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Research article

Epiphytic bacterial community analysis of the macroalgae *Gongolaria barbata* collected from the Sinop region on the Black Sea coast

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Abstract

The purpose of this study was to ascertain the epiphytic bacterial community structure of macroalgae *Gongolaria barbata* (Stackhouse) Kuntze samples taken from seawater using Single Strand Conformation Polymorphism (SSCP) analysis. It also aims to quickly obtain information regarding the composition of communities and the quality of the seawater. *G. barbata* samples were subjected to total DNA extraction, SSCP analysis was conducted with a focus on the V4-V5 region of 16S rRNA, and the bacterial community structure was determined through sequence analysis of a few chosen bands. Upon analyzing the SSCP gel picture and dendrogram, it was seen that the bacterial community structure on the macroalgae varied based on the location as well as within the same species. It was noted that the *Gammaproteobacteria* class accounted for 84.375 percent of the bands that were acquired from the SSCP analysis. The fact that the sequencing data generated from the bands collected at various points largely resembled *Vibrio* and *Klebsiella* genera was notable. This situation highlights the strong link between harmful or opportunistic infectious organisms and macroalgae species, several of which have been suggested for ingestion as food. Furthermore, even if research in the literature suggests that the macroalgae and the microbial load of the nearby water sample do not significantly correlate, we can conclude that this data suggests the possibility of risk.

Keywords: *Gongolaria barbata; bacterial community; SSCP; water quality; infectious agents*

1. Introduction

Macroalgae are multicellular, sessile, photosynthetic eukaryotic organisms that function as primary producers in marine environments by giving a variety of creatures food and shelter (Florez et al., 2017; Xiao et al., 2024). Microbial communities have a place to live on the surfaces of organisms that inhabit the maritime environment. The region of close algae-bacteria interactions is termed the "phycosphere" (Lu et al., 2023). In this sense, microbial communities benefit greatly from the shelter provided by macroalgae. The ability of macroalgae to create oxygen and organic matter, together with the bacterium's availability of minerals and CO_2, determines the connection between macroalgae and bacteria. Additionally, it is known that certain bacteria release regulatory factors resembling auxin and cytokinin, which promote increased plant cell division (Singh and Reddy, 2014; Comba-González et al., 2016).

Exchange activities including waste products, secondary metabolites, and nutrient uptake and release are all carried out on algal surfaces. On these surfaces, bacteria create a biofilm layer and encounter the seaweeds. It impacts the host organism's resistance, performance, and general health in this way. Additionally, it controls the ingress of light, gas, nutrients, pathogens, consumers, and other epibionts that form biofilms in seaweed (Mancuso et al., 2016; Nahor et al., 2024). It is well recognized that the host conditions, in addition to space and time, affect the structure and composition of the microbiota linked to seaweed. For instance, it has been noted that distinct microbial communities are displayed by stressed versus healthy *Ecklonia radiata*. It has also been demonstrated that seasonal

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variations have an impact on the variations in microbial populations. Abiotic variables like water temperature and biotic parameters like the age of the algal tissue and seaweed growth cycle play significant roles in these seasonal fluctuations (Bengtsson et al., 2010; Serebryakova et al., 2018). Macroalgae are regarded as the ecosystem's engineers as they are crucial to the structure of coastal communities. Accordingly, a significant decline in biodiversity is thought to result from the loss of macroalgae in coastal environments (Burke et al., 2011). Bacteria known as the *Vibrio* genus can be found in a wide range of habitats, including aquatic and marine ones. Particularly in humans, these bacteria can lead to illnesses both inside and beyond the gastrointestinal tract. There are almost 200 serogroups of *Vibrio cholerae* in this genus of bacteria. Serogroups O1 and O139 have the potential to become epidemics. When the water is at its hottest, which is in late summer and early autumn, they increase illnesses (Araj, 2019). For thousands of years, wild macroalgae have been consumed by humans. Nowadays, aquaculture methods are used to generate the majority of macroalgae species. Although it has not been assessed, it has been suggested that macroalgae collected from coastal waters might be near a source of potentially harmful bacteria to humans. Fecal coliforms play a crucial role in the digestive system as they are naturally occurring bacterial colonizers and are most prevalent in coastal waters. One of the members of this group, *Escherichia coli*, is regarded as both a pathogen and a sign of fecal contamination. Fecal coliforms are therefore indicative of gastrointestinal pathogen infection in food and drink. In addition to fecal coliforms, nutritional safety is also correlated with the presence of *Vibrio*, a naturally occurring microbe found in coastal waters. This species has certain diseases that affect humans. According to Barberi et al. (2020), *Vibrio parahaemolyticus* is the primary cause of food-borne gastroenteritis in the United States.

To identify bacterial species from environmental samples, numerous techniques are employed. The culture and isolation approach are the most important of them. However, bacterial diversity and isolates that are not identified in that environment are difficult or impossible to detect with this technique. Rather, it was mentioned that molecular methods based on 16S rRNA amplification and sequence analysis would be used to tackle this issue (Agrawal et al., 2015; Franco-Duarte et al., 2019). In addition, many methods based on metagenomics, meta-transcriptomics, metabolomics, and other omics-based techniques have been applied to detect microbial communities in their natural environment (Vigil et al., 2024). Thus, the synergistic use of both culture-dependent and independent approaches will enable the identification of not only dominant microorganisms but also rare taxonomic members (Girão et al., 2024). Here, it is becoming increasingly important to investigate epiphytic bacterial communities in different macroalgae in more detail using new technologies, especially to enable the production of various metabolites through biotechnological applications (Kaur et al., 2023).

In the Black Sea region, edible brown seaweed called *Gongolaria barbata* is typically utilized as a functional food. Because of their high concentration of bioactive compounds, vitamins, minerals, polyphenols, fatty acids, and peptides, edible seaweeds have significant value as functional foods. They are effective against a variety of diseases, including cancer, heart disease, type 2 diabetes, and autoimmune diseases (Trica et al., 2019).

However, Barberi et al. (2020), there is little research on the contamination of sea vegetable products with harmful bacteria in the literature review. Once more, Barberi et al. (2020) revealed that several researchers in Europe found diseases in natural macroalgae. Specifically, they found *Listeria monocytogenes* (Blikra, 2019) in Norway, coliform, enterococci, and *Vibrio* in macroalgae in Japan (Mahmud, 2008). Our analysis of the literature indicates that no study has been conducted in Turkey that thoroughly screens for bacterial pathogens on macroalgae. The purpose of this study was to identify the epiphytic bacterial community of *G. barbata* samples that were taken from the Black Sea-coasting province of Sinop.

2. Materials and methods

2.1. Collection of samples and isolation of DNA

Samples were taken from Sinop's coast at a depth of 0.2-2 meters (Fig. 1) in July 2019. Following collection, the specimens were cleaned, and vouchers were created. DNA was extracted from the apical section of every thallus. A CTAB protocol modification was implemented (Wichachucherd et al., 2014). First, 500 µl of CTAB buffer was used to grind the tissue samples using a tissue grinder. The tissue was ground and then incubated for 20 minutes at 60°C. After thoroughly mixing the aqueous phase twice using CIA (chloroform: isoamyl alcohol, 24:1), the mixture was centrifuged at 15000 g for 10 minutes at +4°C. Lastly, an equivalent volume of cool isopropanol was added to the aqueous phase. At -20°C, the sample was incubated for an entire night. Following the incubation for the entire night, the tube was centrifuged at 15000 g for 20 minutes. After washing the DNA pellet in 70% ethanol, the sample was centrifuged at 15000 g for an additional 10 minutes. The DNA was removed from the ethanol, allowed to dry at room temperature, and then kept in Tris-EDTA buffer until needed.

Fig. 1. Station map where *G. barbata* specimens were collected. S1, Gerze; S2, Sinop inner port; S3, Akliman; S4, Inceburun; S5, Ayancik (Map taken from Google maps).

2.2. SSCP analysis of 16S rRNA gene regions for culture-independent community structure

In a prior study conducted in our lab, the steps for SSCP, DNA recovery from gel, and sequence data processing were thoroughly described (Avsar and Aras, 2020). The methods will be briefly discussed below. Com1 and modified com2-Ph primers specific for the 16S rRNA gene region were utilized for SSCP analysis. The techniques of Schwieger and Tebbe (2000)

and Smalla et al. (2007) were used to achieve the SSCP analysis. Using the Expin Combo GP (GeneAll) purification kit, PCR products were purified. To cleave the phosphorylated chain, 700 ng of the purified PCR product was incubated with 5U Lambda-exonuclease (Thermo Fisher Scientific, California) for 1 hour at 37°C. Using the Expin Combo GP (GeneAll) purification kit, single-chain DNA was purified. A 10 µL purified single-chain DNA sample was placed into four microliters of loading solution, which contained 95% formamide, 10 mmol/L NaOH, 0.025% bromophenol blue, and 0.025% xylene cyanol. After the samples were promptly chilled on ice after being denatured for two minutes at 95°C, 5 µL was put onto the gel. Using Hoofer (SE400, USA) equipment, a mixture of 0.6X MDE (Mutation Detection Enhancement, Thermo Fisher Scientific, Lonza) gel was produced for electrophoresis. For 36 hours, the gel was operated at 5 mA, 200 V, and 20°C. The silver staining approach was used after the DNA profiles from the gel were seen using Byun et al. (2009).

A sterile scalpel was used to cut any dominating or solitary bands found on the polyacrylamide gel after silver staining to prepare them for further examination. After the gel fragments were transferred, 100 µL of elution liquid (0.5 mol/L ammonium acetate, 10 mmol/L Mg^{2+}-acetate, 1 mmol/L EDTA [pH 8.0], and 0.1% SDS) was added to the microtubes. After three hours of incubation at 37°C, the tubes were centrifuged at 12,000×g for one minute at room temperature. Two volumes of cold ethanol (96%) were added to 80 µL of the supernatant, which was then transferred to a micro test tube to precipitate. Following a 7-minute centrifugation at 12,000×g, the DNA was allowed to dry at 30°C for 30 minutes before being dissolved in 10 mmol/L of Tris-HCl at pH 8.0. This solution's target DNA for PCR processing was two microliters (Schwieger and Tebbe, 1998). As previously mentioned, the Com1 and Com2 primer PCR procedure was carried out.

Sequencing was done on amplified products (BM Labs, Ankara). Technelysium Pty Ltd.'s Chromas version 2.24 software was used to alter the sequencing data. The alignment of these sequences was done with Clustal W (Version 2.1). The NCBI GenBank database's BLAST search was used to compare 16S rRNA sequences of bacteria that are phylogenetically related. PyElph version 1.4 was used to produce the UPGMA dendrogram analysis of the SSCP profiles of the bacterial populations. Using Molecular Evolution Genetic Analysis (MEGA), phylogenetic trees were created, and the sequences were corrected and aligned using the Clustal W tool.

3. Results and discussion

The bacterial population from our investigation, which was derived from SSCP analysis of *G. barbata* samples collected from 17 distinct locations, is displayed in Fig. 2. Based on the acquired results, it was noted that samples taken from the same and various sites had both comparable and dissimilar band profiles. Fig. 3 displays the dendrogram that was produced based on the band profiles. It was, therefore, observed that the samples obtained from the 17 sites were split into two fundamental groups (X and Y). These groupings were seen to be split into two subgroups within themselves once more. The distribution of samples 1, 2, and 3 from the closest station, for instance, shows that the band profiles acquired from various samples taken from the same region are included in groups that are separated from one another.

Based on samples collected from 17 distinct sites, as seen

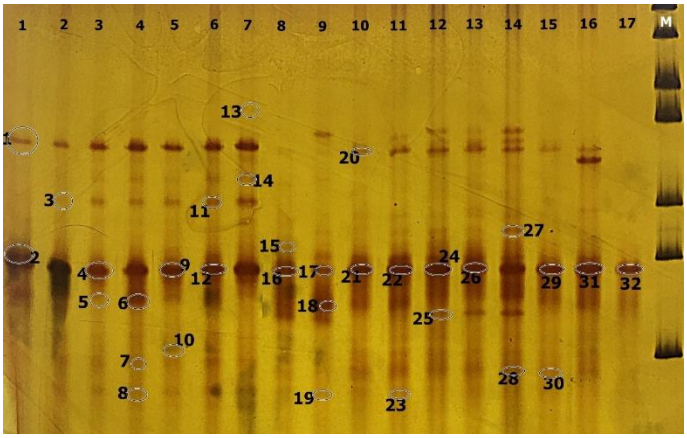

Fig. 2. SSCP band profiles of 17 different points (bands circled are those for which sequence analysis was performed). M, Marker.

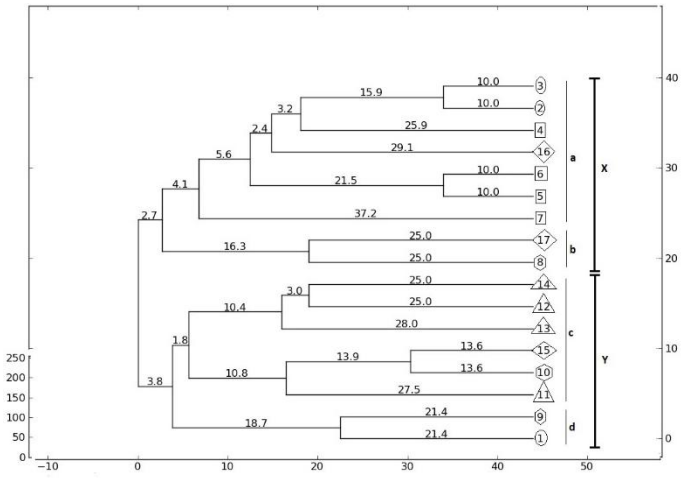

Fig. 3. UPGMA dendrogram analysis of SSCP profiles of bacterial communities from 17 different *G. barbata* samples.

in Fig. 2, 32 dominant and distinct bands were chosen, and the genus and/or species with the highest degree of similarity were identified using sequencing analysis and are listed in Table 1. A closer look at Fig. 2 reveals that certain bands, chosen for their distinctiveness rather than their dominance, represent unique types of microorganisms. Table 1 displays the sequence similarity data for this, which are displayed in bands 7, 8, 10, 19, 23, 25, 28, and 30. This demonstrates the significance of this method. Upon closer inspection of Table 1, it became evident that 84.375 percent of the bacterial groups identified were members of the *Gammaproteobacteria* class. It was discovered that two samples (6.25%) belonged to the phylum *Bacteroidetes*, three samples (9.375%) to the phylum *Cyanobacteria*, and only one sample (3.125%) to the class *Alphaproteobacteria*. Furthermore, it was notable that the sequence data revealed certain species of *Vibrio* and *Klebsiella* to be among the most prevalent opportunistic and marine infections. Furthermore, the phylogenetic tree based on the outgroups from NCBI shows the phylogenetic relationships of the results obtained from the band profiles tested (Fig. 4). In addition, many bacteria that were previously isolated from sediment and marine samples have been identified. Research has indicated that bacterial phylotypes in algae and the surrounding saltwater may differ from one another. In addition to this distinction, it has also been reported that bacteria and their host algae enter into antagonistic relationships with each other (Yang et al. 2023). They believed that the circumstances were connected to the antibacterial

qualities of macroalgae. For instance, whereas *E. coli* was found in the water and surrounding algae in certain investigations, it was not found on seaweed in other examinations. According to their ongoing research (Wiese et al., 2009; Michelou et al., 2013; Barberi et al., 2020), components of the algal bacterial community shown action against *E. coli*. In a prior work, we discovered that macroalgae extracts had microbiological activity against a variety of bacteria, including *E. coli* (Berber et al., 2015), which lends support to these investigations.

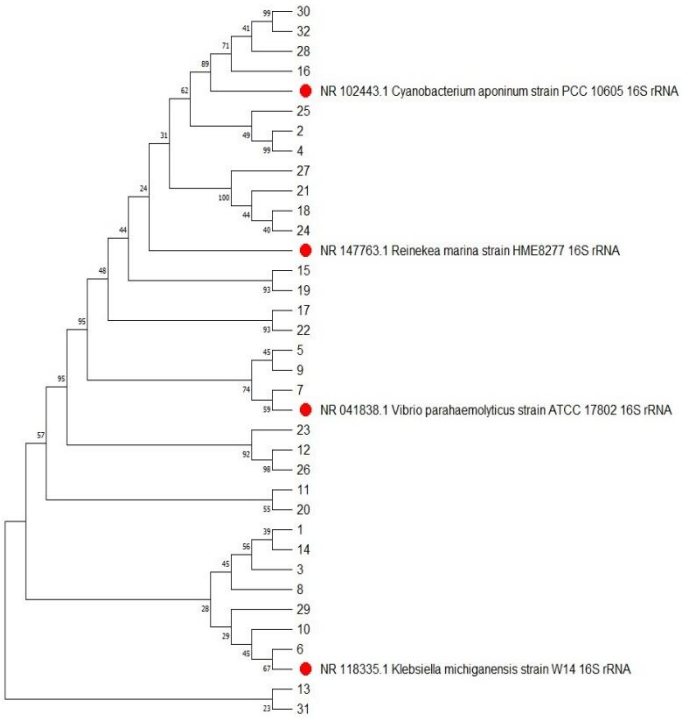

Fig. 4. Phylogenetic tree based on rRNA gene region sequence analysis. Red dots indicate strains outgroups from NCBI as understood from the "accession numbers" in front of their names.

The Neighbor-Joining approach was used to infer evolutionary history (Saitou and Nei, 1987). The ideal tree is shown. The percentage of replicate trees in which related taxa were grouped together in the bootstrap test (1000 replicates) is given next to the branches (Felsenstein, 1985). The tree is shown to scale with branch lengths expressed in the same units as the evolutionary distances used to estimate the phylogenetic tree. Evolutionary distances are shown as base changes per site and were calculated using the Maximum Composite Likelihood approach (Tamura et al., 2004). There were 36 nucleotide sequences in this study. For each sequence pair, all ambiguous sites were eliminated (pairwise deletion option). There was a total of 413 positions in the final dataset. Evolutionary analyses were performed in MEGA11 (Tamura et al., 2021).

According to Lachnit et al. (2011), heteroduplex formations, co-migrations on the gel, and potential variations within multiple copies of 16S rRNA genes can all have an impact on the band motif composition and density, therefore the DGGE technique they used to identify the epiphytic bacterial community only offers a rough overview. We can remark that the SSCP technique we utilize has similar drawbacks to those stated for this circumstance. In their investigation of the bacterial contamination of macroalgae, Barberi et al. (2020) used the membrane filtering approach to identify *E. coli*, *V. parahaemolyticus*, and *V. alginolyticus*. They claimed that the manner of counting was too low. Using molecular techniques, enterohemorrhagic *E. coli* O157:H7, *V. parahaemolyticus*, and *Salmonella enterica* ser. *typhimurium* were identified in the same samples. In their investigation on the microbiological quality of seaweeds, Blikra et al. (2019) found that spore-forming bacteria are present and that the microbial number is modest (1-3 log cfu g^{-1}). Additionally, they stated that while they identified *Bacillus* species, they were unable to find *Listeria monocytogenes*, pathogenic vibrio, coliforms, or enterococci. Burke et al. (2011) used 16S rRNA sequence sequencing in six *Ulva australis* and the surrounding waters to find that *Alphaproteobacteria* and *Bacteriodetes* were prominent in both settings. They did note that in both settings, the resemblance was not at the species level. The identical sponge samples from Lee et al. (2009) and brown algae from Staufenberg et al. (2008) revealed more diversity at the species level but more similarity at higher taxonomic levels in the microbial communities. In a planned investigation, Lachnit et al. (2011) used the DGGE technology to look at the bacterial population of three distinct macroalgae species at different dates. The results of 16S rRNA sequence analyses revealed strong species-level similarity between replicates, but also differences in bacterial communities between three algal species and between summer and winter samples of the same algal species. According to Mancuso et al. (2016), there is a dearth of knowledge regarding the interactions between epiphytic bacteria, despite the abundance of studies on the diverse macrofauna and flora connected to *Cyctoseria* (*sensu lato)*. They examined the interaction between the surrounding water and the bacterial communities in *Cystoseira compressa* in their investigation. The study's findings showed that the predominant sequences on algae and in water samples were those of *Proteobacteria* and *Bacteriodetes*. Furthermore, they discovered that only a few common species varied between the two ecosystems. A study on the connection between the organization of bacterial communities and two distinct *Labophora* macroalgae species was carried out by Vieira et al. (2016). They discovered that the two species' bacterial populations differed. They demonstrated that almost half of the OTU is shared by both species. They stated that *Planctomycetes*, *Proteobacteria*, and *Bacteriodetes* are the taxa to these species-specific OTUs belong. In addition, they identified sixteen culturable isolates from different algae species and discovered that these isolates were connected to corals nearby. The bacterial communities linked to *Ulva rigida* macroalgae, which Califano et al. (2020) gathered in aquaculture and coastal habitats, were shown to be distinct. Additionally, the *Proteobacteria* phylum in algae and the *Bacteriodetes* phylum in water samples were found to be prevalent by the researchers. In a planned investigation, Aires et al. (2016) investigated the association between two invasive seaweed species from the Atlantic Island coastal region and their bacterial communities. They discovered variations between the two species' bacterial communities. They also found that bacterial communities are host-specific and influenced by their surroundings in their investigation. Serebryakova et al. (2018) looked at the temporal and geographical alterations of the microbial community linked to the invasive brown seaweed *Sargassum muticum* in a different study. The researchers noticed that the seaweed displayed alterations based on the structural difference, even though they had anticipated microbial community differences based on the area and the sample month. They therefore show the significance of structural microscale variations in seaweeds, which are hosts connected to microbial populations. Tujula et al. (2010) examined the seasonal dynamics and diversity of bacterial

Table 1
Identify the bands with BLAST scanning in NCBI GenBank database.

Band	Blast top search - GenBank accession number	Phylum - Class	Similarity (%)	GenBank accession number
1	*Klebsiella michiganensis* strain W14 - NR_118335.1	Gammaproteobacteria	99.45	MN134438
2	*Labilibacter sediminis* strain CG51 - NR_169488.1	Bacteroidetes	89.10	MN134439
3	*Klebsiella michiganensis* strain W14 - NR_118335.1	Gammaproteobacteria	98.64	MN134440
4	*Labilibacter sediminis* strain CG51 - NR_169488.1	Bacteroidetes	89.37	MN134441
5	*Vibrio alginolyticus* strain ATCC 17749 - NR_118258.1	Gammaproteobacteria	94.86	MN134442
6	*Klebsiella michiganensis* strain W14 - NR_118335.1	Gammaproteobacteria	96.64	MN134443
7	*Vibrio alginolyticus* strain ATCC 17749 - NR_118258.1	Gammaproteobacteria	98.37	MN134444
8	*Klebsiella michiganensis* strain W14 - NR_118335.1	Gammaproteobacteria	98.66	MN134445
9	*Vibrio alginolyticus* strain ATCC 17749 - NR_118258.1	Gammaproteobacteria	92.06	MN134446
10	*Klebsiella oxytoca* strain NBRC 102593 - NR_114152.1	Gammaproteobacteria	98.91	MN134447
11	*Klebsiella oxytoca* strain NBRC 102593 - NR_114152.1	Gammaproteobacteria	97.84	MN134448
12	*Klebsiella michiganensis* strain W14 - NR_118335.1	Gammaproteobacteria	88.24	MN134449
13	*Klebsiella michiganensis* strain W14 - NR_118335.1	Gammaproteobacteria	98.67	MN134450
14	*Klebsiella oxytoca* strain NBRC 102593 - NR_114152.1	Gammaproteobacteria	98.66	MN134451
15	*Alcanivorax nanhaiticus* strain 19-m-6 - NR_152008.1	Gammaproteobacteria	91.30	MN134452
16	*Nodosilinea alaskaensis* strain L32 - NR_172588.1	Cyanobacteria	83.64	MN134453
17	*Vibrio alginolyticus* strain ATCC 17749 - NR_118258.1	Gammaproteobacteria	90.61	MN134454
18	*Klebsiella michiganensis* strain W14 - NR_118335.1	Gammaproteobacteria	94.91	MN134455
19	*Reinekea marina* strain HME8277 - NR_147763.1	Gammaproteobacteria	90.91	MN134456
20	*Klebsiella michiganensis* strain W14 - NR_118335.1	Gammaproteobacteria	95.04	MN134457
21	*Klebsiella michiganensis* strain W14 - NR_118335.1	Gammaproteobacteria	95.40	MN134458
22	*Vibrio ichthyoenteri* ATCC 700023 - NR_113813.1	Gammaproteobacteria	90.53	MN134459
23	*Kosakonia oryziphila* strain REICA_142 - NR_125587.1	Gammaproteobacteria	83.46	MN134460
24	*Klebsiella michiganensis* strain W14 - NR_118335.1	Gammaproteobacteria	94.01	MN134461
25	*Litoreibacter ponti* strain GJSW-31 - NR_134069.1	Alphaproteobacteria	88.89	MN134462
26	*Klebsiella michiganensis* strain W14 - NR_118335.1	Gammaproteobacteria	88.56	MN134463
27	*Klebsiella oxytoca* strain NBRC 102593 - NR_114152.1	Gammaproteobacteria	90.44	MN134464
28	*Thalassotalea insulae* strain JDTF-40 - NR_163662.1	Gammaproteobacteria	81.74	MN134465
29	*Klebsiella michiganensis* strain W14 - NR_118335.1	Gammaproteobacteria	98.93	MN134466
30	*Mamoreocelis xerophila* strain CATCB5 - NR_172610.1	Cyanobacteria	84.52	MN134467
31	*Klebsiella michiganensis* strain W14 - NR_118335.1	Gammaproteobacteria	98.39	MN134468
32	*Metis fasciculata* strain TAU-MAC 1415 - NR_172573.1	Cyanobacteria	85.32	MN134469

communities based on DGGE in *Ulva australis* macroalgae collected at various periods and places. They found that there are temporal and regional variations in bacterial communities. Using the DGGE approach, they also discovered that *Bacteriodetes* and *Alphaproteobacteria* are significant components of this alga.

Bengtsson et al. (2010) looked at the seasonal changes in the bacterial community in the biofilm that grows on the surface of *Laminaria hyperborea* seaweed in another study. Depending on the outcome they achieved with the DGGE technique, they observed that seasonal variations and water temperature had an impact on the bacterial community. Additionally, they demonstrated that there was little overlap between the bacterial community in the biofilm and the seawater nearby. We find it challenging to assess the water quality of bacterial populations isolated from macroalgae samples considering this reasoning. Even while newer sequencing technologies have nearly entirely replaced approaches like as Sanger sequencing and showing the structure of classical bacterial communities, these techniques are still useful in low-budget labs. Nonetheless, we believe that it should be valued equally regardless of the approach that discloses the structure of the bacterial population without relying on macroalgae growth, which has been increasingly popular for human use in recent years. We do not hold back when highlighting the significance of this work in this regard. Even so, we can still claim that this study is a first for us and serves as the foundation for our more thorough study preparation.

4. Conclusion

According to the bacterial community investigated by a culture-independent technique based on the total DNA sample obtained from the *G. barbata* macroalgae species collected from different stations, it was observed that the majority of the bacteria belonged to the *Gammaproteobacteria* class. Among these, it was noteworthy that members of the potentially dangerous *Klebsiella* and *Vibrio* genera were found. Another important finding was that the bacterial community structure was different in macroalgae collected from different stations. In light of all these and the information given above in the literature, it will be inevitable to plan detailed research on the detection of epiphytic bacteria with more comprehensive techniques, elucidation of their relationships with macroalgae, and what kind of biotechnological benefits/products can be obtained in the context of these relationships.

Conflict of interest: The authors declare that they have no conflict of interests.

Informed consent: The authors declare that this manuscript did not involve human or animal participants and informed consent was not collected.

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