

# Microbiomes associated with cultures of *Gambierdiscus australes* and *Ostreopsis cf. ovata*, two epibenthic dinoflagellates from the NE Atlantic Ocean (Las Palmas, Gran Canaria)

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## Abstract

Our study focuses on bacterial communities associated with two benthic, epiphytic dinoflagellates, *Gambierdiscus australes* and *Ostreopsis cf. ovata*, isolated from coastal waters of Las Palmas, Gran Canaria, and grown in clonal cultures. The goal was to characterize the stable bacterial consortia found within the phycosphere of each dinoflagellate species and establish into which functional group they fell. High-throughput sequencing (HTS) results highlighted a higher bacterial diversity in *O. ovata* compared with *G. australes*. Alphaproteobacteria dominated in both dinoflagellates with *Marivita* and *Labrenzia* as the most represented OTUs in *Ostreopsis* and *Gambierdiscus*, respectively, and *Thalassospira* and *Oceanicaulis* well represented in both species. Based on SIMPER analyses, *Labrenzia* and the Phycisphaerales SM1A02, dominant in *Gambierdiscus* and *Ostreopsis*, respectively, accounted for the most significant difference between the two microbiomes. The microbiomes described here differed from those described for the same dinoflagellate species in other studies, which could depend on differences in environmental conditions, macroalgal substrate, and/or growth stage or bloom phase of the microalgal hosts. The distinct bacterial communities associated with the two potentially toxic dinoflagellates, isolated from the same site and sampling event and cultivated under the same conditions, suggest different modalities of interaction and interexchange between bacteria and their hosts.

## KEYWORDS

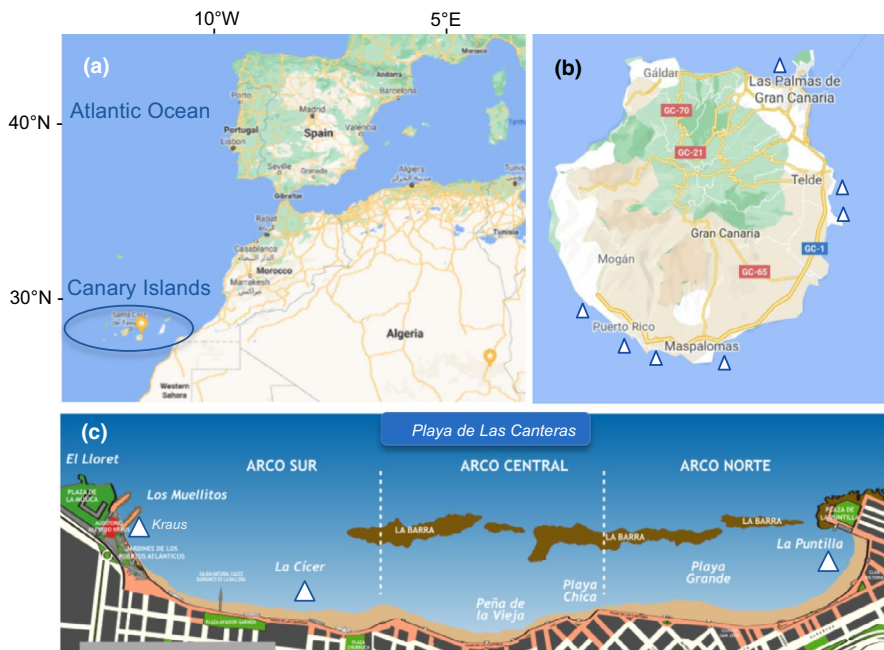
16S rDNA bacterial diversity, algae, dinoflagellates, microbial structure, phycosphere

## 1 | INTRODUCTION

In aquatic environments, bacteria have been recognized as potential regulators of the algal metabolism and behavior, including growth, bloom initiation, maintenance, and decline (Amin et al., 2015; Gonzalez & Bashan, 2000; Kim et al., 2014; Seyedsayamdost et al., 2011). Although algal–bacterial interactions are viewed as discrete

events depending upon environmental factors, many studies report the recurrent co-presence of algae and bacteria in the same micro-environments (e.g., phycosphere, corals, and lichens) where complex mutualistic (Croft et al., 2005), commensal, competitive (Bratbak & Thingstad, 1985), and parasitic relationships (Sachs & Wilcox, 2006) can occur. The interactions between bacteria and algae are essential in regulating algal accumulation and degradation of organic matter

Renata Denaro and Francesca Crisafi contributed equally to the present work.



**FIGURE 1** Location of the Canary Islands in the Atlantic Ocean (a) and sampling stations along the Gran Canaria coast (b). Details are provided on the northern area of sampling, Playa di Las Canteras in Las Palmas (c)

(Cole et al., 1988; Fuentes et al., 2016). Moreover, bacterial communities hosted by dinoflagellates have been associated with their growth through the production of vitamins, iron chelators (siderophores), and cytokinins (Jasti et al., 2005).

Bacteria associated with toxic algae have earned an increasing interest due to their possible influence on the algal toxicity expression. Marine biotoxins produced by microalgae, including dinoflagellates, are known to accumulate through the food chain, with a heavy impact on human health, marine organisms, and aquaculture activity. The phylum Dinophyta includes many species able to produce different toxins, agents of human illnesses. Several planktonic species are responsible for syndromes such as paralytic shellfish poisoning (PSP), neurotoxic shellfish poisoning (NSP), and diarrhetic shellfish poisoning (DSP). Among benthic dinoflagellates, *Ostreopsis* spp., produce isobaric palytoxin (PLTX) and ovatoxins (OVTXs) which are associated with human respiratory illness by exposure to toxic aerosol (García-Altres et al., 2014; Tibirić et al., 2019), whereas *Gambierdiscus* spp. and *Fukuyoa* spp. are responsible for ciguatera fish poisoning (CFP) (Murray et al., 2020; Wang, 2008). The last disorder is a serious problem affecting public health and fishing industries in tropical and sub-tropical regions, and an emerging risk in temperate areas, such as European seas (Aligizaki et al., 2008; Bravo et al., 2015; Kaufmann & Böhm-Beck, 2013; Laza-Martínez et al., 2016; Zingone et al., 2021). In the last decade, ciguatera outbreaks took place in the Canaries and affected ≈100 people following the consumption of local fish (Bravo et al., 2015). In autumn 2016, a bloom of *Gambierdiscus* spp. dominated by *G. caribaeus* was observed in the protected marine reserve El Hierro, the smallest Canarian island, whereas ciguateric fish species (up to 62.5%) were found in the area in December of the same year (Acosta et al., 2018).

Interestingly, bacterial communities associated with toxic dinoflagellates seem to have a function on the regulation of algal toxin

production (Orr et al., 2013). For instance, a threshold number or critical consortium of *Proteobacteria* are needed to promote PSP-toxin production by *Gymnodinium catenatum*, possibly through the supply of sufficient amounts of cofactors or precursors needed in the process (Green et al., 2004). In cultures of *Ostreopsis* and *Gambierdiscus*, bacterial diversity patterns on cell surfaces and in the associated extracellular matrices have been reported to vary with growth phase (Tosteson et al., 1989) whereas in *Ostreopsis*, the degree of toxicity has also been attributed to the composition of the bacterial assemblage (Pérez-Guzmán et al., 2008). Recent co-culturing experiments have highlighted how associated bacteria can influence both growth and toxin production in three Pacific *Gambierdiscus* strains (Wang et al., 2018). Bacterial diversity also varies in *Ostreopsis* cf. *ovata* between different phases of the blooms and over the course of growth cycle (Guidi et al., 2018; Vanucci et al., 2016). Nevertheless, the kind of physiological interaction in dinoflagellates–bacteria associations, as well as the specificity of the associations and the role of environmental conditions, is still unclear.

In this study, we investigated the bacterial microbiomes associated with two potentially toxic, epibenthic dinoflagellates, *Gambierdiscus australes* (GA) and *Ostreopsis* cf. *ovata* (OO), isolated from the Canary Islands (NE Atlantic Ocean) and maintained in culture under the same conditions. Specifically, one GA and one OO isolates obtained from macrophyte hosts collected simultaneously at the same location were cultured under the same conditions and their microbiomes were analyzed by 16S rDNA high-throughput sequencing (HTS). We aimed at comparing the microbiome composition between the two species and, through a literature search, assessing whether the bacterial phycosphere composition of those isolates was unique or overlapped with that observed in the same species from elsewhere and in other dinoflagellate species.

## 2 | MATERIAL AND METHODS

### 2.1 | Sampling and dinoflagellate culturing

Samples were collected in the framework of the EU project MIMAR (INTERREG MAC 2014–2020), aimed at the monitoring, control, and mitigation of changes in the marine ecosystems within the Macaronesian region. The Canaries are the largest and most easterly archipelago of this region, close to the African coasts, and are characterized by volcanic origin, contrasting landscapes and gentle climate. The sampling was conducted in Gran Canaria, which is subject to the influence of trade winds tempering the heat along the coasts during summer; these winds blow more intensely in the afternoon, when they are often moderate or even quite strong.

The cultures of the two potentially toxic, epibenthic dinoflagellates (*Gambierdiscus* and *Ostreopsis* species) were obtained from seaweed samples including more than one macroalgal species, collected in September 2015 at two coastal sites of Playa de Las Canteras (Las Palmas, Gran Canaria): Station 1, close to the Alfredo Kraus Conference Center (28°07'51.6" N, 15°26'49.7" W) and Station 2, at La Cicer beach (28°07'54.6" N, 15°26'42.5" W; Figure 1). Additional macroalgal samples were collected in September and October 2016, that is, in the months of reported *Gambierdiscus* occurrence (Tudò et al., 2020), at the two stations and at another station of the same beach, La Puntilla, as well as in other localities along the Gran Canaria coast: Playa Ojos de Garza, Melenara, Maspalomas beach, Playa de Puerto Rico, Arguineguín, and Playa de Mogán. At all stations, at a depth of about 0.5–1 m, portions of seaweed thalli of 10 to 15 g fresh weight were collected with the surrounding water in 250-ml plastic bottles that were capped under water, according to the European protocol for *Ostreopsis* sampling (ENPI, M3\_HABs, 2015). Sampling bottles were shaken for a few seconds, and macrophytes were rinsed several times with seawater to dislodge epiphytic cells. For each sample, all the seawater resulting from these operations was passed through a 20 µm-meshed filter to concentrate epiphytic cells. The concentrated material was stored in 50-ml tubes. Subsamples were fixed with Lugol's solution for cell counts, which was performed following the Uthermöhl method (1958) or using a Sedgewick Rafter chamber, depending on cell density. Unfixed samples were kept within steel thermos at 22–24°C, according to the water temperature of the sampling site, and brought to CNR IRBIM laboratory in Messina (Italy) in 48 h. There, the samples were observed using an inverted microscope (Axiovert 200 Zeiss), under which individual cells of *Gambierdiscus* or *Ostreopsis* were isolated with a micropipette, washed in sterile medium droplets several times, and finally placed in sterile polystyrene 24-well culture plates kept under the following conditions: 24 ± 1°C, irradiance 60–80 µmole photons m<sup>-2</sup> sec<sup>-1</sup>, and light:dark period 14:10 h. Isolates were then transferred into 6-well culture plates and finally, one month after sampling, to 50 ml culture bottles. Growth media used were K2 Keller or F4 Guillard in sterile seawater diluted at 32 ppt. All cultures were handled aseptically to prevent bacterial contamination and cross-contamination among cultures.

For morphological analyses, culture samples were stained with Calcofluor White M2R according to Fritz and Triemer (1985), which allows to observe the thecal plate pattern.

### 2.2 | DNA extraction and molecular characterization of dinoflagellates

For definitive species identification, culture samples were collected after 15–20 days of growth for 18S-molecular analyses and processed as reported below. Approximately 10 ml of each exponentially growing cultures of the two strains (CNR GA-LP and CNR OO-LP B1) was harvested by centrifugation (4000 rpm, 5 min). Total genomic DNA was extracted using DNeasy Plant Kit (Qiagen), according to Penna and Magnani (1999) and the manufacturer's instructions. The extracted genomic DNA was used as template for PCR amplification of 18S rDNA region using the NS1 and NS8 universal primers (White et al., 1990). The PCR was performed in a final reaction volume of 50 µl containing 2.5 µl of 1X solution Q (Qiagen), 5 µl of 1X Qiagen reaction buffer, 1 µl of each forward and reverse primer (10 µM stock), 1 µl of dNTPs (10 µM stock) (Gibco, Invitrogen Co), 50 ng of DNA, 2.0 U of Qiagen Taq Polymerase (Qiagen), and nuclease-free water up to 50 µl. The PCR amplification was performed in GeneAmp 5700 (PE Applied Biosystems) using the following profile: 3 min hot-start at 95°C; 30 cycles at 1 min 94°C, 1 min 50°C, 2 min 72°C, and a final 10-min extension at 72 °C. PCR products were analyzed by horizontal 1% (w/v) agarose gel electrophoresis (100 V, 1 h) in TAE 1X (Tris 0.04 M, EDTA 0.001 M, pH 8) electrophoresis buffer, to which 1 µl (10000x) of SYBR Safe (Invitrogen) was added. A 1 kb DNA ladder (Bio-Rad) was included as a molecular weight marker. After electrophoretic separation, PCR products were visualized using a UV transilluminator (Bio-Rad). Positive products were purified and sequenced by MacroGen (Amsterdam, The Netherlands) using NS1 and NS8 universal primers.

Fragment sequences were checked by BLAST search in the GenBank database (<http://www.ncbi.nlm.nih.gov>). An 18S phylogenetic tree was obtained from sequences aligned using the latest SILVA databases for ARB (release 138.1 (Nov 02, 2020) SSURef NR99, <http://www.arb-silva.de>). The neighbor-joining algorithm and the Jukes–Cantor distance matrix within the ARB package were used to generate the phylogenetic tree based on distance analysis. One thousand bootstrap re-samplings were performed to estimate the robustness of the tree using the same distance model.

### 2.3 | Analysis of bacteria associated with dinoflagellates: DNA extraction and bacterial community composition

In order to analyze the composition of associated bacterial communities in *Gambierdiscus australes* and *Ostreopsis cf. ovata*, 10 ml of the cultures of the first strains isolated at Playa Las Canteras-Kraus in September 2015 (CNR GA-LP and CNR OO-LP B1) was harvested by

centrifugation (11,000×g for 5 min. according to Pérez-Guzmán et al., 2008). Total genomic DNA was extracted using MasterPure Complete DNA and RNA Purification Kit (Epicentre) following the manufacturer's instructions. The V3-V4 hypervariable regions of the prokaryotic SSU rRNA gene (Forward =5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNGGCWGCAG Reverse =5'GTCTCTGTTGGCTCTCGGAGATGTGTATAAGAGACAGGACTACHVGGGTATCTAATCC, Klindworth et al., 2013) were PCR-amplified, and the amplicons were sequenced on the Illumina MiSeq platform by a commercial company (FISABIO, Valencia, Spain) (<http://fisabio.san.gva.es/en/servicios>). Library preparations followed by Illumina sequencing were performed according to standard protocols (Caporaso et al., 2012). Sequences were trimmed to remove barcodes and primers, then those <150 bp, with ambiguous base calls or homopolymer runs exceeding 6 bp were removed. Pre-processed sequences were analyzed by the NGS analysis pipeline of the SILVA rRNA gene database project (SILVAngs 1.3) (Quast et al., 2013). Each sequence was aligned using the SILVA Incremental Aligner (SINA v1.2.10) for ARB SVN (Pruesse et al., 2012) against the SILVA SSU rRNA SEED and quality controlled database (Quast et al., 2013). After quality control, sequences were subject to dereplication on a per sample basis. Identical reads were identified, and the unique reads were clustered (OTUs 98% similarity) using cd-hit-est (version 3.1.2; <http://www.bioinformatics.org/cd-hit>) (Li & Godzik, 2006). The reference read of each OTU was classified by a local nucleotide BLAST search against the non-redundant version of the SILVA SSU Ref dataset (release 132, Dec 13, 2017; <http://www.arb-silva.de>) using blastn (version 2.2.30+; <http://blast.ncbi.nlm.nih.gov/Blast.cgi>) with default settings (Camacho et al., 2009). For the phylogenetic tree, filtered reads and close relatives were initially aligned using the SILVA alignment tool (Pruesse et al., 2007) and manually inserted in ARB (Ludwig et al., 2004). The sequences were aligned using the latest SILVA databases for ARB (release 138.1 (Nov 02, 2020) SSURef NR99, <http://www.arb-silva.de>). The neighbor-joining algorithm and the Jukes-Cantor distance matrix of the ARB package were used to generate the phylogenetic trees based on distance analysis for 16S rRNA. One thousand bootstrap re-samplings were performed to estimate the robustness of the tree using the same distance model. The Newick format of the tree was imported in iTOL (<https://itol.embl.de/>) and edited to obtain the tree shown in Figure 4. See legend for more details.

## 2.4 | Statistical analyses

PAST PAleontological STatistics V3.25 ([https://palaeoelectonica.org/2001\\_1/past/issue1\\_01.htm](https://palaeoelectonica.org/2001_1/past/issue1_01.htm)) was used to generate the Bray-Curtis similarity matrix obtained from the OTU abundance table and calculate diversity index. The same program was used to calculate the contribution of single OTUs to the observed difference between samples (SIMPER test) (Clarke, 1993). The alpha diversity of each microbiome was estimated by using two richness estimators: observed OTUs (Obs) and FisherAlpha ( $S = a * \ln(1+n/a)$  where S is the number of taxa, n is the number of individuals and a is the Fisher's alpha (Fisher et al., 1943), and two diversity indexes: the Shannon H index

$$H' = - \sum_{i=1}^R p_i \ln p_i$$

where  $p_i$  is the proportion of reads belonging to the  $i^{\text{th}}$  OTUs in the dataset of interest (Spellerberg & Fedor, 2003), and the Simpson index:

$$I = \frac{\sum_{i=1}^R n_i (n_i - 1)}{N(N - 1)}$$

where  $n_i$  is the number of sequences identified in each OTU of  $i^{\text{th}}$  type and N is the total number of sequences in the dataset (Simpson, 1949).

## 3 | RESULTS

### 3.1 | Field samples and dinoflagellate isolates

Macrophytes collected at the sampling sites were mostly Ochrophyta, such as *Halopteris scoparia* and *Dictyota* sp., followed by the Rhodophyta *Jania* sp. The target species of this study, which are epiphytic on these seaweeds, were i) *Gambierdiscus australes*, with the typical antero-posteriorly compressed lenticular shape (Figure 2A-C) and plate formula matching the species descriptions, and ii) *Ostreopsis* cf. *ovata* (Figure 2D-F) with the typical tear-drop shape and numerous gold-brown chloroplasts. The morphological identification of these two taxa was confirmed by the results of the molecular analyses showing 99.88% of identity for *G. australes* with strain I080606\_3 from Japan (accession number AB764308) and 98.10% of similarity for *O. cf. ovata* with strain 1S1D6 from the South China Sea (accession number KX129875) respectively (Figure 3). The two dinoflagellates species were only found in September 2015 at Station 1—Kraus and Station 2—La Cicer of Las Canteras beach, whereas they were not detected at La Puntilla station nor at the other sampling locations southwards along the Gran Canaria coasts. Cell densities at the two stations of Las Canteras ranged between 0 and 100 *G. australes* cells  $\text{g}^{-1}$  macroalga (wet weight, ww) and between 0 and 500 *O. cf. ovata* cells  $\text{g}^{-1}$  macroalga (ww). Macroalgae also hosted other dinoflagellates, such as *Coolia*, *Prorocentrum*, *Amphidinium*, and *Heterocapsa* spp., along with diatoms, mostly *Nitzschia*, *Amphora*, and *Pleurosigma* spp. At the sampling stations, water temperature and salinity were 24.2–24.6°C and 36.8–36.9 in September 2015 and 22.5–23.2 °C and 36.7–36.8 in October 2016, respectively.

*Ostreopsis* cf. *ovata* showed a growth rate of 0.3–0.5 div.  $\text{day}^{-1}$ , which was higher than that of *G. australes* (0.10–0.17 div.  $\text{day}^{-1}$ ).

### 3.2 | Phylogenetic characterization of bacteria associated with *G. australes* and *O. cf. ovata*

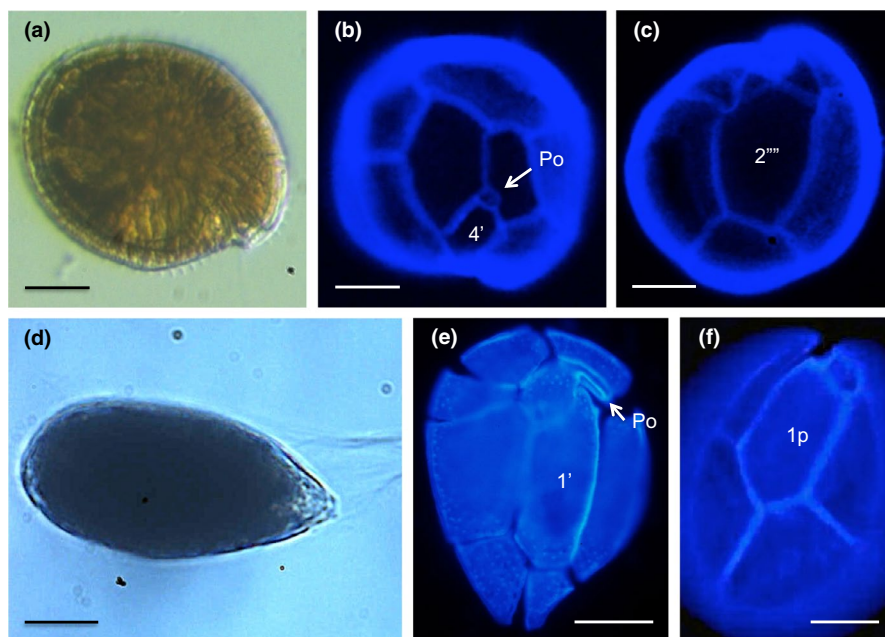
The bacterial 16S rRNA Illumina sequencing resulted in libraries of 31,473 sequences from *O. cf. ovata* (OO) and 13,375 from *G. australes* (GA) cultures. Operational taxonomic units (OTUs) were clustered based on 97% sequence similarity. Alpha-diversity index

values were significantly different between the two microbiomes (Table 1). The number of observed taxa (OBS) in OO was more than double that in GA at all taxonomic levels. The highest diversity value was detected at genus level in OO, along with the highest estimated value of the taxa richness (Fisher- $\alpha$ ), indicating that rare taxa were also well represented in the sample. The lowest diversity value coupled to the highest dominance expressed as invSimpson index was recorded in GA at phylum level.

Bacterial microbiomes were classified into 8 phyla and 15 classes in OO and 4 phyla and 7 classes in GA, respectively. The following description of microbial community structure is mainly based on the relative contribution of the major bacterial signatures, that is, those representing more than 1% in at least one sample. The dominant bacterial phyla in OO were Proteobacteria (49.7%), Bacteroidetes (24.3%), and Planctomycetes (14.9%). The same phyla were detected in GA but with different relative contributions, namely, Proteobacteria (93.3%), Bacteroidetes (5%), and Planctomycetes (1%). Rare phyla (<1%) such as Verrucomicrobia, Actinobacteria, TM6, and SHA-109 detected in *O. cf. ovata* were not recorded in *G. australes*. Moreover, Cyanobacteria were found only in GA as 0.4% of the total retrieved taxa (Figure 4, Table 1). At class level, the bacterial microbiome of OO was dominated by Alphaproteobacteria (40.8%), whereas the rest of the clades were mainly affiliated to Cytophagia (14%), Phycisphaerae (12%), Gammaproteobacteria (6.6%), Flavobacterilia (6.4%), Sphingobacteria (4%), Planctomycetacia (3%), and Deltaproteobacteria (1.4%). In GA, the dominance of Alphaproteobacteria was much more marked (83.5%), whereas the remainder 15.2% was covered by Gammaproteobacteria (9.2%), Flavobacteria (5%), and Phycisphaerae (1%). At genus level, the OO microbiome showed the highest diversity values with 21 detected genera. The most abundant clades were affiliated to the Phycisphaerales clade SM1A02 (11.7%), *Maricaulis* (9%), and *Oceanicaulis* (5.2%). *Thalassospira* were represented by 6.9%

of total OTUs. Moreover, 10.29% of OTUs were attributable to Cytophagales, mostly including unknown families, among which we were able to identify *Fabibacter* (1.6%) and *Reichenbachiella* (1.3%). The 3.7% of the sequences were attributable to *Balneola*. Two genera were closely related to the family Rhodobacteraceae, namely, *Marivita* (6.5%) and *Roseovarius* (4.7%). An unclassified NS11-12 marine group belonging to the order Sphingobacteriales constituted 4% of the total relative abundance. The family Bacteriovoraceae represented 1.2% of the total OTUs, which were closely related to genus *Peredibacter*. OTUs closely related to the genus *Methylophaga* constituted 1.2% of the total abundance in OO. Finally, 1.1% of the OTUs were unclassified genera attributable to the DB1-14 order (Figure 4, Table 2). In GA, 54.5% of total OTUs were affiliated to Rhodobacteraceae, namely, 42.2% *Labrenzia* and 12.3% *Stappia*. Hyphomonadaceae covered 19.3% of the total abundance, with a single genus, *Oceanicaulis*. *Thalassospira* constituted 5.9% of the OTUs representing the family Rhodospirillaceae, together with an uncultured genus (3%). *Marinobacter* and *Pseudospirillum* were 4.5% and 3.4% of the total abundance, respectively (Figure 4, Table 2).

A specific focus on the differences between the two microbiomes was applied by means of the SIMPER analysis which revealed a relatively low number of OTUs (14.8%) shared between *O. cf. ovata* and *G. australes* libraries. Remarkably, shared OTUs included almost 50% of those identified in the GA library. These included four Alphaproteobacteria (*Oceanicaulis*, *Stappia*, uncultured Rhodospirillaceae and DB1-14 group) and two Gammaproteobacteria (*Marinobacter* and *Pseudospirillum* sp.) (Table 2). Moreover, SIMPER analysis highlighted three OTUs as the major contributors to the difference between the two dinoflagellate microbiomes with an Average dissimilarity (Av. dis) >5%: two OTUs belonged to Alphaproteobacteria, namely *Labrenzia* (Av. dis 11.44%), detected only in GA with 38.35% of total sequences, and *Oceanicaulis* (Av. dis 6.8%), more abundant in GA than OO libraries (19.19% and



**FIGURE 2** Specimens of *Gambierdiscus australes* (a-c) and *Ostreopsis cf. ovata* (d-f) from Las Palmas, Gran Canaria (Spain). Light and epifluorescence micrographs with details of epitheca—Po and apical plates 4' (b), 1' (e)—and hypotheca, with antapical plate 2''' (c) and posterior intercalary plate 1p (f), respectively. Scale bars: 20  $\mu\text{m}$  (*Gambierdiscus*) and 10  $\mu\text{m}$  (*Ostreopsis*)

1.9% of sequences, respectively). The third OTU belonged to the Plantomycetes SM1A02 (Av. dis 8.2%), which was detected only in the OO library, where it accounted for 11.73% of sequences (Table 3).

## 4 | DISCUSSION

### 4.1 | Gambierdiscus and Ostreopsis in Gran Canaria

In this study, we have focused on the microbiome associated with two different epibenthic species, *Gambierdiscus australes* and *Ostreopsis cf. ovata*, based on strains obtained during the same sampling event from a locality of the Atlantic Ocean close to Las Palmas de Gran Canaria, Las Canteras.

In Las Canteras beach, *G. australes* and *O. cf. ovata* were isolated from a sample of the ochrophytes *Halopteris scoparia* and *Dictyota* sp, the same macrophytes hosting *Gambierdiscus* spp. in other Canary Islands, such as El Hierro, with the presence there also of *Padina pavonica* (Soler et al., 2016). Various additional Ochrophyta, Rhodophyta, and Chlorophyta were reported from the Macaronesia region (Canaries and Cape Verde), hosting both *Ostreopsis* (predominantly) and *Gambierdiscus* species (Fernández-Zabala et al., 2019). The finding of a single *Gambierdiscus* species in our samples matches previous observations of *G. australes* as the dominant species of the genus in Gran Canaria, with a minor occurrence of *G. excentricus* (Rodríguez et al., 2017).

The 18S rDNA gene analysis supported the morphological identification of the two dinoflagellate species, showing that the *Gambierdiscus* strain from Las Palmas de Gran Canaria, (Spain) has a high similarity with conspecifics from the Pacific Ocean (Japan, Australia) and other Atlantic areas (Florida). *Ostreopsis cf. ovata* was placed in a clade with related species from the Pacific Ocean (China and Australia), but with a lower similarity. This result was expected considering the well-known genetic diversity within this species complex (Penna et al., 2010; 2014; Sato et al., 2011) and also because of the absence of reference 18s rDNA sequences of *O. cf. ovata* strains from the Atlantic-Mediterranean region, to which presumably our strain belongs. Other studies, based on morphology and/or rDNA sequence data from partial nuclear LSU (D1/D2 domains), 5.8S and non-coding internal transcribed spacer (ITS) regions, have shown *O. cf. ovata* as distributed throughout the Mediterranean Sea as well, whereas *G. australes* has only recently been found in the Balearic Islands (Zingone et al., 2021, and papers cited therein).

The study of the microbiomes associated with the two dinoflagellates is particularly intriguing because species of these two genera have been implicated in different harmful algal blooms. Among *Gambierdiscus* species producing lipophilic ciguatoxins and maitotoxins (e.g., Botana, 2014), the species *G. australes* seems to be less toxic, with a very low to no CTX production (Pisapia et al., 2017). However, *G. australes* from Canary Islands was described as a CTX producer (Estevez et al., 2020) and a potential MTX-producer (Reverté et al., 2018).

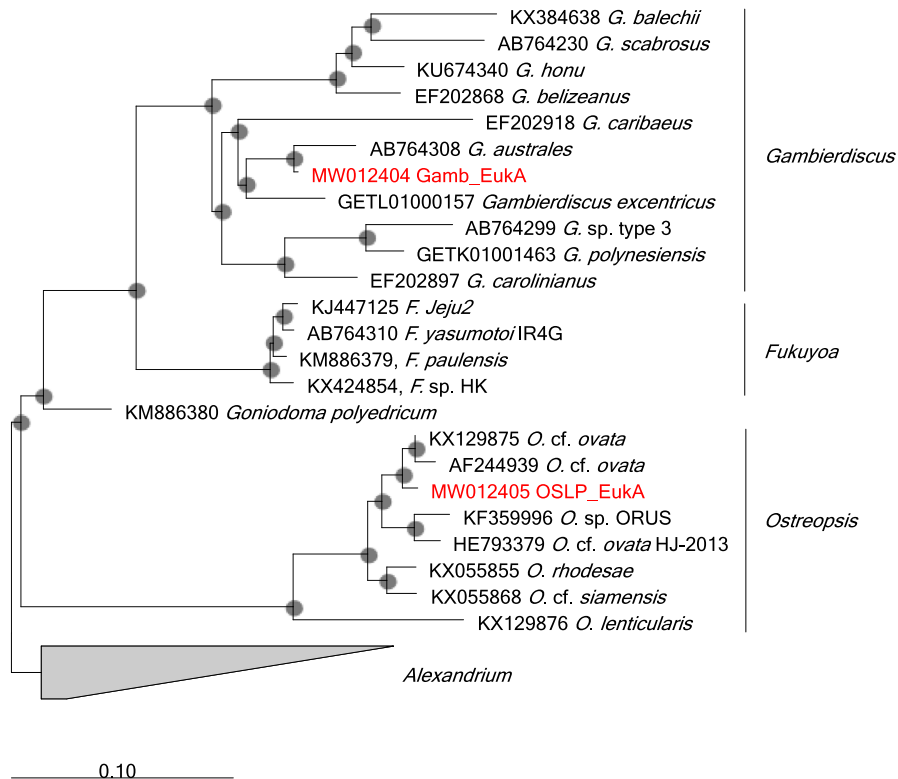
### 4.2 | Associated microbial communities

Bacterial diversity observed in the microbiomes was significantly different between the two cultured strains both in terms of biodiversity pattern and OTUs richness, which was considerably higher in *Ostreopsis*.

The role of the bacteria inhabiting the phycosphere is difficult to define, due to bimodal, synergistic, and/or antagonistic activity they can perform, which can frequently vary under the influence of two main factors: environmental perturbations and/or the state of the host, that is, the growth phase. Moreover, the nature of interchanges between algae and associated bacteria is still object of controversy. An example of different roles played by the microbiomes in our study is provided by SM1A02 (Phycisphaerales), which have frequently been described in macroalgae-associated microbiomes, for example, in *Caulerpa* sp., as the most abundant microbiome component in specimens affected by disease (Liu et al., 2019). However, SM1A02 were also described as facultative aerobic organisms able to metabolize dimethylsulfonylpropionate, an osmolyte produced by several microalgae including *O. cf. ovata* (Vanucci et al., 2016), as well as sulfated polysaccharides (Deschaseaux et al., 2018; Rambo et al., 2020). Another example is provided by the Alphaproteobacteria consortium, which was already reported as dominant over all the growth phases of *O. cf. ovata* cultures, where it mainly included Rhodobacteraceae and Hyphomonadaceae (Guidi et al., 2018). Rhodobacteraceae have been shown to modify their behavior from mutualistic to opportunistic in response to signal molecules, for example, during algal senescence (Guidi et al., 2018; Seyedsayamdost, Carr, et al., 2011; Seyedsayamdost et al., 2011, 2014; Sule & Belas, 2013).

Among other bacteria identified in our study, *Maricaulis* (9% of OTUs in *Ostreopsis*) were found as growth-promoter ectosymbionts in a hydrocarbon-rich green alga (Denaro et al., 2021; Tanabe et al., 2015); *Oceanicaulis*, which we detected in both species and were more abundant in *Gambierdiscus*, were also found in various cultures of other marine microalgae, such as *Alexandrium tamarense* (Strömpl et al., 2003), *Emiliania huxleyi* (Zabeti et al., 2010), *Eutreptiella* sp. (Kuo & Lin, 2013), and *Ostreococcus tauri* (Abby et al., 2014). In *O. cf. ovata* cultures, *Oceanicaulis* were found to be abundant in different growth phases, and they were associated with the biosynthesis of B vitamins and were proposed as candidates to investigate synergic action with *O. cf. ovata* during toxic blooms (Guidi et al., 2018). Bacteroidetes, which we retrieved in high abundance in *Ostreopsis*, possibly have a role in the hydrolysis of biopolymers such as proteins, complex polysaccharides, and glycoproteins (Riedel et al., 2013). In particular, Flavobacteria and Sphingobacteria have been found to be associated with particulate matter/algal detritus contributing to the mineralization processes. In the microbiome associated with *G. australes*, the most abundant reads (>50%) were attributed to *Labrenzia*, which actually were even more abundant considering that the genus *Stappia* (covering 12.3% of the library) was recently reclassified as *Labrenzia*. Previously identified in corals and other microalgal cultures (Chen et al., 2012; Han et al., 2016; Sandhya et al., 2017),

**FIGURE 3** SSU rDNA-based phylogeny of *Gambierdiscus australes* and *Ostreopsis cf. ovata*, strains. Sequences obtained in this study are bold-typed in red. Support of nodes is based on bootstrap values of NJ with 1000 re-samplings. Only values greater than 70 are shown. *Alexandrium* was used as out-group



**TABLE 1** Bacterial alpha diversity in the microbiomes associated with *Ostreopsis cf. ovata* and *Gambierdiscus australes*

Rank	Samples	OBS <sup>a</sup>	Shannon <sup>b</sup>	invSimpson <sup>b</sup>	FisherAlpha <sup>c</sup>
PHYLUM	<i>O. cf. ovata</i>	8	1.28	2.96	0.75
	<i>G. australes</i>	4	0.29	1.15	0.38
CLASS	<i>O. cf. ovata</i>	15	1.88	4.52	1.51
	<i>G. australes</i>	7	0.63	1.41	0.71
GENUS	<i>O. cf. ovata</i>	68	3.14	16.67	8.24
	<i>G. australes</i>	32	1.89	4.17	3.94

<sup>a</sup>number of observed taxa (OTU 97%).

<sup>b</sup>Lande (1996).

<sup>c</sup>Fisher et al. (1943).

*Labrenzia* are notable for their ability to produce dimethylsulfoniopropionate (DMSP) (Curson et al., 2017; Salgado et al., 2014), which plays a key role in stress tolerance in dinoflagellates (Sunda et al., 2002; Williams et al., 2019). They have also been described as aerobic anoxygenic photosynthetic bacteria (Biebl et al., 2005, 2007), advantaged under light–dark carbon-limited regime (Koblížek, 2015; Soora et al., 2015; Wang et al., 2015). *Labrenzia* show a very active metabolic pathway under algal-derived matter remineralization, as during the algal stationary phase (Guidi et al., 2018). However, host-killing, algicidal and antimicrobial activities also seem to be associated with *Labrenzia* (Amiri Moghaddam et al., 2018; Chen et al., 2017; Fiebig et al., 2013), which could determine their success within the microbiome associated with *G. australes* and possibly be related to the considerably lower growth performances of the latter compared with *O. cf. ovata* in our study. An abundance of signatures belonging to Phycisphaerae family has recently been highlighted in the microbiome of two *Gambierdiscus* species, and a possible mutual

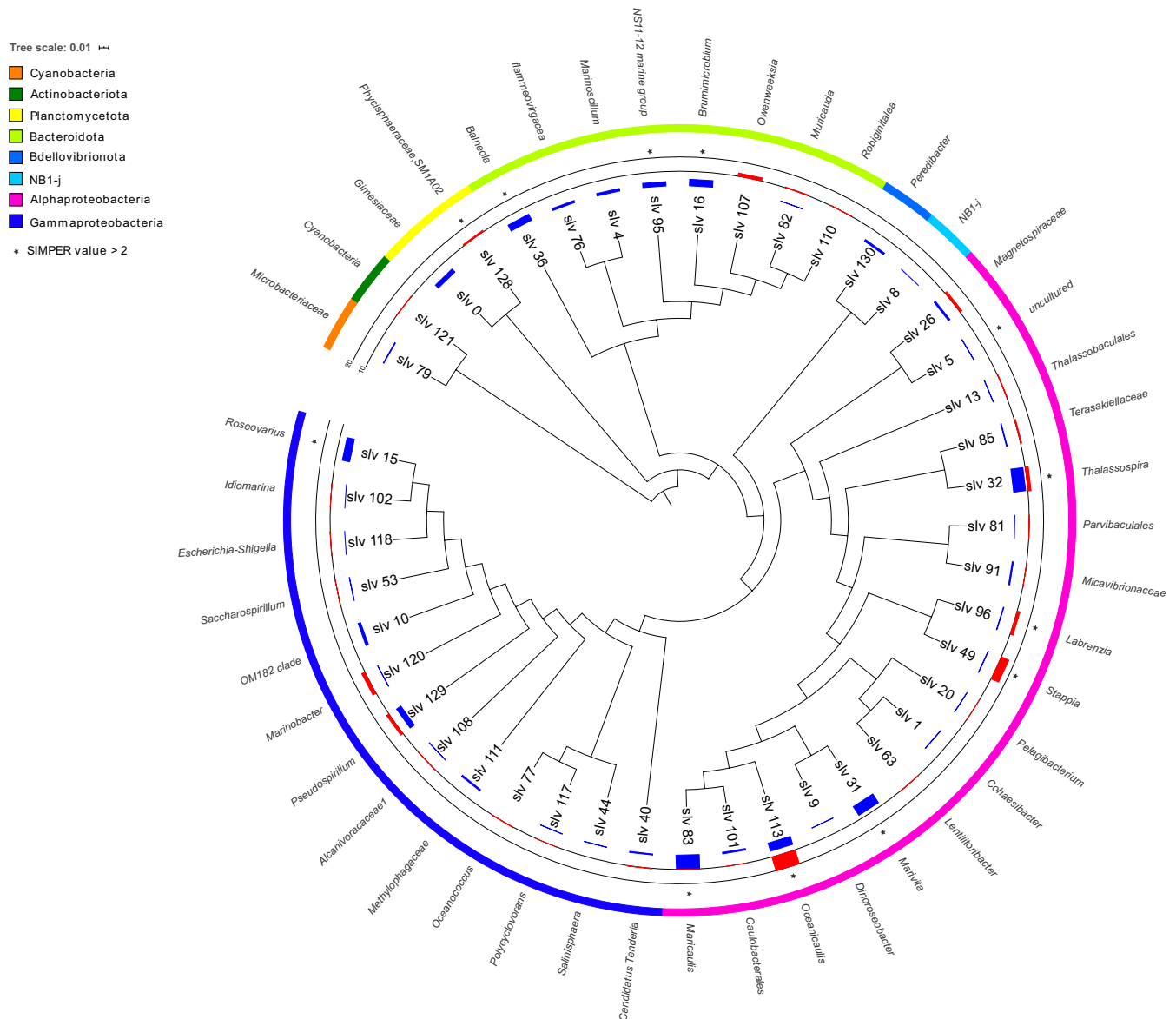
interaction has been hypothesized based on in silico predictions (Rambo et al., 2020). By contrast, we have detected a relatively high abundance of Phycisphaerae family only in *O. cf. ovata*.

Considering the limitations of the method, the bacterial communities revealed in our study may not reflect entirely the original microbiome and should be considered with caution, because culture conditions over a month may have imposed strong constraints on their composition. However, the quite high diversity of bacterial species found through molecular analyses, along with the profound differences between the microbiomes associated with two species handled in the same way, supports the informative value of our results and allows some general considerations. Because the two dinoflagellates species were isolated from the same location, sampling date, and macroalgal samples, the difference between their microbiomes would point at species-specific associations at least in the environment investigated, supporting the “niche” hypothesis (Hardin, 1960): If one algal species offers defined, stable

TABLE 2 List of the identified operational taxonomic units (OTUs) found in libraries from *Gambierdiscus australes* and *Ostreopsis cf. ovata* microbiomes

OTUs	Taxonomy	Relative abundance (%)	
		OO	GA
slv_79	Bacteria; Actinobacteriota; Actinobacteria; Micrococcales; Microbacteriaceae;DS001	0.29	0.00
slv_121	Bacteria; Cyanobacteria; Cyanobacteriia; Chloroplast	0.00	0.41
slv_0	Bacteria; Planctomycetota; Planctomycetes; Planctomycetales; Gimesiaceae; Gimesia	2.89	0.00
slv_128	Bacteria; Planctomycetota; Phycisphaerae; Phycisphaerales; Phycisphaeraceae;SM1A02	11.73	1.17
slv_36	Bacteria; Bacteroidota; Rhodothermia; Balneolales; Balneolaceae; Balneola	3.69	0.00
slv_76	Bacteria; Bacteroidota; Bacteroidia; Cytophagales; Cyclobacteriaceae; Reichenbachella	1.34	0.05
slv_4	Bacteria; Bacteroidota; Bacteroidia; Cytophagales; Cyclobacteriaceae; Marinoscillum	0.54	0.00
slv_95	Bacteria; Bacteroidota; Bacteroidia; Sphingobacteriales;NS11-12 marine group	3.99	0.00
slv_16	Bacteria; Bacteroidota; Bacteroidia; Flavobacteriales; Crocinitomicaceae; Brumimicrobium	5.97	0.00
slv_107	Bacteria; Bacteroidota; Bacteroidia; Flavobacteriales; Cryomorphaceae; Owenweeksia	0.32	4.34
slv_82	Bacteria; Bacteroidota; Bacteroidia; Flavobacteriales; Flavobacteriaceae; Muricauda	0.04	0.58
slv_110	Bacteria; Bacteroidota; Bacteroidia; Flavobacteriales; Flavobacteriaceae; Robiginitalea	0.00	0.16
slv_130	Bacteria; Bdellovibrionota; Bdellovibrionia; Bacteriovoracales; Bacteriovoraceae;uncultured	0.12	0.00
slv_8	Bacteria;NB1-j	0.07	0.00
slv_26	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Magnetospiraceae;uncultured	1.20	3.07
slv_5	Bacteria; Proteobacteria; Alphaproteobacteria;uncultured	0.33	0.12
slv_13	Bacteria; Proteobacteria; Alphaproteobacteria; Thalassobaculales;uncultured	0.13	0.60
slv_85	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Terasakiellaceae;uncultured	0.49	1.45
slv_32	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodospirillales; Thalassospiraceae; Thalassospira	6.90	5.90
slv_81	Bacteria; Proteobacteria; Alphaproteobacteria; Parvibaculales;uncultured	0.00	0.22
slv_91	Bacteria; Proteobacteria; Alphaproteobacteria; Micavibrionales; Micavibrionaceae;uncultured	0.85	0.20
slv_96	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Stappiaceae; Labrenzia	0.38	42.23
slv_49	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Stappiaceae; Stappia	0.22	12.30
slv_20	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Devosiaceae; Pelagibacterium	0.12	0.00
slv_1	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae; Cohaesibacter	0.25	0.00
slv_63	Bacteria; Proteobacteria; Alphaproteobacteria; Rhizobiales; Rhizobiaceae; Lentilitoribacter	0.00	0.09
slv_31	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Marivita	6.54	0.00
slv_9	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Dinoroseobacter	0.07	0.00
slv_113	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Hyphomonadaceae; Oceanicaulis	5.21	19.9
slv_101	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Hyphomonadaceae;uncultured	0.03	0.00
slv_83	Bacteria; Proteobacteria; Alphaproteobacteria; Caulobacterales; Hyphomonadaceae; Maricaulis	9.05	0.00
slv_40	Bacteria; Proteobacteria; Gammaproteobacteria; Tenderiales; Tenderiaceae; Candidatus Tenderia	0.72	0.49
slv_44	Bacteria; Proteobacteria; Gammaproteobacteria; Salinisphaerales; Salinisphaeraceae; Salinisphaera	0.31	0.34
slv_117	Bacteria; Proteobacteria; Gammaproteobacteria; Salinisphaerales; Solimonadaceae; Polycyclovorans	0.13	0.03
slv_77	Bacteria; Proteobacteria; Gammaproteobacteria; Salinisphaerales; Solimonadaceae; Oceanococcus	0.00	0.24
slv_111	Bacteria; Proteobacteria; Gammaproteobacteria; Nitrosococcales; Methylophagaceae; Methylophaga	1.18	0.00
slv_108	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Alcanivoraceae1; Alcanivorax	0.00	0.10
slv_129	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Pseudohongiellaceae; Pseudohongiella	2.30	2.54
slv_120	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Marinobacteraceae; Marinobacter	0.10	4.50
slv_10	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales;OM182 clade	0.88	0.00
slv_53	Bacteria; Proteobacteria; Gammaproteobacteria; Pseudomonadales; Saccharospirillaceae; Saccharospirillum	0.14	0.19
slv_118	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Enterobacteriaceae; Escherichia-Shigella	0.01	0.29
slv_102	Bacteria; Proteobacteria; Gammaproteobacteria; Enterobacterales; Idiomarinaceae; Idiomarina	0.02	0.29
slv_15	Bacteria; Proteobacteria; Alphaproteobacteria; Rhodobacterales; Rhodobacteraceae; Roseovarius	4.73	0.00





**FIGURE 4** Taxonomic composition of the microbiomes associated with *Ostreopsis cf. ovata* and *Gambierdiscus australes* based on sequence libraries. Phylogenetic tree was obtained as described in methods; only OTUs with more than 50 reads per OTU are presented. External color circles represent class affiliation, whereas for every OTUs, the most deepest affiliation up to genus level is reported. Stars indicate OTUs with SIMPER value >2, red and blue histograms indicate relative abundance of OTUs identified associated in OO and in GA respectively. Figure was edited using iTOL. (<https://itol.embl.de/>) (Letunic & Bork, 2019)

conditions, the same well-adapted bacterial species will outcompete the others when present. Species-specific associations of bacteria to algae may depend on various factors such as algal extracellular products, toxicity (Jasti et al., 2005; Schäfer et al., 2002; Šimek et al., 2011) and surface structures and components (Dang & Lovell, 2002). However, environmental conditions and/or the state of the host could also be responsible for the differences observed. As an example, in *Alexandrium catenella* a shift of bacterial dominance occurred from Gammaproteobacteria at the initial bloom stage to Alphaproteobacteria during the bloom decline. Therefore, the dominance of Alphaproteobacteria in *Ostreopsis* and even more in *Gambierdiscus* could also be related to a difference in the phase of

the bloom, which for *O. cf. ovata* in the western Mediterranean generally occurs in July and declines in late summer–autumn (Mangialajo et al., 2011; Rodríguez et al., 2017).

As discussed above, many of the bacterial taxa have been described associated also with other species of dinoflagellates in addition to *Ostreopsis* and *Gambierdiscus*, suggesting a higher relevance of local environmental conditions and/or substrates in determining community patterns of algae-associated microbiomes compared with species-specific interactions (Eigemann et al., 2013). Definitely, further experiments, as well as studies on environmental parameters and seasonal cycles, need to be performed to identify the underlying relationship between dinoflagellates and associated bacteria and to

TABLE 3 Similarity percentage analysis (SIMPER) between *O. cf. ovata* (OO) and *G. australes* (GA) libraries from their respective associated microbiomes

OTU	OO abundance (%)	GA abundance (%)	Mean dissimilarity (%)	Contribution (%)	Cumulative (%)	Taxonomic assignment
35	0	38.35	11.44	12.33	12.33	<i>Labrenzia</i>
24	11.73	0	8.23	8.87	21.21	SM1A02
38	1.93	19.19	6.79	7.32	28.54	<i>Oceanicaulis</i>
91	9.7	0	4.73	5.1	33.64	Unc. Alphaproteobacteria
98	6.74	0	4.7	5.09	38.73	<i>Maricaulis</i>
95	6.73	0	4.59	4.95	43.68	<i>Marivita</i>
108	6.5	0	4.36	4.7	48.39	Unc. Hyphomonadaceae
5	5.9	0	4.18	4.5	52.91	<i>Brumimicrobium</i>
60	5.67	0	3.9	4.2	57.2	<i>Thalassospira</i>
41	0.2	12.29	3.5	3.7	60.99	<i>Stappia</i>

understand the main drivers for the differences observed in the microbiomes of the two species.

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#### CONFLICT OF INTEREST

The authors declare no conflict of interest.

#### AUTHOR CONTRIBUTIONS

**Renata Denaro:** conceptualization, methodology, validation, investigation, data curation, writing original draft, writing review & editing. **Francesca Crisafi:** methodology, investigation, data curation, writing original draft, writing review & editing. **Francesco Smedile:** software, validation, formal analysis, investigation, data curation, writing original draft. **Vittorio Soprano:** writing, review & editing, visualization. **Rachele Rossi:** methodology, investigation, data curation, writing original draft. **Adriana Zingone:** writing review & editing, visualization. **Felix Acosta:** writing review & editing. **Maria Grazia Giacobbe:** methodology, validation, investigation, writing original draft, writing review & editing.

#### DATA AVAILABILITY STATEMENT

N/A

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