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Characterization of persistent marine bacterial community profiles isolated from long-term *Kappaphycus alvarezii* cultures in a closed cultivation system using 16S rDNA analysis

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ABSTRACT

Aims: The study aims to investigate the bacterial community profiles on the surface of red algae (*Kappaphycus alvarezii*) and persistent bacteria that can adapt to long-term cultivation in a closed circulation system.
Methodology and results: *Kappaphycus alvarezii* explants were cultured in a controlled laboratory setting for 30 days to investigate related bacterial adaptability to controlled culture conditions. Bacterial isolates associated with seedlings were subjected to 16S rDNA amplification and sequencing, followed by the construction of a phylogenetic tree using MEGA X software. The results show distinct microbial composition between the first and 30th days. The derived phylogenetic tree features three dominant phyla: Proteobacteria (*Vibrio* and *Thalassospira*), Pseudomonadota (*Pseudoalteromonas*, *Alteromonas*, *Grimontia*, *Ruegeria*, *Phaebacter* and *Bacterioplanes*) and Firmicutes (*Bacillus*). A comparative examination of these two bacterial groups (day 1 and day 30) reveals evidence of persistent marine bacteria, such as the genera *Vibrio*, *Pseudoalteromonas*, *Alteromonas*, *Phaaebacter* and *Bacterioplanes*, that successfully adapt to long-term cultivation within closed circulation systems.
Conclusion, significance and impact of study: The findings of this study contribute to the understanding of bacterial ecology in the controlled red algae cultivation environment and also provide valuable insights into the optimization of an ideal closed cultivation system for sustainable *K. alvarezii* production, benefiting the seaweed industry.

Keywords: 16S rDNA gene sequencing, closed cultivation system, *Kappaphycus alvarezii*, persistent marine bacteria, red algae

INTRODUCTION

The production of marine macroalgae (seaweed) has received considerable attention due to its potential economic, ecological and industrial benefits (Mohammad *et al.*, 2019; Jönsson *et al.*, 2020). Over the past decade, global seaweed aquaculture has grown by more than 200% to 32 million tonnes fresh weight (FW), with *Kappaphycus* and *Eucheuma* accounting for 34% of production (FAO, 2020; Kambey *et al.*, 2021). *Kappaphycus* cultivation has stimulated national economies and improved the livelihoods of millions of South-East Asian farmers, with the species *Kappaphycus alvarezii* standing out as a promising candidate, offering diverse applications ranging from food to bioactive compounds (Baskararaj *et al.*, 2019; Das *et al.*, 2023). Presently, most seaweed cultivation depends on seedlings collected from the wild or vegetative propagation from successive crops of previous harvests (Leandro *et al.*, 2020; Jiksing *et al.*, 2022). However, excessive reliance on natural seaweed resources could lead to the overexploitation of wild seaweed populations, thereby disrupting the balance of the marine ecosystem (Kim *et al.*, 2017). Moreover, reliance on recurrent vegetative propagation of existing cultivars is likely to result in decreased productivity and loss of species diversity (Jiksing *et al.*, 2022; Sultana *et al.*, 2023).

An alternative approach to addressing this challenge entails using a land-based cultivation system to regulate and monitor the seedling production process, aiming to produce high-quality seedlings to meet the demands of the developing farming industry. Land-based cultivation systems offer a distinct advantage in terms of environmental sustainability, as they allow for greater control over growing conditions and reduce the risk of introducing invasive species and diseases into marine ecosystems (Kim *et al.*, 2017). This, in turn, contributes to the conservation of biodiversity and ecological balance. In addition, these systems excel at resource management by efficiently recycling and carefully monitoring water,

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nutrients and waste products, thereby aligning themselves with sustainable agricultural practices (Hurtado *et al.*, 2019; Narvarte *et al.*, 2022). To completely capitalize on these benefits, it is necessary to develop a seaweed cultivation system tailored to the growth requirements of *K. alvarezii* to maximize biomass production (Jiksing *et al.*, 2022).

However, the success of land-based cultivation systems is frequently determined by the complex interactions between macroalgae and associated microorganisms (Kim *et al.*, 2017; Tahiluddin and Terzi, 2021; Rupert *et al.*, 2022). While dynamic interactions do occur between macroalgae and diverse microorganisms in conventional sea-based farming, these interactions take place in open marine environments where their potential impact on seaweed growth may be less predictable or controllable. In contrast, land-based cultivation systems, especially those equipped with closed systems that ensure consistent and predictable water quality, temperature, and nutrient levels, offer an opportunity for more effective management and optimization of these interactions (Narvarte *et al.*, 2022). Such control can lead to improved seaweed growth rates, reduced risks of disease and biofouling, and enhanced overall crop health and quality (Sugumaran *et al.*, 2022). Nevertheless, the lack of a comprehensive understanding of the factors affecting the persistence and adaptation of bacterial communities on the surface of *K. alvarezii* in closed systems hinders the ability to optimize cultivation techniques and predict potential biofouling threats to the macroalgae (Hafting *et al.*, 2012).

The investigation of these persistent bacterial community profiles is essential for advancing our understanding of the microbial ecology within the controlled red algae cultivation environment, which in turn has implications for the success and sustainability of macroalgal cultivation (Hafting *et al.*, 2012). In this study, bacterial isolates from the surface of *K. alvarezii* were subjected to amplification and sequencing of the 16S rDNA gene for taxonomic identification. The 16S rDNA gene was chosen as a marker for bacterial identification due to its well-documented advantages in bacterial taxonomy and phylogeny. The 16S rDNA gene is highly conserved among bacteria, allowing for accurate genus and species identification (Woo *et al.*, 2008; Fykse *et al.*, 2015). Its relatively slow rate of evolution is particularly valuable when working with diverse bacterial populations, enabling discrimination of closely related species (Eren *et al.*, 2013; Beckers *et al.*, 2016). Furthermore, the extensive availability of 16S rDNA sequence data in public databases facilitates accurate taxonomic assignment and comparison of our results with existing literature. Following that, a phylogenetic tree was constructed to further examine the evolutionary relationships and genetic relatedness of various bacterial species associated with *K. alvarezii* (Liu *et al.*, 2023). This approach aligns with established best practices in the field of microbiology and ensures the robustness of our bacterial identification process.

MATERIALS AND METHODS

Isolation of marine bacteria

Kappaphycus alvarezii seedlings, initially obtained from a seaweed production farm located in Kota Belud, Sabah, were cultured for 30 days in the seaweed cultivation laboratory at the Biotechnology Research Institute, Universiti Malaysia Sabah. Throughout the experiment, the seaweed cultures were maintained under optimal growth conditions, as reported by Yong *et al.* (2014). Marine bacteria were isolated from the surface of *K. alvarezii* following the procedure outlined in a previous study (Karthick and Mohanraju, 2018). On the first day following the initiation of the culture, three seedlings were randomly selected for bacteria isolation. The selected seedlings were carefully cleaned and swabbed to collect bacteria present on their surface, and triplicates were performed for each seedling. Swabs from each replicate were placed in separate tubes containing 2 mL of phosphate-buffered saline (PBS), resulting in a total of nine tubes containing bacteria, to preserve bacterial cells in an isotonic environment (Hallmaier-Wacker *et al.*, 2018; Zhang *et al.*, 2022). The seaweed cultures were then cultured for 30 days, and the same sampling techniques were used on day 30 to collect bacteria. Bacterial samples preserved in PBS, collected on day 1 and 30, were plated on Marine Agar (MA) plates. Three replicates of each 200 µL sample were plated and kept at 32 °C for five days of incubation with daily observation for colony formation (Lemos *et al.*, 1985). Bacterial colonies displaying unique characteristics, based on visual examination of colony morphology, were meticulously selected and cultured to obtain pure cultures. Notably, 11 distinct isolates were identified from the seaweed surface on day 1 after culture initiation, while 10 distinct isolates were observed from the seaweed sample on day 30 post-cultivation. These individual isolates were subsequently separated into independent cultures and preserved with 50% glycerol before being stored at -80 °C (Fadanka *et al.*, 2022). The variation in isolate counts between the two time points, day 1 and day 30 post-culture initiation, can be attributed to the distinct colonies observed on the seaweed surface within each respective time frame. Considering that the bacterial populations on day 1 may have been influenced by the conventional farming environment, this variation provides insights into the dynamics of the bacterial community over the 30 days cultivation period, demonstrating how the bacterial community changes over time within the seaweed cultivation system.

Bacterial DNA extraction

Bacterial DNA was extracted using the Wizard Genomic DNA Purification Kit (Promega, USA), following the manufacturer's instructions (Hanum *et al.*, 2018). Cells were collected by centrifuging 1 mL of an overnight culture in a microcentrifuge tube at high speed. The

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pelleted cells were lysed by adding Nuclei Lysis Solution and heated for 10 min at 80 °C. To eliminate RNA, RNase solution was added and incubated at 37 °C for 1 h. For protein removal, Protein Precipitation Solution was added, and the mixture was centrifuged again. The supernatant containing DNA was mixed with an equivalent volume of isopropanol, resulting in visible DNA strands. After centrifugation, the DNA was allowed to air dry at room temperature for 15 min before being rehydrated with DNA Rehydration Solution. The rehydrated DNA was stored at 4 °C until further use. To validate the isolated genomic DNA, electrophoresis on a 1.5% agarose gel was performed, confirming the presence of expected bands and assessing purity (Usama *et al.*, 2023).

Amplification and sequencing of 16S rDNA

The 16S rDNA gene was amplified using the DNA Amplification Kit (Vivantis Technologies, Malaysia), following its associated protocol with some modifications (Tahiluddin *et al.*, 2021). In each reaction, a 1.5 µL aliquot of DNA template (20 ng/µL) was mixed with 1 µL of the forward primer (10 µM), 1 µL of the reverse primer (10 µM), 5 µL of PCR buffer (10×), 3 µL of $MgCl_2$ (50 mM), 1 µL of dNTPs mix (10 mM) and 0.4 µL of DNA polymerase (5 U/µL). This mixture was added to 38.1 µL of double-distilled water in a 0.2 mL PCR tube (Bio-Rad, USA). A pair of universal primers, 27 F (5'-AGA GTT TGA TCM TGG CTC AG-3') and 1492 R (5'-GGT TAC CTT GTT ACG ACT T-3'), was used to target nearly the full length of the 16S rRNA gene, which consists of approximately 1,400 bp (Zhang *et al.*, 2022). Polymerase chain reaction (PCR) amplification was performed using a thermal cycler machine (Bio-Rad PTC-200, USA) with the following cycling conditions: an initial denaturation step at 94 °C for 2 min, followed by 35 cycles of denaturation (94 °C, 30 sec), annealing (60 °C, 30 sec), extension (72 °C, 30 sec) and a final extension step at 72 °C for 7 min. The PCR products were run on a 2% agarose gel for electrophoresis to validate their size before being stored at 4 °C. The products were then sent to Apical Scientific Sdn. Bhd. (formally known as First Base Laboratories Sdn. Bhd., Malaysia) for purification and bi-directional sequencing using the same pair of primers used for amplification.

Sequence analysis

The segments of low-quality sequences were trimmed, followed by the assembly of both forward- and reverse-sequenced DNA fragments (1,200-1,400 bp) using DNA Baser Sequence Assembler (version 5.0), which can be downloaded from https://www.dnabaser.com/download/download.html (Scheublin *et al.*, 2020). The trimmed and assembled sequences were then formatted as FASTA files, submitted to the Basic Local Alignment Search Tool (BLAST) website, http://www.ncbi.nlm.nih.gov, to facilitate bacterial sequence comparison and taxonomic identification (Nurul *et al.*, 2019) and deposited in the GenBank database with accession numbers ranging from MZ570560 to MZ570580. All 21 isolates and their closest matches, which share a high degree of sequence similarity with the isolates studied in the 16S rDNA gene region, suggesting a close genetic relationship, were included in the subsequent phylogenetic analysis. ClustalW was used for sequence alignment, while Molecular Evolutionary Genetics Analysis (MEGA) software was used for cluster analysis. The DNA sequences were aligned, and a neighbor-joining (NJ) phylogenetic tree of the isolates and associated sequences was displayed using MEGA X software, downloadable at https://www.megasoftware.net/downloads/dload_win_gui (Kumar *et al.*, 2018). The phylogenetic tree was constructed using 1,000 bootstraps (Tahiluddin *et al.*, 2021).

RESULTS AND DISCUSSION

K. alvarezii is a widely cultivated red seaweed of economic importance due to its applications in the food and pharmaceutical industries (Amin, 2022; Araújo *et al.*, 2022). Substantial research evidence strongly supports the functional regulation and assistance of seaweed health and resilience by associated bacteria on the seaweed surface, and their ecological role in the seaweed life cycle (Egan *et al.*, 2013). The surface of seaweed serves as a substrate for numerous microbial communities, which can have a significant impact on the overall health and growth of the seaweed (Xu *et al.*, 2022). In controlled land-based seaweed cultivation systems, it is crucial to investigate these microbial communities that affect the growth and survival of the cultivated seaweed. Discovering the bacterial community profiles on the surface of *K. alvarezii* in a closed circulation system is essential for understanding the complex seaweed-microbial interactions within this system (Kaur *et al.*, 2023). Diverse bacterial taxa were identified through sequencing and analysis, revealing dynamic ecological interactions on the seaweed's surface. This information provides preliminary data for improving seaweed cultivation systems, which are valuable for the management of seaweeds in man-made aquaculture settings, ensuring consistent and sustainable seaweed production for various industrial applications. The role of microorganisms in seaweed disease is also gaining attention, and future research should shed light not only on specific seaweed pathogens but also on the possible probiotic effect of the host microbiome.

Bacterial community profiles

The analysis of sequencing data revealed a diverse range of bacteria associated with the surface of *K. alvarezii* on both day 1 and day 30 in a closed cultivation system and their community compositions are outlined in Table 1 and Table 2, respectively. Bacterial prevalence was assessed by considering multiple factors, including bacterial population dynamics, relative abundance and persistence

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Table 1: Bacterial community profiles associated with *K. alvarezii* during day 1 cultivation.

Bacterial ID	Bacterial species	Blast outputs				
		Accession number	Query cover (%)	Identities (%)	Gaps (%)	E-value
1-D1	*Phaeobacter* sp.	MZ570560	99	99.44	0	0.0
2-D1	*Pseudoalteromonas* sp.	MZ570561	98	97.15	0	0.0
3-D1	*Bacterioplanes sanyensis*	MZ570562	96	84.99	2	0.0
4-D1	*Vibrio alginolyticus*	MZ570563	95	99.34	0	0.0
5-D1	*Pseudoalteromonas* sp.	MZ570564	98	99.40	0	0.0
6-D1	*Pseudoalteromonas* sp.	MZ570565	96	98.46	0	0.0
7-D1	*Vibrio alginolyticus*	MZ570566	98	99.63	0	0.0
8-D1	*Vibrio alginolyticus*	MZ570567	98	99.06	0	0.0
9-D1	*Vibrio alginolyticus*	MZ570568	99	99.78	0	0.0
10-D1	*Pseudoalteromonas* sp.	MZ570569	98	93.77	5	0.0
11-D1	*Grimontia celer*	MZ570570	99	99.06	0	0.0

Table 2: Bacterial community profiles associated with *K. alvarezii* during day 30 cultivation.

Bacterial ID	Bacterial species	Blast outputs				
		Accession number	Query cover (%)	Identities (%)	Gaps (%)	E-value
1-D30	*Phaeobacter* sp.	MZ570571	94	98.52	0	0.0
2-D30	*Vibrio* sp.	MZ570572	100	98.34	0	0.0
3-D30	*Ruegeria* sp.	MZ570573	95	99.22	0	0.0
4-D30	*Bacillus aquimaris*	MZ570574	99	99.19	0	0.0
5-D30	*Thalassospira profundimaris*	MZ570575	95	99.47	0	0.0
6-D30	*Alteromonas abrolhosensis*	MZ570576	98	99.41	0	0.0
7-D30	*Pseudoalteromonas* sp.	MZ570577	99	99.70	0	0.0
8-D30	*Vibrio mediterranei*	MZ570578	97	99.27	0	0.0
9-D30	*Bacterioplanes sanyensis*	MZ570579	99	99.04	0	0.0
10-D30	*Alteromonas macleodii*	MZ570580	95	100	0	0.0

over time. Specifically, more prevalent bacteria were defined as those that not only maintained higher population counts but also exhibited a consistent presence from day 1 to day 30. In the present analysis, *Vibrio* emerged as the most prevalent genus, followed by *Pseudoalteromonas*, *Alteromonas*, *Phaeobacter*, *Bacterioplanes*, *Grimontia*, *Ruegeria*, *Thalassospira* and *Bacillus*. Among these genera, *Vibrio*, *Pseudoalteromonas* and *Alteromonas*, known for their carrageenase enzyme production, have been reported to significantly influence carrageenan metabolization in red algae, potentially through extracellular enzymatic activity and the creation of biofilm microenvironments that can affect nutrient availability and carrageenan breakdown (Yusriyyah *et al.*, 2021). The present results are consistent with previous studies, which have reported the presence of *Vibrio*, *Pseudoalteromonas*, *Alteromonas*, *Bacillus*, *Ruegeria*, *Phaebacter*, *Flavobacterium*, *Planctomycetes* and *Bacteroidetes* in association with *K. alvarezii* in both its natural habitat and sea cultivation settings (Syafitri *et al.*, 2017; Riyaz *et al.*, 2019; Kopprio *et al.*, 2021). Nevertheless, it is important to note that a direct comparison of bacterial community profiles between land-based cultivation systems and the natural marine environment remains constrained due to a lack of information in the literature.

Some of the genera identified in this study were associated with both healthy and diseased *K. alvarezii*. This indicates that certain bacterial groups may either benefit from the macroalgae or pose a biofouling threat to them. Throughout the course of this study, numerous *Vibrio* members were detected in the cultivation tanks. Although the cultured seaweeds remained healthy over the 30-day cultivation period with no severe tissue deterioration, it has been documented that while certain *Vibrio* species can have advantageous impacts on seaweed, others could potentially act as pathogens with detrimental effects. For instance, *Vibrio alginolyticus* was isolated from healthy thalli, suggesting that these bacteria may not be related to *Vibrio*-induced ice-ice disease and may be neutral or beneficial to seaweed growth. However, there is a contradiction in other scientific literature in which the same bacterium was identified as a pathogen in *K. alvarezii*, leading to bleaching of the seaweed thallus within the first 12 h post-infection (Azizi *et al.*, 2018; Rahman *et al.*, 2020). Pathogenicity experiments demonstrated in another study that *V. alginolyticus* was a secondary inducer of the ice-ice disease in *Kappaphycus* seaweed. This suggests that the primary induction of ice-ice disease may be attributable to unfavorable environmental conditions, followed by the involvement of a bacterial symbiont or other bacterial

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species on thalli, as opposed to only *Vibrio* (Tahiluddin *et al.*, 2021). In addition, other researchers have reported that *Vibrio* sp. proliferates on stressed *K. alvarezii* thalli, triggering an early onset of ice-ice disease in stressed tissue. However, the increase in *Vibrio* cell density on non-stressed thalli did not instantaneously result in the development of ice-ice disease (Azizi *et al.*, 2018).

Pseudoalteromonas is known for their diverse metabolic capabilities and ability to form biofilms on *K. alvarezii* surfaces (Barzkar *et al.*, 2022). An earlier investigation revealed that members of *Pseudoalteromonas* were primarily detected in *K. alvarezii* that was infected with disease. As observed in cases of kelp decay and in the thallus of *Laminaria japonica*, an overpopulation of *Pseudoalteromonas* sp. could have detrimental effects on the seaweed (Azizi *et al.*, 2018). According to Syafitri *et al.* (2017), *Pseudoalteromonas issachenkonii* was accountable for the symptoms of ice-ice disease in farmed *K. alvarezii*. Additionally, this genus is known for having a considerable impact on carrageenan production in red algae. For example, certain *Pseudoalteromonas* sp. can use both κ-carrageenan and ι-carrageenan as sources of energy (Hettle *et al.*, 2019). According to Yusriyyah *et al.* (2021), all *Pseudoalteromonas* strains were determined to be Gram-negative bacteria and exhibited carrageenan-degrading capacity via Congo red staining. This is corroborated by the research of Chauhan and Saxena (2016), who demonstrated that only Gram-negative bacteria produce the extracellular carrageenase enzyme, allowing them to use carrageenan as an energy source. Moreover, certain strains isolated from marine biofilms demonstrated the ability to produce alginate lyase enzymes for using alginate as a carbon source – a crucial nutrient for marine organisms (Daboor *et al.*, 2021; Barzkar *et al.*, 2022). These findings underscore the importance of monitoring and managing *Pseudoalteromonas* populations in *K. alvarezii* cultivation to ensure optimal seaweed health and carrageenan production.

Alteromonas bacteria, like *Pseudoalteromonas*, are frequently found on the surface of *K. alvarezii*, engaged in interactions with seaweed that can be beneficial or possibly destructive. In a recent study, *Alteromonas* sp. emerged as a prominent species of seaweed-associated bacterium discovered in tissue-cultured samples, potentially harmful to *K. alvarezii* (Azizi *et al.*, 2018). Notably, *Alteromonas* sp. was capable of functioning as a pathogenic agent responsible for inducing ice-ice disease symptoms (Syafitri *et al.*, 2017). For example, this genus was reported to be the causative agent of ice-ice disease symptoms in *K. alvarezii* harvested from Karimunjawa Island, Indonesia. In comparison to other studied strains, *Alteromonas macleodii* exhibited the highest level of pathogenicity to *K. alvarezii* (Azizi *et al.*, 2018). Furthermore, *Alteromonas* sp. has been shown in previous research to be capable of degrading certain polysaccharides found in red algae, such as alginate (Neumann *et al.*, 2015), ulvan (Koch *et al.*, 2019) and ι-carrageenan (Barbeyron *et al.*, 2019). The ability to break down polysaccharides derived from *K. alvarezii* indicated that this genus is predominantly dependent on host cells for nutrition. While the current work focuses primarily on determining the dynamics of bacterial communities associated with *K. alvarezii* cultivation, it is important to recognize synergistic interactions across bacterial groups such as *Vibrio* sp., *Alteromonas* sp. and *Pseudoalteromonas* sp. may play an important role in ice-ice disease and seaweed health. However, researching these interactions involving multiple bacterial combinations is a complicated and intensive study that may necessitate a more extended examination to understand their complexity and specific impacts on seaweed health and disease dynamics.

This study also discovered genera with only a few strains observed on *K. alvarezii*, including *Phaeobacter*, *Bacterioplanes*, *Grimontia*, *Ruegeria*, *Thalassospira* and *Bacillus*. *Phaeobacter* sp. was previously reported to have been detected in the red alga *Tichocarpus crinitus* and was recognized for its ability to degrade carrageenan (Kalitnik *et al.*, 2017; Yusriyyah *et al.*, 2021). Among these, *Ruegeria* sp. was identified as the most frequently occurring bacterium associated with seaweed in tissue-cultured conditions, likely due to its specialized adaptations, competitive advantages and potential symbiotic interactions with the seaweed (Azizi *et al.*, 2018). *Bacillus* sp., such as *Bacillus aquimaris*, was found mostly in nutrient enriched *Kappaphycus striatus*. This bacterial species is moderately halophilic and has the potential to be a source of bioactive compounds with significant biotechnological value (Hernández-González and Olmedo-Álvarez, 2016). Furthermore, the genera *Grimontia*, *Thalassospira* and *Bacterioplanes* are marine bacteria found in diverse marine environments, including seawater (Almeida *et al.*, 2023), deep ocean sediments (Zhao *et al.*, 2023) and marine aquaculture farms (Thiang *et al.*, 2022), but their association with any red algae has not been previously described.

Construction of phylogenetic tree

To assess the evolutionary relationships between bacterial species, a phylogenetic tree depicting the relationships between 21 bacterial species was constructed with *Moritella dasanensis* as the outgroup using the NJ method, as illustrated in Figure 1. The phylogenetic diagram revealed three dominant phyla: Proteobacteria (*Vibrio* and *Thalassospira*), Pseudomonadota (*Pseudoalteromonas*, *Alteromonas*, *Grimontia*, *Ruegeria*, *Phaebacter* and *Bacterioplanes*) and Firmicutes (*Bacillus*). With the exception of Bacteroidetes, which was absent in the current closed system cultivation, this result aligns with previous studies that identified the bacterial phyla associated with *K. alvarezii*, including Proteobacteria, Firmicute and Pseudomonadota (Riyaz *et al.*, 2019; Kaur *et al.*, 2023).

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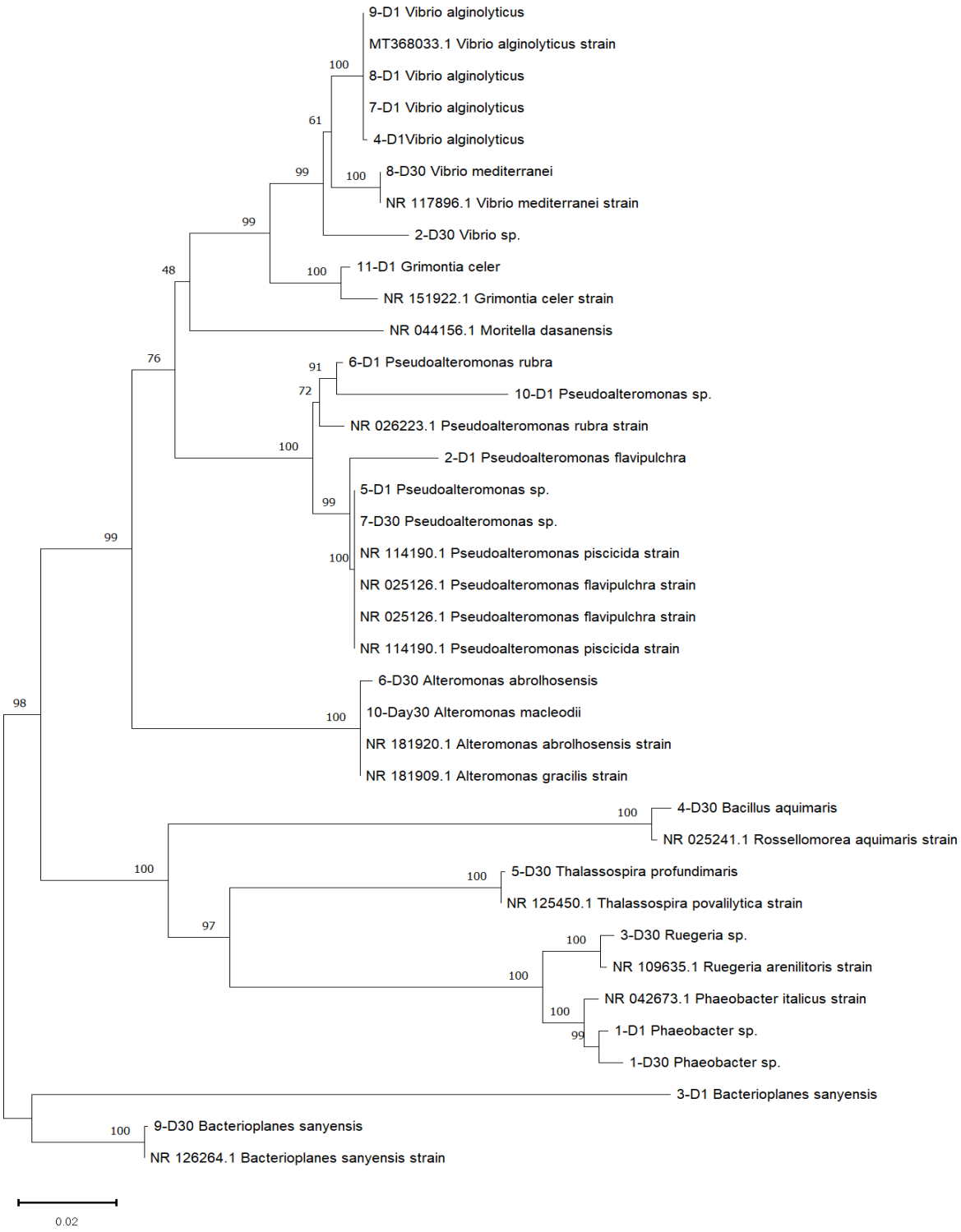

Figure 1: Phylogenetic analysis of the bacterial isolates MZ570560 to MZ570580. Other accession ID except the studied isolates includes MT368033.1, NR117896.1, NR151922.1, NR044156.1, NR026223.1, NR114190.1, NR025126.1, NR114190.1, NR181920.1, NR181909.1, NR025241.1, NR125450.1, NR109635.1, NR042673.1, NR126264.1. Evolutionary history was inferred using the Neighbor-Joining method. This analysis involved 37 nucleotide sequences. Evolutionary analyses were conducted in MEGA X.

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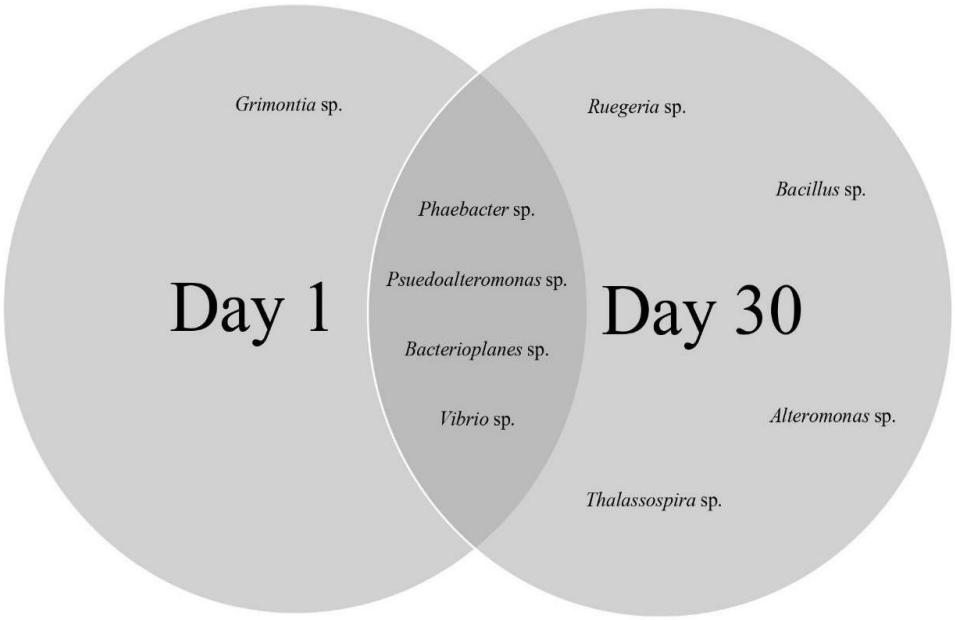

Figure 2: Venn diagram of the overlapped and distinct bacterial community found on day 1 and day 30 of seaweed cultivation in the closed circulation system. The Venn diagram was plotted in Microsoft Excel 365 software.

Comparison of bacterial profiles on day 1 and 30 of cultivation

The data suggests that certain genera observed on day 1 of *K. alvarezii* cultivation remained present on day 30, including *Vibrio*, *Pseudoalteromonas*, *Alteromonas*, *Phaebacter* and *Bacterioplanes*, as depicted in Figure 2. These bacteria appear to demonstrate adaptability within an extended closed cultivation system for *K. alvarezii* cultures. Several of these bacteria exhibit unique capabilities that may contribute to their persistence over the 30-day cultivation period. This adaptability could potentially be attributed to factors such as metabolic versatility and biofilm formation, which are known to facilitate their successful adaptation in extended cultivation environments (Riyaz *et al.*, 2019; Wu *et al.*, 2021). While these hypotheses are founded on existing research, they should be viewed as plausible explanations rather than definitive confirmations solely dependent on the presence of specific bacterial species. Therefore, it is essential to recognize the necessity for further analysis to verify these findings across multiple batches, increasing the robustness of the findings.

The metabolic versatility of these bacteria within long-term *K. alvarezii* cultures allows them to adapt and multiply by metabolizing a diverse range of nutrients or compounds derived from the host for energy and carbon sources (Riyaz *et al.*, 2019). Previous research has proposed that pathogenic *Vibrio* sp. exploit host thalli through motility, rapidly adhering to and colonizing seaweed tissue as an initial step. Subsequently, this genus has the capability to utilize carrageenan found in seaweed thalli as a carbon source. This process involves penetrating the medullary layer of infected branches and establishing mechanisms for long-term residence on host cells, potentially resulting in an antagonistic relationship between *Vibrio* cells and *K. alvarezii* cells that contributes to their coexistence (Tahiluddin and Terzi, 2021). It has also been suggested that the *Pseudoalteromonas* genus can synthesize κ-carrageenase enzymes, allowing for the degradation of κ-carrageenan in *K. alvarezii*, which could serve as a carbon source for the genus. However, it is important to note that such activities have been associated with the induction of ice-ice disease symptoms, leading to thalli whitening (Riyaz *et al.*, 2019). Similarly, the genus *Alteromonas* has been shown to possess the ability to metabolize polysaccharides such as alginate, ι-carrageenan, and κ-carrageenan (Yusriyyah *et al.*, 2021), implying their access to carbon sources for survival while residing on *K. alvarezii* over an extended period. These proposed mechanisms provide potential insights into the adaptability of these bacteria within the seaweed cultivation system. However, further research is required to confirm these hypotheses and establish a more comprehensive understanding of the interactions involved.

Bacterial biofilm plays a crucial role in facilitating the attachment of bacteria to biomaterial surfaces, thereby enhancing interactions between the bacteria and the attached substrate. It is possible that the bacteria identified in this study employ biofilm mechanisms to persist on *K. alvarezii* cultures over the long term (Lage and Graça, 2016). Recent studies have demonstrated that certain genera, including *Vibrio*, *Pseudoalteromonas* and *Alteromonas*, possess robust biofilm-producing capabilities (Wu *et al.*, 2021). Particularly, *Pseudoalteromonas* has consistently been noted as a formidable biofilm producer in marine environments

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(Favre *et al.*, 2018). Experiments investigating the biofilm-producing potential of *Pseudomonas* sp. have suggested that pyruvate and carbon metabolism likely play crucial roles in facilitating the attachment of *Pseudoalteromonas* sp., aiding this genus in adapting to a long-term *K. alvarezii* cultivation system (Wu *et al.*, 2021). Additionally, due to its potential to produce tropodithietic acid and infiltrate pre-existing marine biofilms, the genus *Phaebacter* has been identified as a key contributor to the formation of microbial biofilm communities in natural seawater (Bech *et al.*, 2023). Interestingly, another study has revealed that the genus *Bacterioplanes* has the potential to degrade N-acylhomoserine lactones (AHLs) molecules, which are used to reduce biofilm formation by *Pseudomonas aeruginosa*. Exploring the potential of this genus to act as a prebiotic agent in *K. alvarezii* ecosystems warrants further investigation (Rehman and Leiknes, 2018).

Limitation of the research

While the bacterial species profiles discovered in this study are comparable to those found in previous studies, the scope of isolated bacterial species is limited to those that can be cultured. The methodology used in this study specifically targets the 16S ribosomal DNA gene, which is ubiquitous in both bacteria and archaea (Church *et al.*, 2020). By sequencing this gene, researchers can determine the bacterial species present in a given sample and derive their evolutionary relationships. Though this method provides insights into the composition of microbial communities, it does not directly reveal the functional roles of distinct species (Johnson *et al.*, 2019; Liu *et al.*, 2021).

Advanced approaches with increased throughput, such as metagenomics amplicon sequencing and metatranscriptomics sequencing, are recommended for a more comprehensive understanding. The extraction of DNA or RNA from mixed bacterial samples for metagenomics sequencing provides useful insights into the composition of microbial communities in a range of environments. Furthermore, by analyzing the genes present in the sample, it provides insight into the functional capacities of these microorganisms (Liu *et al.*, 2021). Metatranscriptomics, on the other hand, focuses on the sequencing of RNA transcripts (mRNA) within a microbial community, looking for genes that are actively expressed and so providing information about the functional roles of bacteria under specific environmental conditions (Jiang *et al.*, 2016). These methodologies can be used individually or in combination to acquire a better understanding of the identities and functional roles of various bacterial species found in complicated environmental samples.

Ecological and practical significance

The comparison of these two bacterial groups from *K. alvarezii* cultures on day 1 and day 30 shows that persistent marine bacteria can successfully adapt to long-term cultivation in closed circulation systems, particularly during the early growth or seedling production phase of the seaweed. These persistent bacteria, including those from the genera *Vibrio*, *Pseudoalteromonas*, *Alteromonas* and *Bacterioplanes*, can establish symbiotic relationships with *K. alvarezii* over time. However, in previous studies, *Vibrio* sp. has been identified as a potential pathogen, while *Pseudoalteromonas* and *Alteromonas* were found to contribute to disease infection in stressed tissue but were non-pathogenic in healthy tissue. To prevent overpopulation of these bacteria on *K. alvarezii* cultivation, especially in enhancing defenses against pathogens like *Vibrio*, the introduction of prebiotic microorganisms, such as the *Halomonas* strain, is crucial (Azizi *et al.*, 2018). Furthermore, the genus *Bacterioplanes*, isolated in this study, has previously been shown to be capable of degrading AHLs molecules, enabling them to control disease infections and the formation of biofilms by microorganisms such as *Pseudoalteromonas* and *Alteromonas* through a mechanism known as quorum quenching (Rehman and Leiknes, 2018). These findings suggest that continuous enrichment of *K. alvarezii* cultures can fortify the seaweed's defense system, making it more resistant to pathogens and diseases. For instance, under unfavorable conditions, opportunistic pathogenic bacteria such as *Vibrio* can exploit the vulnerable state of *Kappaphycus* thalli. Thus, maintaining optimal seaweed health through continuous nutrient enrichment may serve as a strategic approach to prevent the infiltration of these harmful bacteria into the seaweed tissue (Largo *et al.*, 1995). Additionally, periodic enrichment with both organic and inorganic nutrients has been shown to enhance the protective mechanisms of seaweed against pathogens like *Vibrio* and numerous diseases (Tahiluddin *et al.*, 2021). These strategies can be particularly significant in the context of land-based seaweed cultivation, where conditions may differ significantly from marine environments. Addressing these differences and proposing strategies for optimizing biomass production and safeguarding seaweed health in land-based systems is crucial for the sustainability of seaweed cultivation. Furthermore, considering the specific challenges and potential pathogens relevant to land-based farming, as well as how identified bacteria can impact seaweed cultures, is essential for a comprehensive approach to seaweed production.

CONCLUSION

This study presents a preliminary identification of persistent marine bacterial community profiles associated with the extended cultivation of *K. alvarezii* in a closed cultivation setting. These findings establish the rationale for refining the *K. alvarezii* cultivation system and expanding our knowledge of the microbial ecology involved in this land-based cultivation technique. However, it is important to note that the identification of bacterial species is limited to those that can be cultured. It is essential to recognize that some bacteria may be

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challenging to culture due to various factors such as specific growth requirements, nutritional needs, or the presence of unculturable or viable but non-culturable cells. Therefore, future research could harness the power of high-throughput sequencing, a cutting-edge technology that allows for rapid and parallel sequencing of numerous DNA or RNA fragments, to gain a more accurate depiction of the overall bacterial diversity and the potential functional roles of specific bacterial species associated with *K. alvarezii*. While this study provides valuable insights into the preliminary identification of bacterial communities associated with *K. alvarezii*, the observed differences in bacterial community profiles between day 1 and day 30 highlight the need for further research to comprehensively understand the dynamic interactions between bacteria and *K. alvarezii* during land-based cultivation.

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