and altered taxonomic compositions compared to healthy, marine-cultured thalli, signifying that environmental changes and conditions associated with bleaching result in substantial restructuring of bacterial communities.

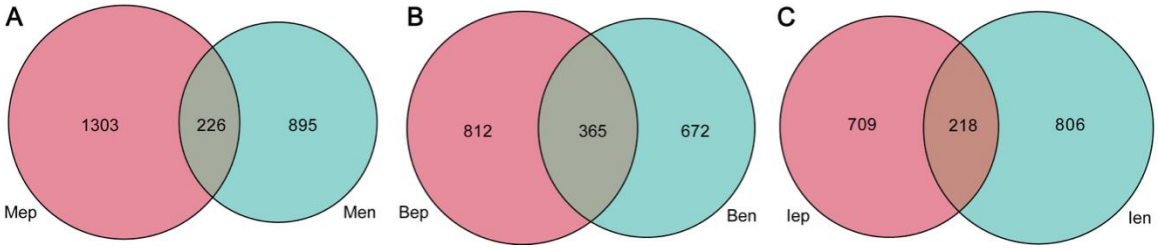


Fig. 5 Venn diagrams of the unique and shared ASVs between three treatments. (A) Mep & Men. (B) Bep & Ben. (C) Iep & Ien. Mep & Men: Mep, epiphytic bacteria of marine-cultured *B. gelatinus*. Mep, Bep, and Iep represent epiphytic bacterial communities from marine-cultured, bleached, and indoor-cultured *B. gelatinus*, respectively. Men, Ben, and Ien represent tissue-associated bacterial communities detected from surface-sterilized tissues of marine-cultured, bleached, and indoor-cultured *B. gelatinus*, respectively.

Predicted functional shifts in bacterial communities

Functional differentiation among host conditions

The predicted functional profiles of both epiphytic and tissue-associated bacterial communities varied among the three host conditions (Fig. 6). At the overall functional level, metabolism-related pathways dominated all bacterial communities, although their relative abundances differed among treatments. In epiphytic bacterial communities, the pathway "Biosynthesis of terpenoids and steroids" accounted for 2.53% of the predicted functional abundance in marine-cultured samples, compared with 0.82% in bleached samples and 1.72% in indoor-cultured samples (Fig. 6A). The abundance of this pathway was significantly lower in bleached samples than in the other two treatments ($p < 0.05$).

PCA based on predicted KEGG functional profiles revealed clear functional differentiation among epiphytic bacterial communities associated with different host conditions (Fig. 6B). The first two principal components explained 75.8% of the total variation. Bleached samples clustered closely together, indicating relatively consistent predicted functional composition, whereas marine-cultured and indoor-cultured samples showed greater within-group variation. Further analysis identified several significantly enriched pathways in epiphytic bacterial communities (Fig. 6C). The "Geraniol degradation" pathway was significantly enriched in marine-cultured samples compared with bleached and indoor-cultured samples ($p < 0.05$). In contrast, bleached samples showed significantly higher predicted abundances of 2-Oxocarboxylic acid metabolism, Arginine biosynthesis, Glycine, serine and threonine metabolism, and Glycolysis/Gluconeogenesis, indicating increased representation of pathways related to amino acid metabolism and central carbon metabolism. The glutathione metabolism pathway was significantly enriched in indoor-cultured samples.

Tissue-associated bacterial communities also exhibited distinct predicted functional profiles among host conditions (Fig. 6D–F). PCA analysis showed clear separation among marine-cultured, bleached, and indoor-cultured samples, with PC1 and PC2 explaining 64.11% and 16.86% of the variation, respectively (Fig. 6E). Compared with epiphytic bacterial communities, tissue-associated communities showed stronger separation along PC1, suggesting pronounced functional restructuring within bacteria detected from surface-sterilized tissues.

Several pathways differed significantly among tissue-associated bacterial communities (Fig. 6F). Marine-cultured samples exhibited significantly higher predicted

abundances of pathways related to biosynthesis of various secondary metabolites, cytochrome P450-mediated xenobiotic metabolism, and carotenoid biosynthesis. In contrast, bleached samples showed enrichment of oxidative phosphorylation and sesquiterpenoid and triterpenoid biosynthesis, whereas indoor-cultured samples exhibited relatively higher abundances of pathways associated with flavonoid biosynthesis and photosynthesis. These results suggest that tissue-associated bacterial communities underwent functional shifts distinct from those observed in epiphytic bacterial communities. Overall, both epiphytic and tissue-associated bacterial communities exhibited host-condition-associated functional restructuring. However, the specific pathways involved differed between the two microbial fractions. Epiphytic bacterial communities were mainly characterized by shifts in amino acid, carbohydrate, and terpenoid-related metabolism, whereas tissue-associated bacterial communities showed greater variation in pathways related to secondary metabolite biosynthesis, energy metabolism, and photosynthesis-associated functions. Together, these findings indicate that changes in host condition were accompanied by substantial alterations in the predicted metabolic potential of both surface-associated and tissue-associated bacterial communities.

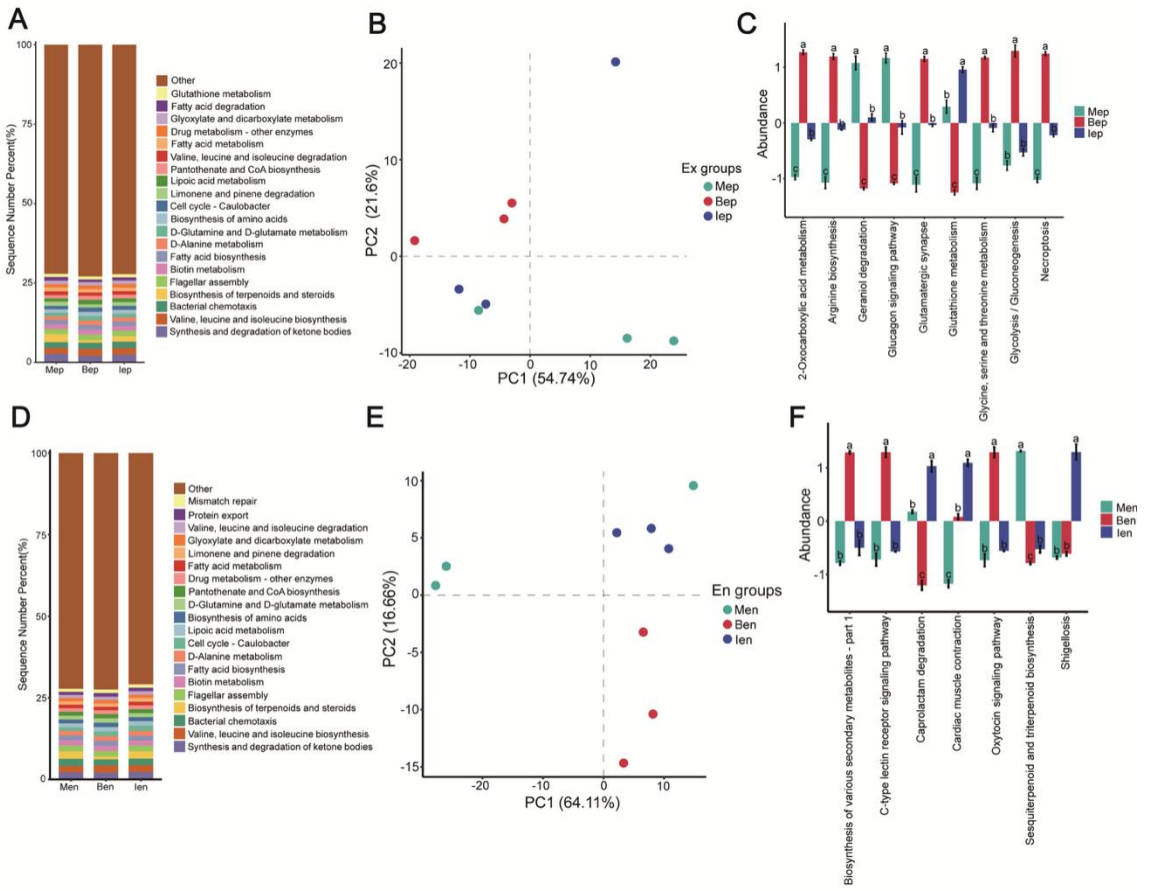


Fig. 6 Functional prediction of epiphytic and tissue-associated bacterial communities associated with *B. gelatinus* under different host conditions. (A) Relative abundance of predicted KEGG pathway composition in epiphytic bacteria. (B) PCA analysis of KEGG pathways in epiphytic bacteria. (C) Differentially abundant KEGG pathways in epiphytic bacterial communities identified by ANOVA followed by Duncan's multiple comparison test. (E) PCA analysis results of KEGG pathways in tissue-associated bacteria. (F) Differentially abundant KEGG pathways in tissue-associated bacterial communities identified by ANOVA followed by Duncan's multiple comparison test. Mep, Bep, and Iep represent epiphytic bacterial communities from marine-cultured, bleached, and indoor-cultured *B. gelatinus*, respectively. Men, Ben, and Ien represent tissue-associated bacterial communities detected from surface-sterilized tissues of marine-cultured, bleached, and indoor-cultured *B. gelatinus*, respectively. Different lowercase letters indicate significant differences among treatments ($p < 0.05$).

Diversity and predicted functions of its2-derived epiphytic eukaryotic communities

Diversity and composition of its2-derived communities

A total of 785,782 high-quality ITS2 sequences were obtained from the epiphytic eukaryotic communities associated with *B. gelatinus* following quality filtering and chimera removal. Although initially intended to target epiphytic fungal communities, ITS2 amplicon sequencing identified several dominant ASVs belonging to non-fungal eukaryotic groups in the reference database. Consequently, the results presented herein describe the ITS2-derived epiphytic fungi and related eukaryotic taxa associated with *B. gelatinus*. The ITS2-derived communities exhibited distinct differences under marine-cultured, bleached, and indoor-cultured host conditions. Across all conditions, 488 ASVs were identified and assigned to 15 phyla, 31 classes, 58 orders, 82 families, and 89 genera.

Alpha-diversity analyses revealed marked differences in diversity patterns among the three host conditions (Figs. 7A). The marine-cultured hosts displayed the highest Chao1 and Shannon indices (Fig. 7B), indicating relatively higher richness and evenness of ITS2-derived epiphytic eukaryotic communities under naturally healthy cultivation conditions. Although the Chao1 index did not show significant differences among the treatments, the Shannon index revealed significant variations between each pair of treatments, illustrating that host condition influenced community evenness and the relative abundance distribution.

Beta-diversity analyses further demonstrated clear differences in community composition among the marine-cultured, bleached, and indoor-cultured samples. The PCoA based on Bray-Curtis dissimilarity explained 85.9% of the observed variation, with the first and second axes accounting for 47.4% and 38.5%, respectively (Fig. 7C). The samples from the three host conditions formed distinct clusters, indicating significant shifts in ITS2-derived community composition among treatments ($p < 0.05$).

Venn analysis also indicated substantial differences in ASV composition among treatments (Fig. 7D). The marine-cultured treatment contained the highest number of unique ASVs (226), followed by the indoor-cultured (142) and bleached treatments (69). Furthermore, the marine-cultured and indoor-cultured treatments shared more ASVs (14) than did the marine-cultured and bleached samples (7) or the bleached and indoor-cultured samples (3), suggesting that bleaching-associated conditions significantly restructured the ITS2-derived epiphytic eukaryotic communities.

Taxonomic composition analysis revealed that the dominant ITS2-derived taxa varied significantly among the host conditions (Figs. 7E-F). At the phylum level, the communities were predominantly comprised of *Ciliophora* and sequences related to *Porifera*. *Ciliophora* showed a relatively higher abundance in bleached samples, while *Chlorophyta* was more abundant in marine-cultured samples, and Rhodophyta in indoor-cultured samples.

At the genus level, the marine-cultured treatment was primarily dominated by *Hymeniacidon* (11.00%), *Pseudovorticella* (6.02%), and *Folliculina* (2.99%). In contrast, bleached samples were predominantly characterised by *Pseudovorticella* (78.59%), followed by *Pseudokahliella* (13.06%) and *Epicarchesium* (6.72%). The indoor-cultured treatment mainly featured *Pseudovorticella* (8.32%). *Hymeniacidon* and *Folliculina* were primarily detected in marine-cultured samples, whereas *Pseudokahliella* and *Epicarchesium* were mostly found in bleached samples. *Pseudovorticella* was present across all host conditions, serving as a common element of the ITS2-derived epiphytic eukaryotic communities.

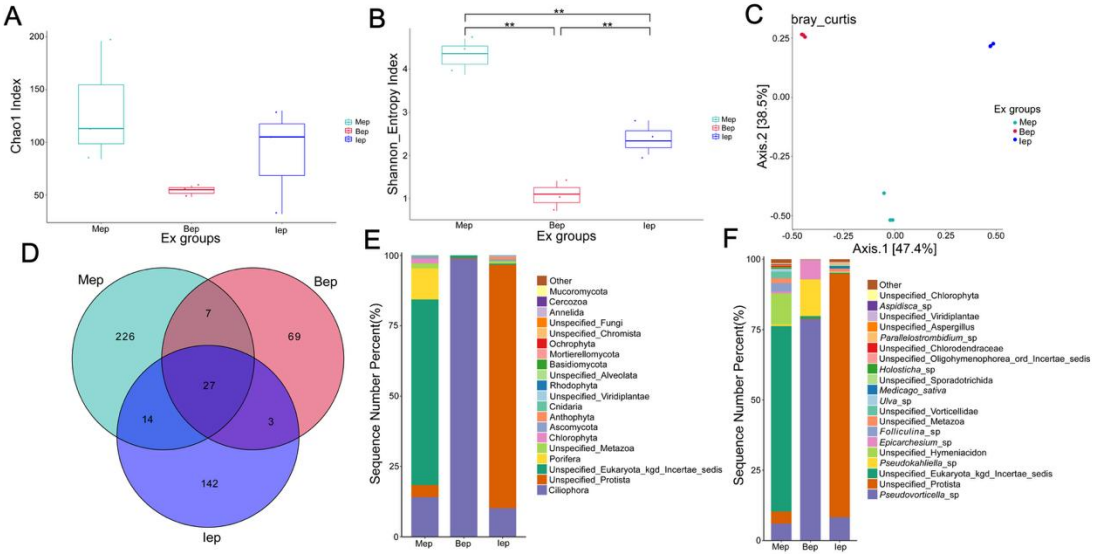


Fig. 7 Diversity and taxonomic composition of ITS2-derived epiphytic eukaryotic communities associated with *B. gelatinus* under different host conditions. (A) Chao1 richness index. (B) Shannon diversity index. (C) Principal coordinate analysis (PCoA) based on Bray–Curtis dissimilarity. (D) Venn diagram showing shared and unique ASVs among treatments. (E) Relative abundance of dominant eukaryotic taxa at the phylum level.

(F) Relative abundance of dominant eukaryotic taxa at the genus level. Mep, Bep, and Iep denote ITS2-derived epiphytic eukaryotic communities from marine-cultured, bleached, and indoor-cultured *B. gelatinus*, respectively. Only dominant taxa are shown, with the remaining taxa grouped as "Other". Different lowercase letters indicate significant differences among treatments, and ** indicates $p < 0.05$.

Functional prediction of ITS2-derived communities

Functional predictions based on the MetaCyc database suggested that the overall functional composition of the ITS2-derived epiphytic eukaryotic communities was relatively uniform across the three host conditions (Fig. 8A). No significant differences were detected in the major predicted MetaCyc pathways among the marine-cultured, bleached, and indoor-cultured treatments. The predicted functional core remained relatively stable across the treatments in terms of pathway prevalence and relative abundance.

However, the PCA indicated some variations in the predicted functional compositions among the three treatments (Fig. 8B). The marine-cultured treatment exhibited greater within-treatment functional variation, whereas the bleached treatment showed more tightly clustered and functionally consistent predicted profiles. Compared to the bacterial functional prediction results, the ITS2-derived communities showed less pronounced functional differentiation among the host conditions.

Overall, the predicted functional profiles of the ITS2-derived epiphytic eukaryotic communities demonstrated limited variation among host conditions, despite notable taxonomic differences in community composition. These findings suggest that bacterial community restructuring may represent a more significant microbial response to bleaching and shifts in cultivation conditions in *B. gelatinus* than changes in the predicted functions of its ITS2-derived eukaryotic communities. Given that the ITS2 primers used in this study may amplify a broad range of eukaryotic taxa beyond fungi, these findings should be interpreted as indicative of changes in surface-associated eukaryotic communities rather than strictly representing fungal community dynamics.

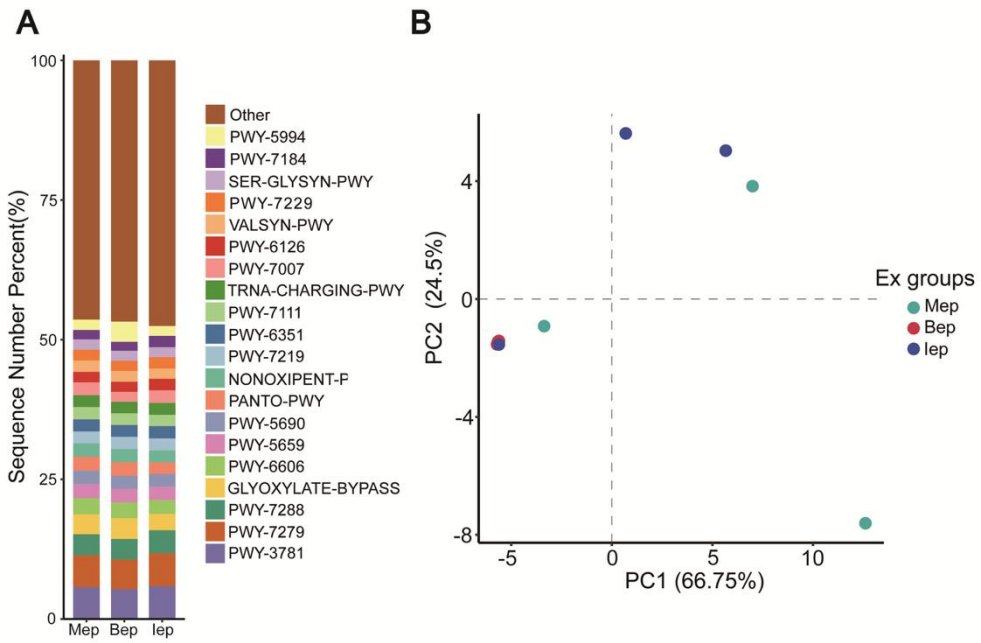


Fig. 8 Functional prediction of ITS2-derived epiphytic eukaryotic communities associated with *B. gelatinus* under different host conditions.. (A) Relative abundance of dominant predicted MetaCyc pathways. (B) Principal component analysis of predicted functional composition based on MetaCyc pathways. Mep, Bep, and Iep denote ITS2-derived epiphytic eukaryotic communities from marine-cultured, bleached, and indoor-cultured *B. gelatinus*, respectively.

Discussion

This study highlighted environmental factors influencing the growth of *B. gelatinus*, demonstrating that phosphate (PO_4^{3-}), iron ions (Fe^{3+}), and the composition of the seawater microbial community significantly impacted its growth. Notably, the significant effect of seawater microorganisms suggests that microbial factors play a crucial role in regulating the growth and health status of *B. gelatinus*. Accordingly, this research further characterized the epiphytic and endophytic microbial communities associated with *B. gelatinus* to explore their potential relationships with various seaweed health conditions.

A significant finding of this study is that changes in host conditions were associated with distinct shifts in the structure of bacterial communities. Marine-cultured thalli, bleached thalli, and indoor-cultured thalli each harbored unique bacterial assemblages,

demonstrating that the *B. gelatinus*-associated microbiome is sensitive to variations in host condition and cultivation environment. In the epiphytic bacterial communities, those from marine-cultured thalli exhibited greater richness but reduced evenness, suggesting a relatively diverse community dominated by specific taxa. In contrast, bleached thalli demonstrated higher Shannon diversity, which might indicate a redistribution of relative abundances and a weakening of the dominant community structure. An increase in diversity should not be straightforwardly interpreted as indicative of a healthier or more stable microbial state. Within the context of macroalgal disease ecology, disturbances in host-associated microbiota can lead to dysbiosis, reduced host filtering, and colonization by opportunistic taxa, ultimately contributing to tissue degradation and disease progression (Murúa et al., 2023; Ward et al., 2020). Hence, the increased evenness observed in the bleached samples may signify microbial community restructuring associated with host deterioration rather than an enhancement of microbial diversity.

The bacterial taxa detected in this research should be more accurately interpreted as associated with host conditions rather than simply labeled as “beneficial” or “harmful” microorganisms. In this study, taxa enriched in marine-cultured thalli, such as *Cupriavidus* and *Ralstonia*, were identified as health-associated candidate taxa because they were more prevalent in thalli without visible bleaching symptoms. Taxa enriched in bleached thalli, such as *Ruegeria* and *Methylophaga*, were classified as bleaching-associated taxa because their presence increased under bleaching conditions. This classification is derived from their distribution patterns across host conditions, not from direct functional validation. Prior studies have indicated that some macroalgal-associated bacteria may support host growth, morphogenesis, nutrient cycling, or defense, whereas others may become opportunistic under stress (Egan et al., 2013; Hollants et al., 2013; Wahl et al., 2012). For instance, *Ruegeria* sp. R11 has been reported to induce bleaching disease in the red alga *Delisea pulchra* under elevated temperature conditions (Case et al., 2011), and shifts in bacterial communities have also been linked to bleaching in *Saccharina japonica* (Ling et al., 2022; Ma et al., 2023). However, genus-level enrichment alone does not suffice to ascertain ecological functions because different species or strains within the same genus may perform contrasting roles. Therefore, the taxa identified herein should be considered as candidates for future isolation and functional assays rather than confirmed causal agents.

Functional prediction analysis of epiphytic bacteria indicated that the “Biosynthesis of terpenoids and steroids” pathway accounted for 2.53%, and the “Geraniol degradation” pathway was predominantly abundant in the marine-cultured treatment of *B. gelatinus*. The biosynthesis of terpenoids and their ecological roles in plant defense have been extensively reviewed (Li et al., 2023a). Similarly, the biosynthesis of phytosterols in plants and their crucial role in maintaining cell membrane integrity have been explored (Du et al., 2022).Research has demonstrated that geraniol promotes seedling growth within a specific concentration range, highlighting its potential to enhance plant development (Choi et al., 2015). However, these results, derived from amplicon-based functional prediction, should be considered indicative of potential functionality rather than direct evidence of gene expression or metabolite production. Such findings propose that the epiphytic bacterial community of the healthy marine-cultured *B. gelatinus* may exhibit metabolic potential related to terpenoids and steroids.

The pattern of bacterial associations observed in bleached specimens in this study could reflect a broader process of microbiome destabilization. It is improbable that a single microbial taxon by itself causes bleaching; rather, the phenomenon may result from the interactions among environmental stress, the weakening of host physiology, and microbial community restructuring. Under stressful conditions, the environment on algal surfaces might change due to altered exudation, tissue damage, diminished defensive capacity, or shifts in nutrient leakage. These alterations can facilitate the emergence of new niches for opportunistic microbes, leading to an increased relative abundance of certain bacterial groups and altered community evenness. Similar dynamics have been documented in macroalgal diseases, where abiotic stressors and microbial agents collaborate to promote disease progression, including ice-ice disease in carrageenophytes and bleaching-associated microbiome shifts in other macroalgae (Ling et al., 2022; Murúa et al., 2023; Riyaz et al., 2019; Tahiluddin and Terzi, 2021). Consequently, the enrichment of bleaching-associated taxa in *B. gelatinus* should be interpreted as evidence of microbial community restructuring during bleaching, rather than as direct proof that these taxa cause bleaching. Ma et al. (2024) also reported that bleaching in *B. gelatinus* was accompanied by tissue damage, pigment loss, reduced photosynthetic performance, and shifts in epiphytic bacterial communities. Consistent with these findings, our results indicated that bleached

thalli harbored distinct bacterial communities, supporting the link between bleaching and microbiome restructuring. However, the dominant bleaching-associated taxa differed between studies, potentially due to variations in sampling time, environmental conditions, cultivation practices, or analytical procedures. Unlike previous studies that focused predominantly on healthy and bleached field samples and epiphytic bacteria, our study expanded the scope to include environmental-factor screening, tissue-associated bacteria, ITS2-derived eukaryotic communities, predicted microbial functions, and provided a more comprehensive analysis.

The predicted functional profiles suggest that bacterial communities associated with different host conditions differ in their metabolic potential. Marine-cultured samples exhibited relatively higher predicted abundances of terpenoid- and steroid-related pathways, whereas bleached samples showed enrichment in pathways related to amino acid and central carbon metabolism. These disparities could indicate functional restructuring of bacterial communities under varying host conditions. However, these results, derived from 16S rRNA gene-based functional predictions, should be considered indicative of putative functional potential rather than direct evidence of gene expression or metabolite production. Predictions based on amplicon sequencing have inherent limitations as they estimate functional profiles from marker-gene information instead of directly measuring genes, transcripts, proteins, or metabolites (Langille et al., 2013). Therefore, observed differences in predicted metabolic pathways generate hypotheses for future metagenomic, metatranscriptomic, metabolomic, or culture-based studies rather than providing definitive functional conclusions. Similar caution has been emphasized in macroalgal microbiome studies, where host-associated microorganisms are believed to play significant ecological roles, yet their functions necessitate experimental validation through isolation, reintroduction, or multi-omics approaches (Deutsch et al., 2023; Egan et al., 2013; Li et al., 2023b).

The composition of tissue-associated bacterial communities also varied among host conditions, displaying some similarities with epiphytic bacterial patterns. Bleached thalli exhibited higher richness and evenness in these communities, along with the enrichment of taxa also observed in the epiphytic fraction. This observation suggests that bleaching is associated not only with changes on the algal surface but also with shifts in the bacteria

detected from surface-sterilized thallus tissues. Previous research has demonstrated that macroalgal internal or tissue-associated bacterial communities can differ from those of epiphytic and surrounding seawater communities, indicating that these microbial fractions may represent distinct host-associated niches (Hollants et al., 2011; Longford et al., 2007; Lachnit et al., 2009). However, because the effectiveness of surface sterilization was not independently verified by culturing or sequencing the final rinse solution, these communities should be interpreted as surface-sterilized, tissue-associated bacteria rather than strictly confirmed endophytes. The overlap between epiphytic and tissue-associated ASVs further suggests that residual surface bacteria or closely associated tissue-surface communities cannot be fully excluded. Future studies should employ final-rinse controls, culture-based validation, fluorescence microscopy, or fluorescence in situ hybridization to confirm the true localization of endophytic bacteria, as highlighted in studies exploring macroalgal endophytes and their reintroduction into algal hosts (Deutsch et al., 2021; Deutsch et al., 2023; Hollants et al., 2011).

The eukaryotic communities derived from ITS2 on epiphytic hosts demonstrated distinct taxonomic shifts across different host conditions, with a notable dominance of ciliate-related taxa in bleached samples. Although ITS2 sequencing initially targeted fungal communities, it identified several dominant taxa within non-fungal eukaryotic groups, such as *Ciliophora* and Porifera-related taxa. Consequently, these data should be regarded as representing ITS2-derived surface-associated eukaryotic assemblages, rather than exclusively fungal communities. The prevalence of taxa such as *Pseudovorticella* and *Pseudokahliella* in bleached samples likely indicates colonization by opportunistic eukaryotes on altered or deteriorated thallus surfaces. Similar interactions between host-associated eukaryotes and fungi have been observed in macroalgae, although their ecological roles often necessitate further investigation through microscopic, isolation-based, or chemically mediated studies (Rousseau et al., 2025; Vallet et al., 2018). Moreover, the annotation of fungi and eukaryotes in environmental ITS datasets frequently suffers from the limitations of incomplete reference databases, potentially resulting in a significant proportion of unclassified or non-target taxa within microbiome datasets (Yao et al., 2019). However, the exact contribution of these taxa to bleaching remains uncertain without microscopic observation or isolation-based confirmation, raising questions as to whether

these organisms actively contribute to bleaching or merely respond to alterations in surface microhabitats. Additionally, the predicted functional profiles of the ITS2-derived communities revealed minimal differences across host conditions, suggesting that taxonomic shifts were more pronounced than changes in predicted functions.

Overall, this study indicates that the health status of *B. gelatinus* is intricately linked to the restructuring of host-associated microbial communities, implicating a complex interplay among environmental conditions, host physiological status, bacterial community stability, and opportunistic colonization in bleaching events. This pattern aligns with broader macroalgal disease studies which suggest that environmental stress disrupts host-associated microbiota, promotes microbial dysbiosis, and facilitates opportunistic colonization during disease development (Egan et al., 2013; Murúa et al., 2023; Spagnuolo and Genovese, 2024; Ward et al., 2020; Zozaya-Valdes et al., 2015). In carrageenophytes, phenomena such as ice-ice disease suggest a combined role of abiotic stressors and opportunistic microbial agents in disease development, further supporting the notion that algal diseases reflect multifaceted interactions among host conditions, environmental factors, and microbiota (Tahiluddin and Terzi, 2021; Riyaz et al., 2019). Consequently, the key contribution of this study is the identification of microbial patterns associated with host conditions and the delineation of candidate taxa for future research. Future studies should isolate representative bacterial strains from healthy and bleached thalli, undertake reinoculation or co-culture experiments, monitor microbiome dynamics during the progression of bleaching, and incorporate host physiological indicators such as photosynthetic efficiency, pigment content, oxidative stress markers, and tissue damage to ascertain whether microbial shifts are drivers, consequences, or co-occurring indicators of health deterioration in *B. gelatinus*.

Conclusion

In conclusion, this study investigated the effects of selected abiotic conditions and seawater treatment on the growth of *B. gelatinus*, and characterized the microbial communities associated with both epiphytic and surface-sterilized tissues under varying host conditions. The findings demonstrated that the growth of *B. gelatinus* was sensitive to nutrient conditions, particularly phosphate and iron, as well as to seawater sterilization treatments. Additionally, thalli cultured in marine environments, those subjected to

bleaching, and thalli grown indoors exhibited distinct structures of host-associated microbial communities. This suggests that alterations in the health status of the algae and in cultivation conditions are closely linked to changes in microbial community structures. Rather than elucidating the direct beneficial or harmful impacts of specific microorganisms, the study identified various microbial taxa associated with different host conditions and predicted functional patterns. These may serve as preliminary candidates for further investigation. The shifts observed in the microbial communities provide a foundation for further exploration into the links between the health status of *B. gelatinus*, changes in the community related to bleaching, and the stability of the host-associated microbiome. However, the current study primarily relied on amplicon sequencing and predicted functional profiles, thus the ecological roles of these candidate taxa have yet to be experimentally confirmed. Future research should integrate culture-based isolation, re-inoculation or co-culture experiments, measurements of host physiology, and multi-omics approaches. These methods would help to ascertain whether changes in microbial communities act as drivers, consequences, or merely indicators of the health deterioration of *B. gelatinus*. Such investigations will enhance our understanding of the microbial ecology of *B. gelatinus* and provide a scientific basis for the health monitoring and cultivation management of this carrageenophyte.

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