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# Bacteria associated with the alga *Ulva lactuca* (Ulvaceae) from the Colombian Caribbean and their laccase activity

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## ABSTRACT

**Introduction:** Marine macroalgae, their associated bacteria and the environment, interact to produce compounds that aid the holobiont in adapting to biotic and abiotic challenges. These compounds include several novel enzymes with industrial applications and with less environmental impact than industrial chemical reactions. Laccases are an example of enzymes that are of interest due to their wide range applications, and their versatility is a subject of research and exploration. Despite the abundance of macroalgal holobionts in the Caribbean region of Colombia, little is known about the microorganisms associated with these hosts and their potential for biotechnology.

**Objective:** To evaluate the epibiont and endobiont bacteria associated with the macroalga *Ulva lactuca* present in Santa Marta, Colombian Caribbean, and to search for laccase producers among them.

**Methods:** Culture techniques were used to isolate bacteria from *U. lactuca* collected on February 27, 2023. The 16S rRNA region was sequenced to determine the identity of the different isolates. Lacase production was screened by inoculating the isolates in guaiacol medium, which was later confirmed in nutrient agar with 0.2 % dimethoxyphenol.

**Results:** 118 isolates were obtained, of which 64 were epibionts and 54 were endobionts. 75 % were identified to genus and species level. The predominant epibiont isolates were Proteobacteria, especially *Vibrio*, while Firmicutes, with *Bacillus*, had a higher representation in the endobiont isolates. Laccase activity was found in 42 isolates including *Enterobacter*, *Halomonas*, *Paenibacillus*, *Priestia*, *Pseudomonas*, *Shewanella*, and *Vibrio*. Among them, endobionts related to *Bacillus* had the highest number of isolates positive for laccase.

**Conclusions:** Proteobacteria and Firmicutes dominated the culturable bacterial community of *U. lactuca*. This study indicates that several bacterial genera associated with *U. lactuca* in the Colombian Caribbean are positive for laccase activity. Further research is needed to explore the potential industrial applications of these enzymes.

**Key words:** seaweed; culturable marine bacterial diversity; biological substance of interest; laccase screening.

## RESUMEN

**Bacterias asociadas con el alga *Ulva lactuca* (Ulvaceae) del Caribe colombiano y su actividad lacasa**

**Introducción:** Las macroalgas marinas, sus bacterias asociadas y el medio ambiente interactúan para producir compuestos que ayudan al holobionte a adaptarse a desafíos bióticos y abióticos. Estos compuestos incluyen enzimas novedosas con aplicaciones industriales y con un menor impacto ambiental que las reacciones químicas

industriales. Las lacasas son enzimas que suscitan interés debido a sus aplicaciones y versatilidad, por lo que son objeto de investigación y exploración. A pesar de la gama de holobiontes macroalgales en el Caribe colombiano, poco se conoce sobre los microorganismos asociados a estos hospederos y su potencial biotecnológico.

**Objetivo:** Evaluar las bacterias epibiontes y endobiontes asociadas a la macroalga *Ulva lactuca* presente en Santa Marta, Caribe colombiano, y buscar productores de lacasas.

**Métodos:** Se emplearon técnicas de cultivo para aislar bacterias de *U. lactuca* recolectadas el 27 de febrero de 2023. Se secuenció la región 16S ARNr para determinar la identidad de los aislamientos. La actividad lacasa se comprobó inoculando los aislamientos en medio suplementado con guayacol y posteriormente se confirmó en agar nutritivo con 0.2 % de dimetoxifenol.

**Resultados:** Se obtuvieron 118 aislamientos, siendo 64 bacterias epibiontes y 54 endobiontes. El 75 % de ellas se identificaron a nivel de género y especie. Los aislamientos epibiontes predominantes pertenecen a las Proteobacterias, en particular *Vibrio*, mientras que los Firmicutes, con *Bacillus*, tuvieron una mayor representación en los aislamientos endobiontes. Se encontró actividad lacasa en 42 aislamientos, incluidos *Enterobacter*, *Halomonas*, *Paenibacillus*, *Priestia*, *Pseudomonas*, *Shewanella*, y *Vibrio*. Entre ellos, los endobiontes afiliados a *Bacillus* presentaron el mayor número de aislamientos positivos para lacasas.

**Conclusiones:** Proteobacterias y Firmicutes predominaron en la comunidad bacteriana cultivable de *U. lactuca*. Este estudio indica que varios géneros de bacterias asociadas a *U. lactuca* en el Caribe colombiano son positivas para actividad lacasa. Se requiere investigación adicional para explorar los potenciales usos de estas enzimas.

**Palabras clave:** algas marinas; diversidad bacteriana marina cultivable; sustancia biológica de interés; cribado de lacasas.

## INTRODUCTION

Macroorganisms provide a habitat for microorganisms, which can live on the surface of the host as epibionts, and within their tissues as endobionts (Kumar et al., 2017; Kumar et al., 2019; Tadych & White, 2009). Together, a macroorganism and its associated microbial community, along with their interactions, form a holobiont. The microorganisms involved in this association have the potential to be either latent pathogens or mutualistic symbionts that form a mutually beneficial ecological relationship with the host organism (Bordenstein & Theis, 2015; Tadych & White, 2009; van der Loos et al., 2019).

The symbiotic microorganisms in the holobiont produce metabolites that promote the growth and overall health of the host by enhancing its nutrient uptake and strengthening its defenses against pathogen attack (Azevedo et al., 2000; Armstrong et al., 2001; Chen et al., 2022; Saha & Weinberger, 2019). They also enhance host resistance to abiotic stressors, such as drought and salinity (Arnold et al., 2003; Devarajan et al., 2021; Hardoim et al., 2015; Kannadan & Rudgers, 2008; Waller et al., 2005).

Notably, within these communities, microorganisms produce enzymes and other compounds that enhance microbial resilience to host defenses against colonizing microorganisms (Egan et al., 2013; Kumar et al., 2017) and substances that prevent biofouling and predation by inhibiting the growth of other microorganisms (Sánchez-Rodríguez et al., 2018). They also play an active role in decomposing senescent host biomass (Kumar et al., 2017; Kumar et al., 2019). Furthermore, ecological studies have documented substantial genetic variability among microorganisms in holobionts (Guyomar et al., 2018), thereby reflecting their extensive biochemical capabilities and remarkable involvement in ecosystem processes through their participation in the decomposition of senescing biomass. These microbial communities play a crucial role in the biogeochemical cycles of carbon, nitrogen, and phosphorus (Abril et al., 2005; Bell, 2008; Fürnkranz et al., 2008; Moyes et al., 2016; Pita et al., 2018; Purahong et al., 2010; Sun et al., 2011; Voříšková & Baldrian, 2012).

Understanding the ecological relationships between hosts, microbial epibionts, and endobionts is crucial for understanding ecosystem

functioning and for discovering novel secondary metabolites, antimicrobials, and enzymes with biotechnological potential (Newman & Cragg, 2015; War Nongkhla & Joshi, 2014). The biotechnological potential of epibionts and endobionts communities has been evaluated in marine organisms (Pita et al., 2018, Sánchez-Rodríguez et al., 2018). However, the coexistence of these two communities within a single host (i.e., from a holobiont perspective) has not yet been thoroughly investigated. The study of the entire microbial community associated with a host is necessary, as it lays a foundation for understanding holobiont interactions and allows the relationship between these microbial communities and the physiological function of the host to be assessed. Furthermore, holobiont interactions are important in bioprospecting, as many substances of interest to industry are part of the traits that result from such interactions (Pita et al., 2018).

An ideal holobiont for the study of its associated microbial diversity and biotechnological potential is the marine macroalga *Ulva lactuca*. *U. lactuca* is a sessile organism that lives in environments with wide variations in temperature, radiation, salinity, and osmotic stress, such as rocky coastlines. Due to these environmental conditions, the microorganisms associated with *U. lactuca* produce substances with a wide range of biochemical properties and functions (Beygmoradi & Homaei, 2017; Busetti et al., 2017). Furthermore, the microbial communities associated with macroalgae, which reach average densities between $10^6$ and $10^9$ bacteria cm$^{-2}$ (Egan et al., 2013), represent microbial diversity hotspots that are a promising source of new molecules.

Most reports of bacteria associated with *Ulva sp.*, have come from studies conducted in Australia, the Baltic and North Seas in Germany, and the North Atlantic in the United Kingdom, Portugal, and Spain (Singh & Reddy, 2014). Until recently, there were no published studies on the ecology and diversity of microorganisms associated with marine macroalgae in the Caribbean, nor on the potential biotechnological applications of these communities.

A first study by Comba-González et al. (2018) investigated the epibiont bacterial community associated with the macroalga *U. lactuca* off the coast of Santa Marta, Colombia, and showed that these epibiont bacteria synthesize amylases, lipases, cellulases, agarases, and siderophores. However, as this pioneering study only addressed epibiont characteristics, the biological aspects (such as phylogenetics, functional diversity and biotechnological potential) of the joint assessment of epibionts and endobionts of macroalgal hosts in the Caribbean remain to be investigated.

Currently, fungal, and bacterial enzymes with industrial applications are in high demand because their use has a lower environmental impact than industrial oxidation reactions that lead to undesirable side reactions and the production of hazardous pollutants (Fernández & Gómez-Dégano, 2017). Enzyme types currently in demand include amylases, glycosidases, pullulanases, agarases, lyases, galactosidases, cellulases, xylanases, chitinases, cresolases, proteases, lipases, hydrogenases, and laccases (Kennedy et al., 2008). Laccases are polyphenol oxidases (PPOs) that belong to the family of multicopper blue oxidases (MCOs). These enzymes perform substrate oxidation, reducing molecular oxygen to water without producing harmful by-products. Current applications of laccases include dye degradation and detoxification, biobleaching of paper pulp, wastewater pretreatment, biodegradation of environmental xenobiotics, ethanol production, biosensor production, and drug synthesis (Agarwal et al., 2022; Harris, 2017).

Bacterial laccases have gained interest due to their sustained activity at elevated temperatures, stability at extreme pH, and exceptional stability in the presence of inhibitors such as halides, organic solvents, and at high salt concentrations (Agarwal et al., 2022). Nevertheless, despite these encouraging properties, bacterial laccases have not been adequately studied and produced (Maharsiwi et al., 2020). Only laccases of fungal origin have been produced on a large scale (Agarwal et al., 2022). However, their applications have been limited due to the slow

growth rate of fungi, low tolerance to adverse conditions, unsuitable growth in liquid media, preference for low pH ranges, and long fermentation times (Chandra & Chowdhary, 2015).

Laccase bioprospecting in bacteria associated with marine macroorganisms is a promising endeavor because enzymes are most likely to catalyze reactions under alkaline pH and fluctuating temperature conditions in the marine environment. Applications in the textile industry (Subash & Muthiah, 2021), biofuels (Ragauskas et al., 2006), and degradation of residual crop biomass (Galić et al., 2021) require laccases that are stable under harsh conditions such as ionic liquids, extreme pH, and high temperatures. Such conditions occur, for example, in the degumming of high lignocellulose natural fibers (Barber-Zucker et al., 2022).

Therefore, the objectives of the present study were, first, to evaluate the microbial community associated with the macroalgae *U. lactuca* present in the Colombian Caribbean, assessing the composition of its epibiont and endobiont bacteria based on a culture approach, and second, to continue the bioprospecting work looking for laccase-producing bacteria.

## MATERIALS AND METHODS

**Sampling site:** Samples of *U. lactuca* were collected on January 27, 2023, from the site "La Punta de la Loma" (11°07'00.9" N & 74°14'01.3" W) in Santa Marta, Colombia (Fig. 1). The sampling site is primarily a fossil coral reef with sand cover and scattered batholith rocks from the nearby Sierra Nevada de Santa Marta (Márquez & Patiño, 1986). The climate in this area is determined by the prevailing patterns that affect the Colombian Atlantic coast. The Intertropical Convergence Zone (ITCZ) causes a dry season, typically between December and April, followed by a rainy season from May to December (García-Hoyos et al., 2016). The rocky platform found in this location provides ideal conditions to support a rich diversity of macroalgae, among which the *U. lactuca* species is dominant (Diaz-Pulido & Díaz-Ruíz, 2003; García & Diaz-Pulido, 2006).
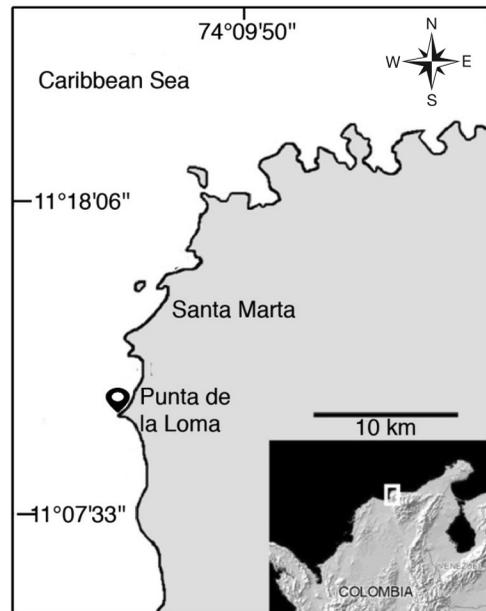


**Fig. 1.** Study area "Punta de la Loma" (Colombian Caribbean), adapted from Camacho and Montaña-Fernández (2012).

**Sample collection:** Sterile tweezers were used to detach 36 whole macroalgal thalli from rocks in the constant wave intertidal zone (Fig. 2). These thalli were washed with sterile sea water to remove surface solids and macroorganisms (Tujula et al., 2010). They were then



**Fig. 2.** Morphological characteristics of *Ulva lactuca* (Ismail & Mohamed, 2017).

placed in 200 ml sterilized containers, which were transported to the laboratory in a portable cooler at 4 °C. Once in the laboratory, each of the collected thalli was rinsed with a sterile 0.85 % saline solution to remove any remaining sand or embedded macroorganisms.

**Isolation of epibiont bacteria:** Three methods were used to isolate epibiont bacteria from *U. lactuca* to maximize the number of isolates: i) Epibiont resuspension. This was performed by obtaining three 1 g wet-weight fragments from the three macroalgal samples. These fragments were then placed in 50 ml of a sterile saline solution (0.85 % concentration) and shaken at 130 rpm for 25 min. Then, 100 μl of the resulting solution was added in triplicate to a Petri dish containing nutrient agar with NaCl (1 % w/v). ii) Swabbing. The macroalgal surface was scraped with a sterile swab, then nutrient agar with NaCl (1 % w/v) was inoculated by exhaustion with it. iii) Direct inoculation. Three 1 g fragments of macroalgal thallus were placed in the center of Petri dishes containing Zobell marine agar.

After inoculation, all cultures were incubated at 29 °C, like the temperature recorded at the collection site. The different isolates were reinoculated on nutrient agar with NaCl (1 % w/v) until pure isolates were obtained.

**Isolation of endobiont bacteria:** Two protocols were followed to obtain endobiont bacteria from *U. lactuca* thalli: i) Bacterial extraction. A 5 g wet-weight macroalgal thallus sample was immersed in 70 % ethanol for 50 s. This sample was then immersed in 0.85 % sterile NaCl solution to wash off residual ethanol. Each thallus was then homogenized in 20 ml of deionized water for 10 min using a sterile blender. The resulting homogenate was then inoculated onto Zobell marine agar in triplicate, using 100 μl of each sample. This procedure was performed on six macroalgal samples. ii) Petri dish isolation. 32 fragments from the thallus were immersed in 70 % ethanol for 50 s, then immersed in 0.85 % sterile NaCl solution (Flewelling et al., 2013) and finally placed on Zobell marine agar. This

procedure was performed on three macroalgal samples. A total of 96 fragments were placed on Zobell marine agar.

Endophytic bacterial isolates were grown in the above media and incubated at 29 °C, then reinoculated on nutrient agar plus NaCl (1 % w/v) until pure isolates were obtained.

For cryopreservation of isolated pure strains, each isolate was cultured at 30 °C for 24 h in 5 ml Zobell medium. Each culture was then centrifuged at 6 000 rpm, the supernatant discarded, and the pellet resuspended in 5 ml Zobell medium supplemented with 20 % glycerol. Aliquots of 1 ml were placed in sterile cryovials and stored at -80 °C.

**Qualitative screening of laccase activity in *Ulva lactuca* epibiont and endobiont bacterial isolates:** Laccase production capacity was qualitatively assessed in each of the *U. lactuca* bacterial isolates by inoculating the isolated colonies in culture medium supplemented with guaiacol (5 mM) and NaCl (1 %) w/v (Ali et al., 2022). Isolates positive for laccase activity were identified by the appearance of a brown coloration in the colonies approximately eight days after the start of incubation. A confirmatory test for laccase activity was then performed by inoculating the strains preliminarily identified as laccase producers onto nutrient agar supplemented with 0.2 % dimethoxyphenol (DMP). If the colony turned brown orange after at least eight days of incubation, it was confirmed as a laccase producer (Neifar et al., 2016). *Bacillus subtilis* was used as a positive control as it is a confirmed laccase producer (Muthukumarasamy et al., 2015).

**Identification of epibiont and endobiont bacteria:** Identification of isolates began with Gram staining followed by amplification and sequence analysis of the 16S rRNA region of each isolated bacterium. PCR amplification was performed using the universal primers 8F 5'-AGA GTT TGA TCC TGG CTC AG-3'- and 1541R 5'-AAG GAG GTG ATC CAG CCG CA-3'. Inoculum from a colony of all isolated bacteria was used as the source of template

DNA for the reaction. Each inoculum was suspended in 10 μl of molecular grade water. Amplification reactions were performed in a thermal cycler with a final reaction volume of 50 μl. The reaction mixtures contained 1X PCR reaction buffer, 2 mM $MgCl_2$, 0.2 mM of each dNTP, 250 nM of forward and reverse primers, 1U Taq polymerase (Promega, Madison, WI, USA), 400 ng/μl BSA (Bioline, Taunton, MA, USA) and 1 μl of bacterial suspension. The following thermocycling program was used for the PCR reactions with the 8F and 1541R primers: initial denaturation at 94 °C for 4 min; followed by 30 cycles at 94 °C for 50 s, 55 °C for 45 s, and 72 °C for 50 s; with one final extension step at 72 °C for 5 min.

Amplicons were sequenced at the Genecore Sequencing Center of the Universidad de Los Andes using the Sanger method. The resulting sequence files were evaluated and edited using the FinchTV 1.4.0 software (Geospiza Inc.; Seattle, Washington). After editing the sequence files and curation procedures, resulting sequences were compared with those available in the National Center for Biotechnology Information (NCBI) and the EzTaXon platform for taxonomic classification of the isolated bacterial strains (Chun et al., 2007).

The 16S rRNA sequences were aligned using Clustal W to determine the relationship of the epibiont and endobiont bacterial isolates. To construct the distance tree of the aligned sequences, the maximum parsimony method was used (Nei & Kumar, 2000), implemented in MEGA (Tamura et al., 2021). Bootstrap analysis was performed on 1 000 resamplings to evaluate the tree topologies, as described by Tamura et al. (2021).

Finally, three subgroups were defined for the studied culturable marine bacterial community: (i) genera present as epibionts and endobionts; (ii) genera present exclusively as epibionts; and (iii) genera present exclusively as endobionts. These subgroups were represented in a Venn diagram generated by the online tool Venny (Oliveros, 2007).

**Sequence submission to gene Bank:** Nucleotide sequences were deposited in NCBI's GenBank and accession numbers were retrieved.

## RESULTS

**Epibiont and endobiont bacterial isolates:** A total of 118 pure isolates were obtained, of which 64 were epibionts bacteria and 54 were endobionts. Several of these isolates had similar morphotypes and colors. Gram-negative bacilli with non-pigmented colonies predominated among the epibionts (52 isolates) and gram-positive bacilli with white colonies among the endobionts (32 isolates). A gram-positive coccus was observed in both endobiont and epibiont isolates.

**Identity of the epibiont and endobiont bacterial isolates:** Based on 16S rRNA analysis, we were able to identify 41 epibiont and 48 endobiont isolates. However, 29 of the isolates did not give any amplification product within the 16S rRNA region. It is likely that the unidentified bacterial isolates associated with *U. lactuca* belong to taxa whose 16S rRNA regions cannot be amplified with the primers used in this study. Amplification of the 16S rRNA gene via PCR primers shows a bias towards certain taxa, resulting in the inability to detect others that could be identified using a metagenomic approach (Brown et al., 2015; Eloe-Fadrosh et al., 2016).

It should be noted that although most of the sequences analyzed showed identity similarities higher than 98 % (Table 1 and Table 2), the limitations associated with the low resolution of 16S rRNA sequences (González et al., 2013; Mulet et al., 2010; Rodicio & Mendoza, 2004) make it impossible to obtain a reliable identification at the species level. For this reason, it is necessary to be cautious with the taxonomic identification of isolates at the species level, and therefore we focus the discussion of the results mainly on the higher taxonomic levels. The genera and species assigned to the identified isolates are detailed in Table 1 and Table 2. Sequences were submitted to the

**Table 1**

Identity of *Ulva lactuca* epibiont and endobiont isolates (Phyla Proteobacteria and Actinobacteria).

| Phylum | Class | Order | Genus | Epibionts | | Endobionts | |
|---|---|---|---|---|---|---|---|
| | | | | Specie | Percent Identity | Specie | Percent Identity |
| Proteobateria | Gamma | Vibrionales | *Vibrio* | *Vibrio halioticoli* (2) | 99.53 | *Vibrio* sp. (66) | 99.72 |
| | | | | *Vibrio* sp. (3) | 99.22 | *Vibrio rumoiensis* (91) | 100 |
| | | | | *Vibrio* sp. (4) | 94.44 | | |
| | | | | *Vibrio* sp. (11) | 94.02 | | |
| | | | | *Vibrio* sp. (12) | 99.86 | | |
| | | | | *Vibrio alginolyticus* (13) | 89.41 | | |
| | | | | *Vibrio alginolyticus* (14) | 99.26 | | |
| | | | | *Vibrio* sp. (16) | 99.85 | | |
| | | | | *Vibrio* sp. (19) | 99.45 | | |
| | | | | *Vibrio* sp. (21) | 99.94 | | |
| | | | | *Vibrio* sp. (24) | 98.96 | | |
| | | | | *Vibrio* sp. (28) | 99.86 | | |
| | | | | *Vibrio* sp. (29) | 99.06 | | |
| | | | | *Vibrio brasiliensis* (30) | 99.86 | | |
| | | | | *Vibrio brasiliensis* (31) | 100 | | |
| | | | | *Vibrio Ponticus* (32) | 99.72 | | |
| | | | | *Vibrio tubiashii* (35) | 99.10 | | |
| | | | | *Vibrio* sp. (45) | 99.58 | | |
| | | | | *Vibrio* sp. (56) | 99.3 | | |
| | | | | *Vibrio parahaemolyticus* (62) | 98.01 | | |
| | | | | *Vibrio alginolyticus* (63) | 99.81 | | |
| | | | | *Vibrio alginolyticus* (64) | 99.18 | | |
| | | | *Photobacterium* | *Photobacterium* sp. (9) | 99.86 | | |
| | | Pseudomonadales | *Pseudomona* | *Pseudomonas putida* (42) | 100 | *Pseudomonas* sp. (74) | 100 |
| | | | | *Pseudomonas* sp. (52) | 100 | *Pseudomonas* sp. (79) | 100 |
| | | | | *Pseudomonas* sp. (60) | 100 | | |
| | | | Psychrobacter | | | *Psychrobacter* sp. (96) | 100 |
| | | Alteromonadales | *Shewanella* | *Shewanella baltica* (17) | 99.74 | *Shewanella baltica* (77) | 98.68 |
| | | | | *Shewanella algae* (58) | 100 | *Shewanella baltica* (89) | 100 |
| | | | | | | *Shewanella baltica* (90) | 100 |
| | | | | | | *Shewanella baltica* (92) | 100 |
| | | Oceanospirillales | *Halomonas* | *Halomonas* sp. (57) | 99.7 | *Halomonas* sp. (101) | 100 |
| | | Xanthomonadales | *Stenotrophomonas* | | | *Stenotrophomonas* sp. (72) | 99.71 |
| | | | | | | *Stenotrophomonas* sp. (73) | 99.03 |
| | | | | | | *Stenotrophomonas* sp. (75) | 99.85 |
| | | Enterobacterales | *Pantoea* | | | *Pantoea eucrina* (80) | 97.55 |
| | Beta | Urkholderiales | Ralstonia | | | *Ralstonia* sp. (67) | 100 |
| | | | | | | *Ralstonia* sp. (68) | 99.86 |
| Actinobacteria | Actinomycetia | Micrococcales | *Kocuria* | *Kocuria gwangalliensis* (5) | 99.86 | | |
| | | | *Pseuduclavibacter* | *Pseudoclavibacter* sp. (18) | 100 | | |

Numbers in parentheses are isolate identification numbers.

**Table 2**

Identity of *Ulva lactuca* epibiont and endobiont isolates (Phylum Firmicutes).

| Phylum | Class | Order | Genus | Enpibionts isolates | | Endobionts isolates | |
|---|---|---|---|---|---|---|---|
| | | | | Specie | Percent Identity | Specie | Percent Identity |
| Firmicutes | Bacilli | Bacillales | *Bacillus* | *Bacillus altitudinis* (38) | 100 | *Bacillus xiamenensis* (65) | 100 |
| | | | | *Bacillus* sp. (39) | 100 | *Bacillus* sp. (69) | 100 |
| | | | | *Bacillus altitudinis* (40) | 100 | *Bacillus* sp. (70) | 100 |
| | | | | *Bacillus* sp. (43) | 100 | *Bacillus xiamenensis* (71) | 100 |
| | | | | *Bacillus licheniformis* (44) | 100 | *Bacillus* sp. (82) | 100 |
| | | | | *Bacillus* sp. (48) | 99.87 | *Bacillus subtilis* (85) | 100 |
| | | | | *Bacillus cereus* (53) | 100 | *Bacillus* sp. (93) | 98.88 |
| | | | | | | *Bacillus* sp. (94) | 97.77 |
| | | | | | | *Bacillus pumilus* (95) | 94.27 |
| | | | | | | *Bacillus altitudinis* (97) | 100 |
| | | | | | | *Bacillus* sp. (98) | 100 |
| | | | | | | *Bacillus tequilensis* (99) | 99.43 |
| | | | | | | *Bacillus* sp. (100) | 99 |
| | | | | | | *Bacillus* sp. (102) | 99.28 |
| | | | | | | *Bacillus xiamenensis* (103) | 100 |
| | | | | | | *Bacillus altitudinis* (104) | 100 |
| | | | | | | *Bacillus* sp. (106) | 99.4 |
| | | | | | | *Bacillus* sp. (107) | 100 |
| | | | | | | *Bacillus glycinifermentans* (108) | 98.1 |
| | | | | | | *Bacillus altitudinis* (109) | 100 |
| | | | | | | *Bacillus toyonensis* (110) | 100 |
| | | | | | | *Bacillus velezensis* (112) | 100 |
| | | | | | | *Bacillus* sp. (115) | 100 |
| | | | | | | *Bacillus* sp. (116) | 100 |
| | | | | | | *Bacillus* sp. (117) | 95.38 |
| | | | | | | *Bacillus paramycoides* (118) | 100 |
| | | | | | | *Bacillus paramycoides* (119) | 100 |
| | | | *Priestia* | *Priestia megaterium* (46) | 100 | *Priestia flexa* (81) | 100 |
| | | | | | | *Priestia megaterium* (105) | 100 |
| | | | *Cytobacillus* | *Cytobacillus oceanisediminis* (49) | 99.87 | | |
| | | | Paenibacillus | | | *Paenibacillus illinoisensis* (84) | 99.71 |
| | | | | | | *Paenibacillus* sp. (84) | 100 |
| | | | Staphylococcus | | | *Staphylococcus warneri* (83) | 100 |
| | | | | Uncultured bacteria (47) | 90.11 | | |

Numbers in parentheses are isolate identification numbers.

NCBI database and assigned accession numbers OR863796-OR863885.

Most of the epiphytic bacterial isolates belonged to the phylum Proteobacteria, representing 70.7 % of all epibiont isolates (Table 1). These isolates were all from the class Gammaproteobacteria with the order Vibrionales (53.6 %) being the most abundant. The phylum Firmicutes (24.4 %) contained only members of the class Bacilli and the order Bacillales (Table 2). Epibiont isolates also included Actinobacteria with members of the class Actinomycetia and the order Micrococcales (4.9 %) (Table 1). The most abundant endobiont bacteria were from

the phylum Firmicutes, accounting for 66.7 % of all endobiont isolates. These bacteria were exclusively from the class Bacilli and the order Bacilliales. The second most common phylum was Proteobacteria, which represented 33.3 % of the isolates. The majority of Proteobacteria were from the class Gammaproteobacteria (29.2 %), dominated by isolates from the orders Alteromonadales and Pseudomonadales. The class Betaproteobacteria represented less than 4.1 % of the isolates.

The relationships studied among the bacterial isolates, based on 16S rRNA gene analysis, showed that both epibiont and endobiont isolates were present in each of the two main branches corresponding to the Proteobacteria and Firmicutes (Fig. 3). Only epibionts were present in the Actinobacteria. The results also show that the 16S rDNA region did not discriminate between bacteria isolated from the macroalgal surface and those present in the macroalgal tissue. A grouping of epibiont and endobiont isolates belonging to the same genus was observed.

The most common bacterial genus in the community of cultivable epibionts was *Vibrio*, followed by *Bacillus*, *Pseudomonas*, *Shewanella*, *Pseudoclavibacter*, *Kokuria*, *Photobacterium*, *Priestia*, *Cytobacyllus*, *Halomonas* and the uncultured bacteria. The genera *Pseudoclavibacter*, *Kocuria*, *Photobacterium*, and *Cytobacyllus* and the uncultured bacteria, were isolated only as epibionts; the presence of these genera was not evidenced in the taxonomic identifications of the isolated endobionts. On the other hand, the most common genus in the community of cultivable endobionts was *Bacillus*, followed by *Shewanella*, *Stenotrophomonas*, *Priestia*, *Pseudomonas*, *Ralstonia*, *Vibrio*, *Paenibacillus*, *Pantoea*, *Psychrobacter*, *Halomonas* and *Staphylococcus*. The genera *Ralstonia*, *Paenibacillus*, *Pantoea*, *Staphylococcus*, *Psychrobacter*, and *Stenotrophomonas* were isolated only as endobionts (Fig. 4).

The genera *Vibrio*, *Bacillus*, *Pseudomonas*, *Shewanella*, *Priestia*, and *Halomonas* were among the bacterial isolates living equally inside and on the algae. However, there was a higher proportion of *Vibrio* isolates among the epibionts, and more *Bacillus* isolates among the endobionts (Fig. 4).

The genus *Vibrio* accounted for the highest percentage of gram-negative epibionts (53 %) among the identified Gram-isolates, while the highest percentage of gram-positive isolates belonged to *Bacillus* (57 %).

**Laccase production by bacterial isolates:** 42 bacterial isolates associated with *U. lactuca* were found to be laccase producers, the majority of which belonged to the genus *Bacillus*. 17 epiphytes and 25 endophytes showed positive laccase activity (Table 3 and Fig. 5).

## DISCUSSION

**Epibiont and endobiont bacterial composition:** In this study, we characterized the culturable epiphytic and endophytic bacteria isolated from *Ulva lactuca*, a marine macroalga found in the Caribbean region of Colombia. We also determined the laccase activity of these bacteria. The culturable microbial community associated with *U. lactuca* was dominated by Proteobacteria and Firmicutes. Isolates collected from the macroalgal surface indicated that Gammaproteobacteria were the most abundant taxon. This finding is consistent with previous reports of *Ulva* sp. from Germany, Australia, Spain, Portugal, and the United Kingdom, which also showed a significant number of Gammaproteobacteria.

Although Gammaproteobacteria are found at high frequencies in other global locations, the dominant microbial groups in these other locations are Alphaproteobacteria, Deltaproteobacteria, Bacteroidetes, Actinobacteria, and Planctomyces (Singh & Reddy, 2014). While there is evidence suggesting a specificity for the microorganisms and their algae host (Egan et al., 2013), it is not unexpected that the dominant microbiota of the Caribbean holobiont may differ from those documented in other regions. Invasive macroalgae, such as those in
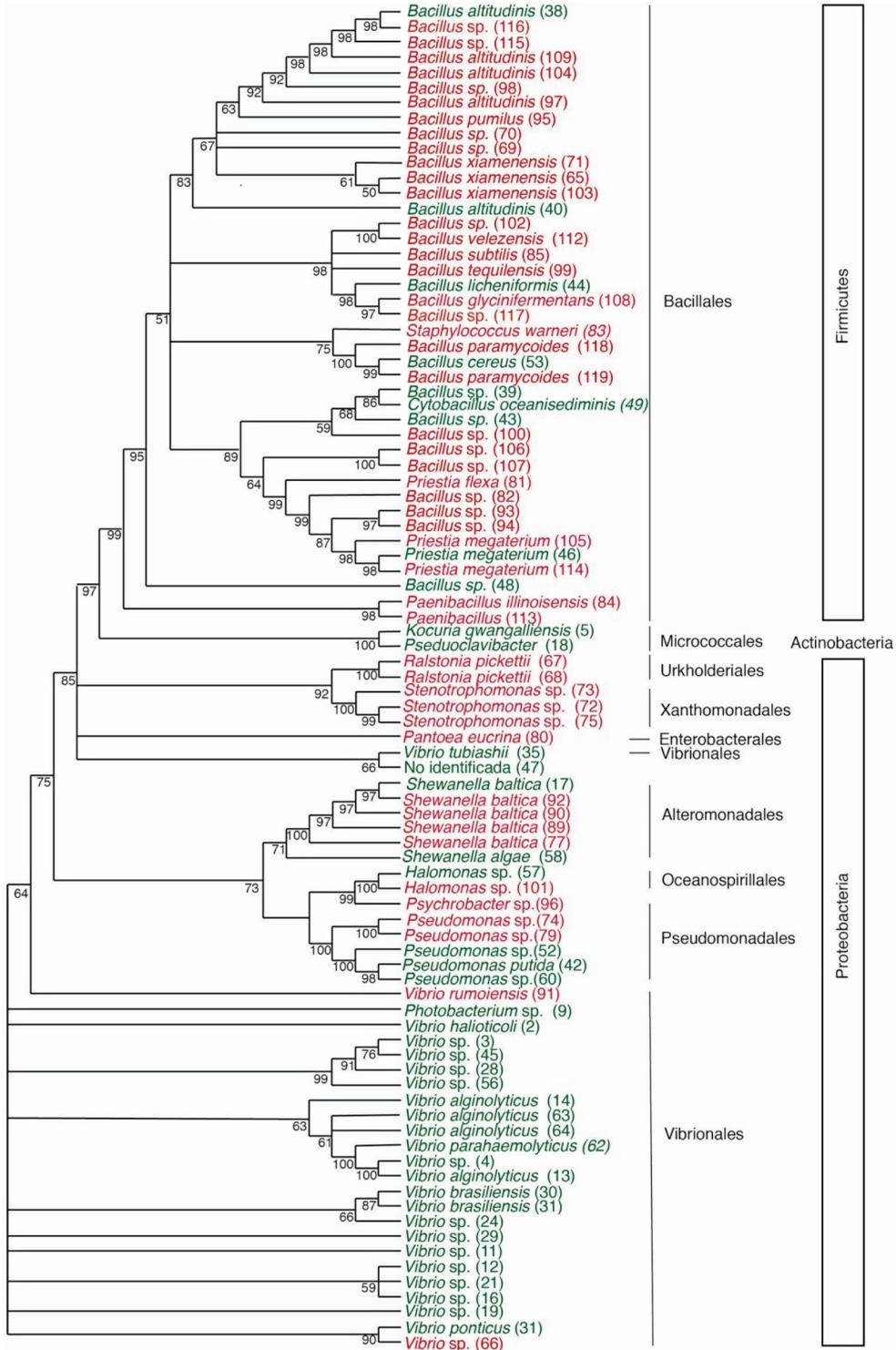
**Fig. 3.** Distance tree of the identified bacterial isolates from *Ulva lactuca* based on 16S rRNA. Green labels = epibiont isolates, red labels= endobiont isolates.
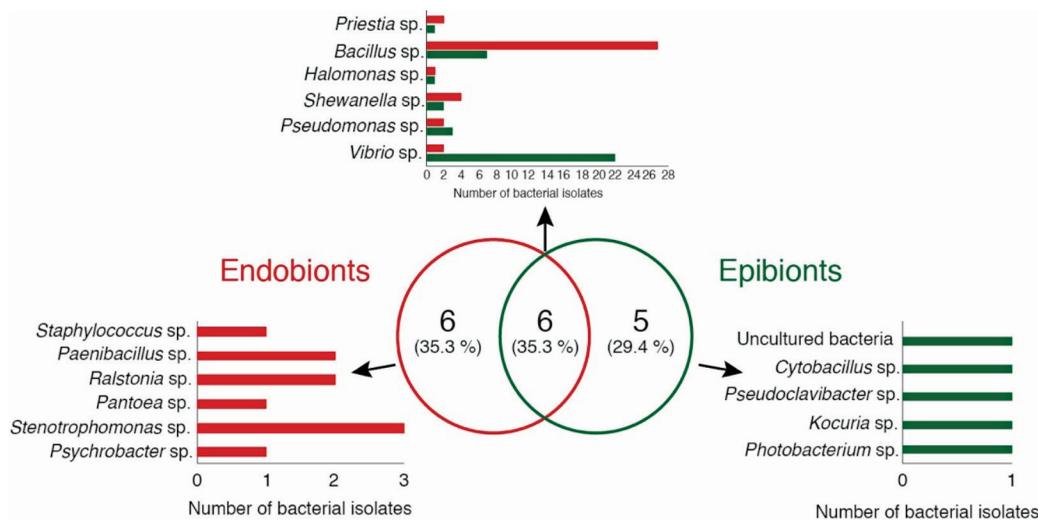
**Fig. 4.** The Venn diagram illustrates the distribution of genera both within (Endobionts) and on the algae (Epibionts). The number of isolates associated with each genus is shown in the bar graph.
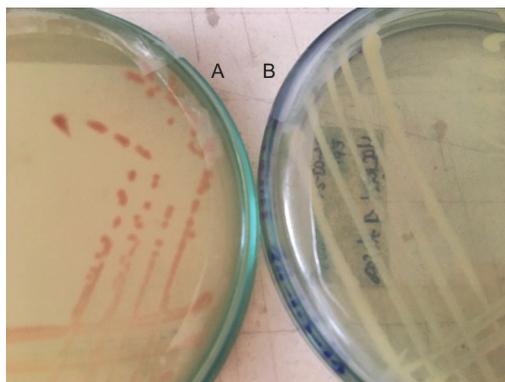


**Fig. 5.** *Shewanella baltica*, an example of an endobiont isolate positive for laccase activity. **A.** Growth in nutrient agar supplemented with guaiacol (5 mM) and NaCl (1 % w/v). **B.** Growth on nutrient agar is supplemented with NaCl (1 % w/v) without adding guaiacol.

the family Ulvaceae (Williams & Smith, 2007), have been shown to have a strategy of colonizing new habitats without relying on specific taxa. Instead, they have the ability to reconfigure their associated microorganisms using a wide variety of microorganisms from the immediate environment (Bonthond et al., 2021). Functional diversity, rather than taxonomic diversity, has recently been emphasized in holobiont relationships. A metagenomic study by Burke et

al. (2011) showed that microbial communities of *Ulva australis* from nearby sites, although differing in taxonomic composition, shared a core of functional genes common to all hosts. Consequently, although the composition of the dominant bacterial community in the Caribbean differs from that in other regions, the ecological functions provided to the host by these microbial communities may be maintained. It is important to note that the discrepancy between microbial community data for the Caribbean and other areas may also be a result of the methodology used to assess microbial composition. Previous studies have used the 16S RNA gene metabarcoding technique to identify microbial diversity, including taxa that cannot be grown in culture media (Steen et al., 2019; Theron & Cloete, 2000). In contrast, we used a cultural approximation method, which may be biased towards certain communities (Montalvo et al., 2014; Wang et al., 2020).

Due to insufficient data on the endobiotic microbiota of *Ulva* sp. from other latitudes, we are unable to make any comparisons with our results.

The genus *Vibrio*, which is commonly found in marine, estuarine, and freshwater environments (Sampaio et al., 2022), accounted

**Table 3**
*Ulva lactuca* epibiont and endobiont bacterial genera positive for laccase activity.

| | Number of isolates | Genus | Species (Isolates per species) |
|---|---|---|---|
| | | Bacterial isolates positive for laccase activity. | |
| Epibionts | 17 | *Bacillus* | *Bacillus altitudinis* (2) |
| | | | *Bacillus* sp. (3) |
| | | | *Bacillus licheniformis* (1) |
| | | | *Bacillus cereus* (1) |
| | | *Halomonas* | *Halomonas* sp. (1) |
| | | *Priestia* | *Priestia megaterium* (1) |
| | | *Pseudomonas* | *Pseudomonas putida* (1) |
| | | | *Pseudomonas* sp. (2) |
| | | *Vibrio* | *Vibrio* sp. (3) |
| | | | *Vibrio parahaemolyticus* (1) |
| | | Unidentified | (1) |
| Endobionts | 25 | *Bacillus* | *Bacillus* sp. (9) |
| | | | *Bacillus xiamenensis* (1) |
| | | | *Bacillus subtilis* (1) |
| | | | *Bacillus altitudinis* (3) |
| | | | *Bacillus velezensis* (1) |
| | | | *Bacillus paramycoides* (2) |
| | | *Enterobacter* | *Enterobacter* sp. (1) |
| | | *Halomonas* | *Halomonas* sp. (1) |
| | | *Paenibacillus* | *Paenibacillus illinoisensis* (1) |
| | | *Priestia* | *Priestia megaterium* (1) |
| | | *Pseudomonas* | *Pseudomonas* sp. (1) |
| | | *Shewanella* | *Shewanella baltica* (3) |

for the majority of the culturable epibiont bacteria isolated from *U. lactuca*. *Vibrio* species can either grow as free-living organisms in the water column or be associated with organic particles and macroorganisms (Froelich et al., 2013; Lyons et al., 2007; Sorroza-Ochoa et al., 2017; Thompson et al., 2004) such as corals, fish, mollusks, seagrasses, sponges, shrimp, and zooplankton. This genus can thrive in the water column, showing dominance in its microbial communities (Gilbert et al., 2012) and increasing in abundance with temperature and salinity (Takemura et al., 2014). *Vibrio* species are opportunistic and can exploit a wide range of resources. They have the ability to attach to surfaces, form biofilms (Hood & Winter, 1997), and readily colonize the surfaces of macroorganism, resulting in the development of symbiotic relationships with their hosts (Wahl et al., 2012). Several studies have shown

that members of the Vibrionaceae family living as epiphytes on macroalgae can play multiple roles, such as inducing macroalgal morphogenesis, promoting macroalgal spore colonization, exerting antimicrobial activity, and degrading algal compounds (Florez et al., 2017).

Therefore, it is common to observe *Vibrio* as the primary culturable genus among bacterial epibionts in algae. In our research, *Vibrio* accounted for 53.6 % of *U. lactuca* epibiont isolates in the Caribbean region of Colombia. This finding is consistent with previous research showing *Vibrio* as the dominant cultivable group found in epibiont isolates collected from brown macroalgae such as *Ascophyllum nodosum*, *Laminaria* spp. (Takemura et al., 2014), *Saccharina japonica* (Wang et al., 2008), and *Splachnidium rugosum* (Albakosh et al., 2016), as well as from the red macroalgae *Hypnea* spp. and *Polysiphonia lanosa*, and the green macroalgae

*Chaetomorpha* spp., *Enteromorpha intestinalis*, and *U. lactuca* (Takemura et al., 2014).

Interestingly, *Vibrio* does not appear to be as successful in colonizing *U. lactuca* tissues as it is in living as an epibiont, with its relative abundance as an endobiont being lower (8 %). While many studies have documented the presence of *Vibrio* on macroalgal surfaces, there are only a few reports indicating the presence of this genus within macroalgae. Singh et al. (2015) reported a low occurrence of *Vibrio* as an endobiont of the red macroalga *Gracilaria corticata* on the coast of India. This reduced incidence of *Vibrio* as an endobiont could be attributed to the inhibitory effect of bioactive compounds produced by the macroalgae and its associated epibionts and endobionts. Macroalgal lipid extracts from the red macroalga *Gracilariopsis longissima* have shown inhibitory activity against various *Vibrio* species (Cavallo et al., 2013). In addition, chlorophyte marine macroalgae, including *U. lactuca*, produce bioactive molecules such as steroids, alkaloids, and terpenes, that exhibit antimicrobial activity (Shah et al., 2020). Microorganisms with inhibitory activity against various *Vibrio* species have been discovered, such as *Bacillus amyloliquefaciens*, an epibiont of the red macroalgae *Laurenciae papillosa* and *Padyna gymnospermaha* (Chakraborty et al., 2017; Chakraborty et al., 2018), *Bacillus subtillis* associated with the brown macroalga *Anthofolphycius*, the endobiotic fungus *Aspergillus terreus* isolated from the marine macroalga *Laurencia okamurai* (Li et al., 2019), and *Penicillium citrinum*, another endobiotic fungus obtained from the mangrove *Bruguiera sexagula* var. rhynchopetala (He et al., 2017). Polyketides, terpenes, and nitrogen-containing compounds are the three main structural types of active molecules produced by microorganisms to inhibit aquatic bacteria (Guo et al., 2022). There is limited literature on the effect of *U. lactuca* on *Vibrio* growth, but studies examining pathogenic *Vibrio* species have found that incorporation of *U. lactuca* as a feed supplement and water bioremediation agent in shrimp farms effectively reduces *Vibrio alginolyticus* levels in water (Mangott

et al., 2020). Additionally, research has shown that residues of *Ulva prolifera* increase disease resistance to *Vibrio parahaemolyticus* in white shrimp (*Litopenaeus vannamei*) (Ge et al., 2019).

Our distance analysis showed that one of the endobiont *Vibrio* isolates clustered with epibiont isolates of the same genus and *Vibrio rumoiensis*, the other endophytic vibrio, is distant from the grouping formed by this genus (Fig. 3). Genetic variation within the genus *Vibrio* may allow certain species to inhabit macroalgal tissues, which warrants further investigation through genome sequencing and subsequent whole-genome sequence comparisons between epibiont and endobiont Vibrios in future studies. This method may help to identify specific and common functions of the endobiotic *Vibrio* isolates discovered in this research.

*Bacillus* was the most common genus (56.3 %) among the endobiont bacterial isolates in our study. This genus has also been commonly isolated as an endobiont from *Sargassum sabrepandum*, a brown macroalga (Ahmed et al., 2016), as well as from several macroalgal species in different water sources in Israel (Deutsch et al., 2021). One of the rare studies that assessed both epibionts and endobionts also reported *Bacillus* as the most common endobiont bacterium in *Sargassum honeyri* from the Yellow Sea, China (Mei et al., 2019). The diverse association of *Bacillus* with different macroorganism taxa (Saxena et al., 2020) can be attributed to its rapid growth and ability to thrive under challenging environmental conditions, including temperature, salinity, pH, and nutrient levels (Xiao et al., 2022). The presence of *Bacillus* bacteria as endobiont microorganisms in marine macroalgae is associated with their ability to enhance the colonization and growth of macroalgal zoospores (Singh & Reddy, 2014; War Nongkhla & Joshi, 2014).

In contrast, *Bacillus* was a less common macroalgal epibiont in our study, with only a few epibiont isolates (17 %) belonging to this genus. Similarly, Mei et al. (2019) evaluated both endobionts and epibionts and did not find *Bacillus* in the epibiont microbial community.

However, Kumar et al. (2022) found *Bacillus* to be one of the most abundant epibionts in several macroalgal species on the central West coast of India. Although this genus has been reported in numerous marine and terrestrial macroorganisms (Gao et al., 2021; Korsten et al., 1995; Xiong et al., 2018), it is difficult to determine whether *Bacillus* species are preferentially endobionts because most studies are limited to assessing the presence of microorganisms in one of two niches, the host surface, or internal tissues.

The genera *Pseudomonas*, *Halomonas*, *Shewanella,* and *Priestia* were rare in our work, but were also present as *U. lactuca* endobionts and epibionts. *Pseudomonas* has shown a wide range of biotechnological potential in the production of antimicrobials and industrially valuable enzymes (Bollinger et al., 2020). Species of this genus form biofilms in water, which allows them to exist as epibionts (Millas & France, 2020) in both aquatic and terrestrial environments (Krimm et al., 2005; War Nongkhla & Joshi, 2014). *Pseudomonas* species are known endobiotic in terrestrial plants (Chen et al., 2014; Fu et al., 2018). However, their presence in macroalgae has been poorly reported. In one such work, *Pseudomonas stutzeri* was found in the macroalga *Ulva prolifera* (Fu et al., 2018). In addition, *Halomonas* and *Shewanella* are commonly found in macroalgae as well as marine and terrestrial organisms. The genus *Halomonas* has been identified in epibiont bacterial communities associated with macroalgae, specifically *Saccharina japonica* (Zhang et al., 2020), *Laminaria japonica* (Wang et al., 2008), and *Ulva* sp. In this last macroalga, *Halomonas* has an important function in the induction to the normal morphological development (Kaur et al., 2023). This species has also been found as an endobiont of the terrestrial plant *Mesembryanhemum crystallinum* (Zhang et al., 2018; Zhang et al., 2020). *Shewanella* has been observed as an epibiont of marine organisms including sponges, invertebrates, and brown macroalgae such as *Bifurcaria bifurcata* (Horta et al., 2014). In addition, strains isolated from the surfaces of *Ulva* sp. were reported

to stimulate the zoospore settlement of this macroalgae (Kaur et al., 2023). *Shewanella* species have also been discovered as endobiont in Indonesian seagrasses (Fitri et al., 2017). While the genus *Priestia* has been documented as an epibiont on several macroalgae from the central west coast of India (Kumar et al., 2022), there are few reports of this genus as an endobiont.

Although our study detected *Pseudoclavibacter*, *Kocuria*, *Photobacterium*, and *Cytobacillus* exclusively on the surface of *U. lactuca* and *Ralstonia*, and *Paenibacillus*, *Pantoea*, *Staphylococcus*, *Enterobacter*, *Psychrobacter*, and *Stenotrophomonas* exclusively in macroalgal tissues, these genera are not restricted to either niche. Previous studies indicate that they are also found worldwide as both epibionts and endobionts in marine and/or terrestrial hosts (Table 4). Exceptions are *Photobacterium*, which is mainly found in marine environments (Fuertes-Perez et al., 2021), and *Ralstonia*, which has not been reported as an epibiont or endobiont of macroalgae. However, Sarr et al. (2010) were able to isolate *Ralstonia* from the nodules of cowpea, a type of bean.

Interestingly, *Kocuria,* a common epibiont of marine hosts, especially macroalgae, has shown antibacterial activity against other bacteria associated with macroalgae (Leiva et al., 2015). Furthermore, it has been found to produce an extracellular polymer against the colonization of macroalgal surfaces by bacteria and barnacle larvae (Ba-Akdah & Satheesh, 2021). *Stenotrophomonas* is an opportunistic pathogen in corals, reported by Meyer et al. (2014). However, certain species of this genus found in terrestrial plants have shown the ability to produce chitinases that can inhibit the growth of the pathogenic *Fusarium oxysporum* and *Leptinotarsa decemlineata*, both of which pose a threat to agricultural production (Aktas, 2022). *Paenobacillus,* an endobiont of *Lonicera japonica*, can inhibit the growth of phytopathogenic fungi and promote the growth of its host plant by producing siderophores and solubilizing phosphorus (Zhao et al., 2015).

The results of this preliminary study show that most of the bacterial genera obtained

**Table 4**

Bacterial genera associated with marine and terrestrial hosts as epibionts or endobionts.

| Genus | Habitat | Host | Niche | World region | Source |
|---|---|---|---|---|---|
| *Pseudoclavibacter* | Marine | *Mnemiopsis leidyi* (Comb jelly) | Epi | Kiel Bight, Baltic Sea | Weiland-Bräuer et al., 2020 |
| | Terrestrial | *Panax ginseng* C. A. Meyer (Ginseng) | End | Korea | Cho et al., 2007 |
| | Terrestrial | *Glycyrrhiza uralensis* (Perennial herb) | End | North-West China | Li et al., 2016 |
| *Kocuria* | Marine | *Ulva lactuca* (Macroalgae) | Epi | Central Red Sea, S. Arabia | Ba-Akdah & Satheesh, 2021 |
| | Marine | Antarctic marine macroalgae | Epi | South Shet- land Islands, Antarctica Peninsula | Leiva et al., 2015 |
| | Marine | Marine macroalgae (*Sargassum*, *Ulva*, *Padina*, *Dictyota* and *Pterocladia* sp.) | Epi | Central West coast of India | Kumar et al., 2022 |
| | Terrestrial | *Coffea arabica* L | End | Colombia | Vega et al., 2005 |
| | Terrestrial | *Ficus carica* L | End | Southern Bulgaria | Lozanova et al., 2022 |
| *Brevundimonas* | Marine | Marine macroalgae (*Sargassum*, *Ulva*, *Padina*, *Dictyota* and *Pterocladia* sp.) | Epi | Central West coast of India | Kumar et al., 2022 |
| | Terestrial | *Olea europaea* L (Olive trees) | End | Mediterranean Basin | Mina et al., 2020 |
| | Terrestrial | *Buxus sempervirens* (Bush) | End | North Carolina, U.S.A. | Li et al., 2023 |
| *Photobacterium* | Marine | *Fucus serratus* (Seaweed) | Epi | Kiel Bight, Western Baltic Sea | Nasrolahi et al., 2012 |
| | Marine | *Cyanea capillata* (Jelly Fish) *Tubularia indivisa* (Hydrozoa) *Sagartia elegans* (Sea anemone) | End | Orkney Islands, Northeastern coast of Scotland | Schuett & Doepke, 2010 |
| | Marine | *Asparagopsis armata* (Red algae) | Epi | Peniche, Portugal | Horta et al., 2019 |
| *Cytobacillus* | Terrestrial | *Oryza sativa* L. (Rice) | End | South Korea | Dutta et al., 2022 |
| | Marine | Hydroclathrus sp (Brown Macroalgae) | Epi | South Sulawesi, Indonesia | Ethica et al., 2023 |
| *Paenibacillus* | Seashore | *Rhizocarpon geographicum* L (Lichen) | Epi | La Pointe de Crozon, France | Miral et al., 2022 |
| | Marine | *Enhalus acroides* (Seagrass) | Epi | Papua New Guinea | Hassenrück et al., 2014 |
| | Terrestrial | *Lonicera japonica* (*Medicinal plant*) | Endo | Eastern China | Zhao et al., 2015 |
| *Pantoea* | Terrestrial | *Brassica oleracea* var. capitata *Spinacia oleracea* | Epi | Japan | Oie et al., 2008 |
| | Terrestrial | *Pisumsativum* *Gossypium hirsutum* L. | End | Netherlands Alabama (U.S.A) | Elvira-Recuenco & van Vuurde, 2000 McInroy & Kloepper, 1995 |
| *Psychrobacter* | Marine | *Anoxycalyx joubini* (Sponge) *Lissodendoryx nobilis* (Sponge) *Haliclonissa verrucosa* (Sponge) | End | Antartic | Papaleo et al., 2012 |
| | Marine | *Sargassum polycystum* *Padina antilarum* *Dictyota* sp. | Epi | Anjuna, Kunkeshwar and Malwan (India) | Kumar et al., 2022 |
| *Stenotrophomonas* | Terrestrial | *Cucumis sativus* L. (Cucumber plants) | End | Alabama: U.S.A. | Ryan et al., 2009 |
| | Marine | *Porites astreoides* (Coral) | End | Belize | Meyer et al., 2014 |
| | Marine | *Laminaria saccharin* (Macroalga) | Epi | Baltic Sea: Germany | Lage & Graça, 2016 |

(Epi = Epibiont), (Endo = Endobiont).

from *U. lactuca* in the Colombian Caribbean are present in microbial communities associated with brown and red macroalgae, marine invertebrates, and terrestrial plants from different regions of the world. However, to determine the unique functional and taxonomic assemblages of microbial epibiont and endobiont communities in *U. lactuca*, the use of metagenomics and the 16S rRNA metabarcoding approach is necessary.

The advantage of using conventional culture methods to assess microbial community composition is that the genetic material of the isolated microorganisms can be sequenced, thereby improving the ability to explore the biotechnological potential of these isolates through genomic bioprospecting. Genome mining, an *in silico* bioprospecting method (Ziemert et al., 2016), is ideal for bioprospecting the genomes of the isolates. It will facilitate the exploration of metabolic pathways, secondary metabolites, and antimicrobials that contribute to different aspects of holobiont interactions, such as (i) the synthesis and production of enzymes that degrade sugars produced by the macroalga (Barbato et al., 2022), (ii) microorganisms engaging in antagonistic interactions through secondary metabolites, including antibiotics (Goecke et al., 2010; Ortega-Morales et al., 2008; Velupillaimani & Muthaiyan, 2019), (iii) the production of phytohormones (Ulrich et al., 2022), and motility/chemotactic substances (Colin et al., 2021) that promote biofilm formation, and (iv) siderophores for iron scavenging (Zoccarato et al., 2022). Today, this genomic bioprospecting is possible because of access to a global repository of genome and metagenome datasets through platforms such as the Integrated Microbial Genomes and Microbiomes System (IMG/M) (Chen et al., 2020). In addition, IMG/M provides the Genomes OnLine Database (GOLD), a manually curated database with a metadata reporting system that allows users to tabulate and search the associated metadata associated with each submitted genome (Mukherjee et al., 2021).

**Laccase production:** Laccases are found in in plants, animals, insects, and both eukaryotic and prokaryotic microorganisms (Janusz et al., 2020). These polyphenol oxidases (PPOs) perform essential oxidase functions in various biological systems. It has been shown that fungal laccases are involved in lignin degradation, sporulation, pigment production, morphogenesis, stress defense and plant pathogenesis (Thurston, 1994). Plant laccases have been implicated in the biosynthesis of lignin polymers (Sterjiades et al., 1992; Tobimatsu & Schuetz, 2019; Zhao et al., 2013), elongation (Balasubramanian et al., 2016; Liang et al., 2006), stress response (Cho et al., 2014), repair of damaged plant tissues, iron accumulation and the polymerization of phenolic compounds (Gałązka et al., 2023). Insect laccases participate in cuticle sclerotization and pigmentation, as well as other processes such as wound healing and the development and maintenance of the immune system (Dittmer & Kanost, 2010).

Bacterial laccases, on the other hand, are involved in pigmentation processes, endospore coat protein synthesis, morphogenesis, toxin oxidation and protection against oxidants and UV light (Sharma et al., 2007). These enzymes have been reported for many gram-negative and gram-positive bacterial genera such as *Azospirillum*, *Streptomyces*, *Bacillus*, *Aeromonas*, *Pseuomonas*, *Oscillatoria*, *Thermus*, *Escherichia and Haloferax,* and have been detected in soils, rhizospheres, deep-sea sediments, and extreme and marine environments (Janusz et al., 2020; Singh et al., 2011). In addition, a previous study identified genes encoding laccases by analyzing the genome of the *U. lactuca* epibiont bacterium *Achromobacter denitrificans* strain EPI24 (Niño et al., 2023). Therefore, although there are no reports of laccase activity for microorganisms associated with *U. lactuca*, we were not surprised to find that out of 16 cultivable genera associated with *U. lactuca*, eight were positive for laccase activity. Furthermore, *Bacillus* sp., the genus with the highest number of isolates, also had the highest number of epibiont and

endobiont isolates positive for this enzyme (70 %). In this genus, laccases are part of the endospore walls and are involved in the biosynthesis of a brown spore pigment, a melanin-like polymer responsible for protection against UV radiation and heavy metals (Janusz et al., 2020).

Although *Vibrio* had a high number of isolates, especially epibionts, only 16 % showed laccase activity. These results are consistent with the rare reports in the literature of positive laccase activity by *Vibrio. Enterobacter*, *Halomonas, Pseudomonas*, *Paenibacillus, Priestia* and *Shewanella*, were also found to be positive for laccase production. These genera have been previously reported to be laccase producers by Edoamodu and Nwodo (2021), Kurian and Kumar (2015), Mathews et al. (2016), and Sinirlioglu et al. (2013). Conversely, to the best of our knowledge, *Priestia* and *Halomonas* have not been previously reported to produce this enzyme.

In this study, endobionts exhibited a higher number of isolates positive for laccase production in comparison to epibionts. This imbalance may indicate a significant biological function for the laccase-producing endophytic bacteria associated with *Ulva lactuca*. The microbial laccase catalyzes the oxidation of small organic compounds (including phenolic compounds, diamines, and aromatic amines), producing radicals that can be utilized by the host in anabolic and catabolic pathways (Jeon & Chang, 2013) linked to processes that enhance the performance of the algae, such as the neutralization of toxic by-products released in biochemical reactions, morphogenesis, toxin oxidation and protection from UV light (Gałązka et al., 2023).

Evaluating the potential use in biotechnology of laccases produced by the many isolates detected in this study is worthwhile. Firstly, bacterial laccases have advantages over those produced by fungi. Comparative studies have shown that the active sites of bacterial, fungal and plant laccases are highly conserved, reflecting a common reaction mechanism for copper oxidation and $O_2$ reduction in these enzymes (Dwivedi et al., 2011). However, analysis of laccase molecular surface areas, volumes and cavity residues, revealed that bacterial laccase has the largest putative substrate binding site cavity, as compared to fungi and plants, which could explain the different functions of laccases (Dwivedi et al., 2011). Another important difference of these polyphenol oxidases (PPOs) between different biological groups is their redox potential (E°) at the T1 site of the enzyme (Gałązka et al., 2023; Morozova et al., 2007; Singh et al., 2014). This is a fundamental physicochemical property of laccases, as the rate of a laccase oxidation reaction depends on the difference in redox potential ($\Delta$ E°) between the T1 site of the enzyme and the substrate (Xu et al., 1996). Laccases of fungal origin tend to have higher E° values (0.34 to 0.81 V vs. NHE, Normal Hydrogen Electrode) (Munk et al., 2015; Singh et al., 2014) compared to bacterial laccases (0.4 to 0.5 V vs. NHE), which can withstand more challenging conditions than fungal laccases (Agarwal et al., 2022). Bacterial polyphenol oxidases are active at high pH values and much more stable at high temperatures, in the presence of organic solvents and over a range of salt concentrations (Janusz et al., 2020). Although data for plants are scarce, a few studies report that their laccases have low E° values (Janusz et al., 2020; Munk et al., 2015). Second, biotechnology now makes it possible to modify biological properties. Even if we find bacteria in the Caribbean with laccase activity that has significant advantages over fungal laccases, none of these bacterial enzymes may be optimal for industry. However, genetic engineering can help overcome these hurdles by modifying some biological properties of microorganisms so that their enzymes can be used by industry. For example, most microorganisms have limited laccase yields, which limits industrial production of the enzyme. Many screens have been conducted to explore laccase-producing microorganisms in different environments to identify efficient hypersecretory microorganisms for large-scale production or laccase-producing microorganisms that can be improved using recombinant DNA tools (Yang et al., 2017).

This study shows that a significant proportion of microorganisms associated with *U. lactuca* in the Colombian Caribbean can produce laccases. It is then worthwhile to search for enzymes applicable in industrial settings in the microbial community associated with *U. lactuca*. It is worth further investigating whether endobiont bacteria are more inclined to synthesize laccases with biotechnological potential and exploring the impact of their interaction with the macroalgal host on enzyme production.

The present study showed that among the culturable microorganisms associated with *Ulva lactuca* from the Colombian Caribbean, a large proportion belongs to the genera *Vibrio*, which grow mainly on the algal surface, and *Bacillus*, which grows in the tissues of the macroalga. These epibiont and endobiont bacteria, especially *Bacillus*, can produce laccases.

## ACKNOWLEDGMENTS

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