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Cultivable epiphytic bacteria of the Chlorophyta Ulva sp.: diversity, antibacterial, and biofilm-modulating activities

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Abstract :

Aims

Macroalgae harbor a rich epiphytic microbiota that plays a crucial role in algal morphogenesis and defense mechanisms. This study aims to isolate epiphytic cultivable microbiota from Ulva sp. surfaces. Various culture media were employed to evaluate a wide range of cultivable microbiota. Our objective was to assess the antibacterial and biofilm-modulating activities of supernatants from isolated bacteria.

Methods and results

Sixty-nine bacterial isolates from Ulva sp. were identified based on 16S rRNA gene sequencing. Their antibacterial activity and biofilm modulation potential were screened against three target marine bacteria: 45%, mostly affiliated with Gammaproteobacteria and mainly grown on diluted R2A medium (R2Ad), showed strong antibacterial activity, while 18% had a significant impact on biofilm modulation. Molecular network analysis was carried out on four bioactive bacterial supernatants, revealing new molecules potentially responsible for their activities.

Conclusion

R2Ad offered the greatest diversity and proportion of active isolates. The molecular network approach holds promise for both identifying bacterial isolates based on their molecular production and characterizing antibacterial and biofilm-modulating activities.

Keywords : Ulva sp, epiphytic bacteria, antibacterial activity, biofilm assays, cultivable microbiota, molecular network analysis

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21 **Significance and impact of the study** : Some of these isolates may be of biotechnological

22 interest, such as *Kocuria* sp. VNSS-35 exhibiting a strong probiofilm effect and three isolates

23 (*Cellulophaga* sp. R2A-50, *Paraglaciecola* sp. R2A-57, *Pseudoalteromonas* sp. R2A-38)

24 showing antibiofilm activity but no antibacterial effect.

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1. Introduction

Macroalgae are known to harbor on their submerged surface a rich microbiota among which bacteria constitute the predominant occupants with mean densities varying from 10^6 to 10^8 bacteria/cm^2 depending on the thallus section, season and macroalgal species (Armstrong *et al*., 2000, Burke *et al*., 2011, de Oliveira *et al*., 2012). The epiphytic bacteria inhabiting these ecological niches differ from those present in the marine environment and undergo selection by the macroalgae. *Gammaproteobacteria,* CFB group *(Cytophaga-Flavobacterium-Bacteroides), Alphaproteobacteria* and *Actinomycetes* are among the most commonly found epiphytic bacteria associated with different macroalgal groups (Hollants *et al*., 2012). Interactions between *Ulva* sp. and their associated bacteria have been well-characterized over the last years. They reflect the mutualism role of the *Ulva* association with bacterial epiphytes on algal growth, development and morphogenesis (Spoerner *et al.,* 2012). Associated epiphytic bacteria may deploy defensive strategies to maintain their ecological niche from opportunistic bacteria colonization since the macroalgae surface constitutes a desirable habitat for nutrients and protection for the bacteria (Armstrong *et al*., 2001). In marine ecosystems, infochemicals are the main underwater means of communication in biological systems, providing a cross-kingdom chemical language between the macroalgal host and its associated bacteria (Saha *et al.,* 2019). The macroalgae surface is in constant interaction with biotic and abiotic factors that influence the dynamic of the large diversity of bacteria associated with it (Bolinches *et al*., 1988, Van der Loos *et al*., 2022) and may stimulate or decrease the release/production of algal defense compounds. Macroalgae surfaces constitute a bioactive molecule reservoir, whose antibacterial and antifouling potentialities can be explored through bacterial culture-based approaches. For instance, the marine algal surface-associated genus *Pseudoalteromonas* is known to produce biologically active compounds with antibacterial, antifungal, algicidal and agarolytic properties (Holmström and Kjelleberg, 1999).

56 Although access to Next Generation Sequencing methods have led to a better understanding
57 of the structure and function of the macroalga-associated bacterial communities, a culture-
58 based approach allows for the screening of bacterial activities and the characterization of both
59 cultivable bacteria and molecules of interest. This can simultaneously identify a portion of the
60 macroalgae microbiota and evaluate its potential interaction with the host along with possible
61 biotechnological applications (Lian *et al.,* 2018).
62 Responsible for numerous strandings, green tides, or blooms worldwide, *Ulva* species are
63 viewed negatively by coastal populations. Algal blooms and strandings are becoming
64 increasingly significant globally due to climate change and anthropogenic activities (Ye *et al.,*
65 2011). They are considered a nuisance due to their decomposition and the production of toxic
66 vapors, which affect the tourism industry in coastal areas and marine ecosystems (resulting in
67 the inhibition of seaweed zygote germination and decreased growth rates of algal species).
68 These occurrences lead to financial losses for resort operators, who must also bear the costs of
69 removing and disposing of the beached algae. (Charlier *et al.,* 2008, Ye *et al*., 2011, Louis *et*
70 *al.,* 2023).
71
72 This study aims to assess the epiphytic cultivable bacterial community of three *Ulva* sp.
73 (*Ulva lacinulata, Ulva australis* and *Ulva rigida*) collected from three locations on the
74 Kerleven beach in south Brittany (France). Various culture media were employed to evaluate
75 a wide range of cultivable bacteria. In the subsequent phase, our objective was to explore the
76 agarolytic potential, biofilm modulation capacities and antibacterial activities of supernatants
77 produced by isolated bacteria. Furthermore, the nature of the molecules responsible for these
78 activities was investigated for four isolates of interest.
79

2. Experimental procedures

1. Sampling site

Ulva lacinulata, Ulva australis and *Ulva rigida* identified by sequencing *tufA* gene (Olivier De Clerck, Ghent University, Belgium) were collected at high-coefficient low tide the 2nd of December 2021 at Kerleven beach along three stations in the southern coast of Brittany (France) : station 1 (47°54'36"N; 3°58'17"O), station 2 (47°54'36"N, 3°58'17"O) and station 3 (47°54'36"N; 3°58'17"O). *Ulva lacinulata* were found in stations 1 and 3; *Ulva rigida* and *Ulva australis* in station 2. A pool of 18 algae per station were detached from their rocky support with gloves, placed into 3 sterile Schott bottles (6 per bottle) filled with the seawater from the station and transferred within 2 hours at the laboratory in isothermal containers.

Kerleven beach is a wind-sheltered bay approximately one kilometer in length consisting of fine sand with a rocky foreshore. Three watersheds surround the bay of La Forêt and several rivers contribute to nitrogen flow into the bay; the topography of the bay and the constant nitrogen input favor significant development of green algae. Station 1 is located on a rocky dyke on the sandy foreshore and is influenced by the port la Forêt and the mudflat upstream, while stations 2 and 3 are puddles located on the rocky infralittoral**.**

In order to further characterize the sampling stations, a multiparameter probe (NKE MP7, NKE Instrumentation France) was placed in the seawater during sampling. Data collected at each station are reported in **Table 1.**

106 Table 1 : Physical parameters at each station
107

Stations	Conductivity (ms/cm)	pH	Fluorescence	Depth (m)	Temperature (°C)	Turbidity (NTU)	Dissolved oxygen (mg/L)
Station 1	36.8	7.9	5.7	0.3	9.4	9.6	10.3
Station 2	35.5	7.9	1.7	0.2	7.9	0.2	11.1
Station 3	36.1	7.9	2.0	0.4	8.5	0	11.6

108

109

110 **2. Bacterial isolation and identification**

111

112 Upon arrival at the laboratory, the 54 sampled algae were rinsed twice with sterile
113 Artificial Sea Water (ASW) (Sea salt 30 g.L^{-1}, Sigma-Aldrich, St. Louis, USA) to remove
114 transient bacteria. The epiphytic bacteria were recovered by swabbing the algal surfaces with
115 sterile cotton swabs (18 algae per station). Swabs from each station were then placed in 10 mL
116 of sterile ASW to generate suspensions of recovered microbes. The 3 suspensions (one per
117 station) were 10-fold serially diluted in ASW. Then 100 µL of 4 successive dilutions (10^0 to
118 10^{-3}) were inoculated in duplicate onto the 4 following media for enumeration and isolation:
119 oligotrophic bacteria in 1/20 diluted R2A (R2Ad) (Reasoner and Geldreich, 1985) adapted for
120 marine bacteria (Suzuki *et al.,* 1997 ; Kleinjan *et al*., 2017) and the seawater-mimicking
121 medium Väätänen Nine Salt Solution agar (VNSS, Marden *et al*., 1985, Hermansson *et al.,*
122 1987), copiotrophic bacteria in Marine Agar (MA, Grosseron), while the growth of
123 actinobacteria/actinomycetes was favored by Actinomycetes medium (Strength, G.
124 Actinomycete Isolation Agar DM738). The agar plates were incubated for 2 to 5 days at 20°C
125 ± 2°C. Different bacterial colonies were selected according to their characteristics (color, size
126 and shape) and further isolated until clonal cultures were obtained (at least 4 successive
127 streaking on their respective medium). Colonies grown on R2Ad were further streaked on R3A

128 medium to promote rapid bacterial growth (Reasoner and Geldreich, 1985). The isolates were
129 examined by Gram staining and stored at -80°C in 30% glycerol.
130
131 **3. DNA extraction from isolates and PCR condition**
132
133 Bacterial isolates were identified by partial sequencing of the 16S rRNA gene. Genomic
134 DNA was extracted by thermal lysis. Briefly, bacterial isolates (5 ml) were grown in their
135 isolation medium for 24 h (25°C, 180 rpm). From 2 mL of the centrifuged sub-cultures (6000g,
136 10 min), pellets were washed twice with phosphate-based buffer (Hurt *et al.*, 2014). Pellets
137 were then resuspended in 0.1 ml of ultra-pure water, incubated 15 min at 100°C and the PCR
138 was done on the supernatants (1 µl) using the universal 16S rRNA gene primers B8F and 1492r
139 (Durand *et al.*, 2010). PCR were performed using a thermal cycler (Biosystem GeneAmp PCR
140 System 9700), in 25 µl final reaction mixtures containing 1X GoTaq buffer (with $MgCl_2$), 0.2
141 mMol dNTP, 0.4 µMol of each primer, 0.024 U of GoTaq polymerase (Promega,
142 Charbonnières, France) and 1 µL of template DNA. The PCR assay started by an initial
143 denaturation at 94 °C for 5 min, followed by 30 cycles at 94 °C for 1 min, 49 °C for 1 min 30s,
144 and 72 °C for 2 min, and a final extension step at 72 °C for 6 min. Each PCR reaction was run
145 together with positive (genomic DNA from *Vibrio tapetis* CECT 4600 (Borrego *et al.,* 1996)
146 and negative controls (PCR mixture with molecular grade water instead of DNA).
147
148 **4. Sequencing**
149
150 The amplified 16S rRNA gene amplicons were verified by electrophoresis (0.8%
151 agarose, 1 × TAE running buffer containing 40 mM Tris acetate and 2 mM EDTA), cleaned
152 using the Wizard® Genomic DNA Purification kit (Promega, France) and sent for sequencing
153 to GENEWIZ Laboratories (Leipzig, Germany). The sequences were first trimmed with
154 Geneious (version 2023 1.2, Biomatters, New Zealand) and compared using BLAST search

155 using EzBioCloud database (https://www.ezbiocloud.net/) (Yoon *et al*., 2017) to determine the
156 closest relatives. A sequence similarity greater than 94.5% is considered sufficient to allocate
157 the taxonomic identity at genus level (Yarza *et al*., 2014).
158
159 **5. Evaluation of biological activities**
160
161 **a. Antibacterial activity evaluation**
162
163 Bacteria were reactivated from -80°C storage and subcultured at least twice in their
164 isolation medium (25°C, 180 rpm) before use. First, the isolates were screened for their
165 antibacterial activity against pioneer biofilm bacteria *Paracoccus* sp. 4M6 and *Algibacter* sp.
166 1M6 (Grasland *et al.*, 2003) and an opportunistic pathogen of marine organisms *Vibrio harveyi*
167 BB120 (Bassler *et al.*, 1997). The antibacterial evaluation was performed through the agar well
168 diffusion method (Tagg *et al*., 1976; Devi *et al*., 2011). Supernatants from 10 mL of 48h
169 subculture isolates were centrifuged (10 000 g, 10 min), the pH was recorded and the
170 supernatant was sterile filtered (0.2 µm).
171 Fresh bacterial suspension of target-bacteria was adjusted to a density of 0.75 Mac Farland
172 with a densitometer DEN-1 and DEN-1B (Grant Instruments, United Kingdom) in a NaCl
173 solution (20 g.L-^{1}), inoculated (0.1%) in sterile VNSS soft agar (0.8 % w/v) and poured in Petri
174 dishes. After solidification of the medium, wells of 5 mm diameter were made and filled with
175 80 µl of the cell-free supernatant to be tested. Agar plates were then incubated at 30°C ± 2°C
176 for 48h. Inhibition diameters were measured at 24 h and 48 h. *Vibrio harveyi* BB120, which
177 exhibits antibacterial activity, was used as positive control against target bacteria *Paracoccus*
178 sp. 4M6 and *Algibacter* sp. 1M6.
179 The same protocol was also carried out with concentrated supernatants. A small volume
180 of culture medium (300 µL) was added to a 24 hours old bacterial culture pellet and grown
181 overnight. Supernatants from the overnight cultures (25°C, 180 rpm) were prepared and

182 evaluated according to the agar well diffusion method. In parallel, *Ulva* sp. homogenate made
183 of 5 mg.L^{-1} of 72 hours lyophilized *Ulva* sp. (Alpha 1-4 LSC basic, Christ, Germany) and NaCl
184 solution (20 g.L-^{1}) was used to evaluate the production of antimicrobial molecules when the
185 isolates were in contact with the alga. The protocol was the same as the one used to test
186 concentrated supernatants, with 300 µL of *Ulva* sp. homogenate added to a 24 hours old
187 bacterial culture pellet before overnight growth. The *Ulva* sp. homogenate was autoclaved and
188 tested as a negative control to ensure the absence of antimicrobial activity.
189
190 **b. Agarolytic capacity evaluation**
191
192 The agarolytic capacity of the isolates was assessed by the demonstration of
193 liquefaction or shallow depressions appearing around the colonies, as described by
194 Vijayaraghavan and Rajendran (2012).
195
196 **c. Antibiofilm activity evaluation by microtiter plate assay**
197
198 Inocula of biofilm-forming strains *Paracoccus* sp. 4M6 and *Algibacter* sp. 1M6 were
199 prepared from fresh 24-hour cultures (VNSS broth at 25°C) and adjusted to a density of 0.5
200 Mac Farland in a NaCl solution (20 g.L^{-1}). Supernatants from 24-hour test cultures, grown in
201 their isolation medium, were prepared by 0.2 µm sterile filtration and 45 µL were added per
202 well to the 10 µL inoculum in 96-well microtiter flat-bottom polystyrene plates (Corning-
203 Falcon, Dutscher, France). Plates were incubated overnight in VNSS medium at 25°C without
204 shaking to enhance the adhesion step (Klein, 2011). Culture liquid was then removed by
205 aspiration and the wells were rinsed three times with artificial sea water. Biofilms were stained
206 with 0.8% crystal violet solution for 20 minutes at room temperature (Doghri *et al.*, 2020). The
207 wells were rinsed with distilled water until the wash-liquid was clear. The stained biofilm was
208 eluted with 96% ethanol and solubilized in the pipette reflow. After 10 minutes of

209 solubilization, the OD_{595} was determined with a plate reader (POLARstar Omega, BMG
210 Labtech, Germany). Positive control (biofilm-forming bacteria, no supernatants) and negative
211 control (sterile medium) were made for each plate with the same protocol, adding either MB,
212 VNSS or R3A because of the different media growth of *Ulva* sp. isolates. The biofilm biomass
213 was determined by measuring the absorbance at OD_{595}.
214
215
$$\left(\frac{OD595\,nm\,isolate\ -\ OD595\,nm\,negative\ control}{OD595\,nm\,positive\ control\ -\ OD595\,nm\,negative\ control}\right) \times\ 100$$
216

217 **d. Statistical analysis**

218
219 All tests in microtiter plates were carried out in three biological replicates from three
220 different subcultures of isolates and biofilm-forming bacteria. For each plate, 3 technical
221 replicates were carried out. The statistical significance between the supernatants was
222 determined by a one-way Anova on the OD_{595} averages per plate and a Dunett test was then
223 performed for comparison to the positive control of each plate (p values of < 0.05 were
224 considered significant). All the tests were calculated using Matlab software (Mathworks
225 Inc.,Natick, Massachusetts, USA).
226

227 **e. Molecular network analysis**

228

229 **i. Bacterial strains, growth condition and supernatants preparation**

230
231 Four isolates of interest were selected for molecular network analyses: *Dokdonia* sp.
232 VNSS-42, which displayed antibacterial activity after contact with *Ulva* sp. homogenate only;
233 *Paraglaciecola* sp. R2A-48 and *Sulfitobacter* sp. R2A-59, which both showed antibacterial and
234 antibiofilm properties and *Kocuria* sp. VNSS-35, which harbored the strongest probiofilm

235 effect on both *Algibacter* sp. 1M6 and *Paracoccus* sp. 4M6. Supernatants of bacterial cultures
236 were recovered after 48 hours of growth in 5mL of their respective culture medium at 25°C
237 under orbital shaking (180 rpm). Then, the bacterial suspensions were inoculated in 150 mL of
238 their respective culture medium under the same growth conditions. The cultures were
239 centrifuged at 10 000 g for 10 min before sterilization of the supernatants by 0.2 µm filtration
240 and stored at -80°C. The final volume of supernatants from *Dokdonia* sp. VNSS-42,
241 *Paraglaciecola* sp. R2A-48, *Sulfitobacter* sp. R2A-59 and *Kocuria* sp. VNSS-35 were
242 respectively 101 mL, 125 mL, 146 mL and 130 mL. The bacterial activity initially observed in
243 each of these supernatants was checked after freezing.
244
245 **ii. Sample preparation before analysis**
246
247 Then entire volume of each strain supernatant and 100 mL of each sterile culture
248 medium were fractionated through solid-phase extraction (SPE) columns C18-E Strata (55µm,
249 70A), 2 g / 12 mL (Phenomenex, USA), using a vacuum manifold. First, columns were
250 preconditioned with 12 mL of methanol, then 12 mL of distilled water and samples were
251 applied at 1 $mL.min^{-1}$. The columns were washed with 24 mL of distilled water and dried during
252 5 min before elution with successively 24 mL of methanol followed by 24 mL of
253 dichloromethane. Solvents of each fraction were then evaporated under vacuum using a
254 Rotavapor (Buchi B-491, Marshall Scientific, USA) to give two fractions labeled M and D
255 respectively. After resolubilization at a concentration of 1 $mg.mL^{-1}$ with methanol for fraction
256 M and ethanol for fraction D, each fraction was filtered on a 4 mm, 0.2 µm RC syringe filter
257 (Phenex, Phenomenex, USA). To obtain 1 mg of the final sample, 900µL of the filtered fraction
258 M and 100µL of fraction D were taken, pooled together and the solvents removed under
259 vacuum (Mivac, Genevac, UK). Dried samples were stored at 4°C until analysis.
260

261 **iii. LC-MS/MS analysis**

262

263 Positive and negative LC-ESI-HR MS/MS analyses were performed with an Elute
264 UHPLC system (Bruker, USA) combined with a TOF tims mass spectrometer (Bruker
265 Daltonics, USA). The analytes were separated on an analytical C18 column InfinityLab
266 Poroshell 120SB-C18 (2.1 x 150mm, 2.7µm, Agilent, USA) thermostated at 40°C. Methanol
267 was used to resuspend dried samples at 1 $mg.mL^{-1,}$ and a volume of 2µL were injected in
268 positive ESI and 5 µL in negative ESI respectively. The mobile phase was composed of A:
269 H2O + 0.1% formic acid (FA); B: CH3CN + 0.1% FA. The gradient was performed at 0.4
270 $mL.min^{-1}$ with 5% B to 100% B for 20 min. Mass spectra were recorded in positive and
271 negative ion mode, according to the parameters in **Table 2**. Agilent's tuning mix was used for
272 calibration, with a mass measurement accuracy of less than 3 ppm.

273

274 Table 2 : Parameters of the mass spectra

	Positive ESI	**Negative ESI**
Source parameters		
Capillaire voltage (V)	4200	4500
Gas temperature (°C)	250	250
Drying gas (N2) flow rate ($L.min^{-1}$)	8.6	8.6
Nebulizer pressure (Bar)	3	3
Collision at source (eV)	0	0
MS		
Gamme de masse (m/z)	20-1350	50-1350
MS collision energy	5eV	5eV
Spectra rate MS	10Hz	10Hz
AutoMS/MS preference	m/z 99.5-1350	m/z 99.5-1350
Collision energy	20 eV	20 eV
Spectra rate MS/MS	5Hz	5Hz
Number of precursors	4	4
State of charge	2-1	2-1

275

276

277 **iv. Molecular networks generation**

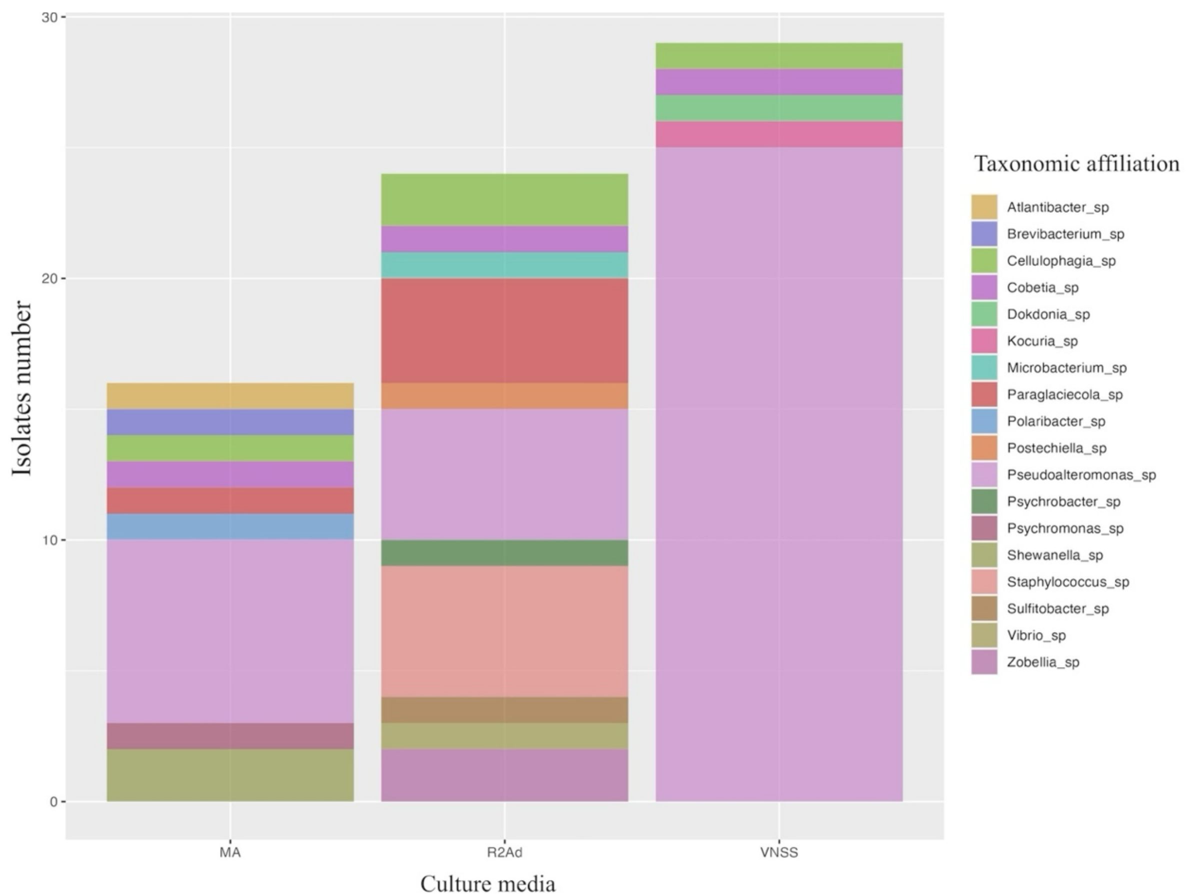
278
279 MSconvert software belonging to Proteowizard (Chambers *et al.,* 2012) package was used
280 for data conversion from Bruker constructor format to mzML files. The converted data files
281 were processed using the online workflow at GNPS (Wang *et al*., 2016) with the following
282 settings for network generation: minimum pairs cos, 0.7; parent ion mass tolerance, 0.02 Da;
283 fragment ion mass tolerance, 0.5 Da; network topK, 10; minimum matched peaks, 6; minimum
284 cluster size, 2. Then, the data were imported into Cytoscape 3.10.1 (Shannon *et al.,* 2003) for
285 nodes and edges visualization. Node colors were determined according to the identity or
286 biological activity of the samples, and edge thickness was defined according to cosine
287 similarity scores, with thicker lines corresponding to higher similarity.
288

289 # 3. Results
290

291 ## 1. Cultivable diversity
292

293 Bacterial levels recovered per medium were obtained by colony enumeration on Petri
294 dishes after a serial dilution of the original sample. The bacterial levels were 1.4 $10^6 \pm 6.5$ 10^5
295 UFC.mL in MA; 5.5 $10^5 \pm 4.5$ 10^5 UFC.mL in VNSS; and 2.1 $10^5 \pm 9.3$ 10^4 UFC.mL in the
296 R2Ad medium. No growth was observed on Actinomycetes medium. Between 16 and 29
297 isolates per medium (MA, VNSS, R2Ad) with different colony morphologies (color, shape,
298 colony size) were obtained in pure culture by repeated transfers (**Figure 1**).

299



300 Figure 1: Bacterial communities associated with *Ulva* sp. grown on three different media:
301 MA (Marine agar), VNSS (Väätänen Nine Salt Solution agar), R2Ad (1/20 diluted, Reasoner
302 and Geldreich, 1985; KleinJan *et al.,* 2017)

303

304 From the isolates that initially grew on the three media, 69 isolates were identified, 89%
305 of which were Gram-negative and belonged to the *Gammaproteobacteria* (75%),
306 *Flavobacteriia* (13%) and *Alphaproteobacteria* (1%). The Gram-positive ones (11%) were
307 affiliated to the *Actinomycetes* (4%) and to the *Bacilli* (7%). The taxonomic affiliations are
308 shown in **Table 3.** Species with sequence similarity below species threshold value of 98.7%
309 (Stackebrandt and Ebers, 2006) were considered to the genus level.

310

311

312 Table 3 : Phylogenetic affiliation of heterotrophic bacteria isolated from the surface of *Ulv*a
313 sp.. Isolates were named according to the culture medium. Their number corresponds to the
314 30 isolates/medium initially isolated from the initial cultures (30-60) in December 2021.
315 Taxonomic affiliations were based on 16S rRNA gene sequencing. Whole genome
316 sequencing was performed on two isolates of interest (*Dokdonia* sp. VNSS-42 and *Kocuria*
317 sp. VNSS-35).
318

Isolates	Colors	Agarolytic	Size 16rRNA amplicon (bp)	Closest matching strain	Accession number of the closest matching strain	Identity (%)	Family	Class	Phylum
MB-46	orange		1328	*Brevibacterium* sp.	/	97.44	*Brevibacteriaceae*	*Actinomycetes*	*Actinomycetota*
MB-47	yellow		1325	*Polaribacter* sp.	/	97.50	*Flavobacteriaceae*	*Flavobacteriia*	*Bacteroidota*
MB-48	yellow iridescent		1208	*Cellulophaga PHUL_s*	PHUL01000001	99.42			
MB-38	white		1207	*Paraglaciecola chathamensis/agarilytica*	BAEM01000005/BAEK01000058	98.83	*Alteromonadaceae*	*Gammaproteobacteria*	*Pseudomonadota*
MB-33	white		1214	*Cobetia* sp.	/	95.7	*Halomonadaceae*		
MB-34	light pink	+	80	*Atlantibacter* sp.	/	86.11	*Enterobacteriaceae*		
MB-32	light pink		1176	*Pseudoalteromonas issachenkonii*	CP013350	98.98	*Pseudoalteromonadaceae*		
MB-37	light pink		1241	*Pseudoalteromonas* sp.	X82136	98.11			
MB-43	white		1166	*Pseudoalteromonas carragreenovora*	X82136	99.13			
MB-52	light pink		1127	*Pseudoalteromoas carragreenovora*	X82136	99.37			
MB-53	white		285	*Pseudoalteromonas agarivorans*	CP011011	100			
MB-55	grey		1439	*Pseudoalteromonas* sp.	/	97.32			
MB-58	white		1174	*Pseudoalteromonas carragreenovora*	X82136	99.49			
MB-45	white		80	*Psychromonas* sp.	/	91.43	*Psychromonadaceae*		
MB-42	pink		1248	*Shewanella* sp.	FR744784	96.78	*Shewanellaceae*		
MB-49	pink		1132	*Shewanella* sp.	FR744784	98.05			
VNSS-35	light pink		WGS	*Kocuria rhizophila*			*Micrococcaceae*	*Actinomycetes*	*Actinomycetota*
VNSS-36	yellow	+	298	*Cellulophaga sp.*	KF270632.1	99.65	*Flavobacteriaceae*	*Flavobacteriia*	*Bacteroidota*
VNSS-42	yellow		WGS	*Dokdonia* sp.					
VNSS-60	white		287	*Cobetia* sp.	/	94	*Halomonadaceae*	*Gammaproteobacteria*	*Pseudomonadota*
VNSS-31	white	+	1053	*Pseudoalteromonas atlantica/tetraodonis*	BJUT01000111/CP011041	99.91	*Pseudoalteromonadaceae*		
VNSS-44	yellow	+	1137	*Pseudoalteromonas carragreenovora*	X82136	99.91			
VNSS-45	white		289	*Pseudoalteromonas carragreenovora*	X82136	99.65			
VNSS-46	yellow		1054	*Pseudoalteromonas carragreenovora*	X82136	99.9			
VNSS-47	yellow	+	781	*Pseudoalteromonas* sp.	/	80.46			
VNSS-49	yellow		1135	*Pseudoalteromonas carragreenovora*	X82136	99.12			
VNSS-51	yellow		1218	*Pseudoalteromonas carragreenovora*	X82136	99.09			
VNSS-57	yellow		1216	*Pseudoalteromonas* sp.	CP011034	92.14			
VNSS-58	white		1175	*Pseudoalteromonas carragreenovora*	X82136	99.49			
VNSS-41	white		913	*Pseudoalteromonas neustonica*	BDDS01000056	99.89			
VNSS-32	yellow		1233	*Pseudoalteromonas nigrifaciens*	CP011036	99.78			
VNSS-33	orange		1269	*Pseudoalteromonas nigrifaciens*	CP011036	99.05			
VNSS-38	yellow		1209	*Pseudoalteromonas nigrifaciens*	CP011036				
VNSS-40	orange		1054	*Pseudoalteromonas nigrifaciens*	CP011036	99.14			
VNSS-48	yellow		1042	*Pseudoalteromonas nigrifaciens*	CP011036	100			
VNSS-52	white		1099	*Pseudoalteromonas nigrifaciens*	CP011036	99.55			
VNSS-53	yellow		1228	*Pseudoalteromonas nigrifaciens*	CP011036	98.86			
VNSS-56	white		1226	*Pseudoalteromonas nigrifaciens*	CP011036	99.26			
VNSS-59	yellow		1264	*Pseudoalteromonas nigrifaciens*	CP011036	98.89			
VNSS-34	light pink		1107	*Pseudoalteromonas* sp.	/	99.28			
VNSS-54	orange		287	*Pseudoalteromonas* sp.	/	100			
VNSS-39	yellow	+	1190	*Pseudoalteromonas tetraodonis*	CP011041	99.07			
VNSS-43	white	+	1048	*Pseudoalteromonas tetraodonis*	X82136	100			
VNSS-55	white		1051	*Pseudoalteromonas tetraodonis*	CP011041	100			
VNSS-37	white		1214	*Pseudoalteromonas undina*	X82140	99.08			
R2A-31	light yellow		1216	*Microbacterium* sp.	/	98.1	*Microbacteriaceae*	*Actinomycetes*	*Actinomycetota*
R2A-35	light yellow	+	1261	*Cellulophaga* sp.	/	97.68	*Flavobacteriaceae*	*Flavobacteriia*	*Bacteroidota*
R2A-50	yellow	+	1260	*Cellulophaga* sp.	/	79.6			
R2A-56	light yellow		1214	*Postechiella* sp.	/	97.93			
R2A-47	yellow		1240	*Zobellia russellii*	AB121976	99.27			
R2A-52	yellow	+	900	*Zobellia* sp.	/	97.05			
R2A-37	yellow		1245	*Staphylococcus edaphicus*	KY315825	99.19	*Staphylococcaceae*	*Bacilli*	*Bacillota*
R2A-46	white		1110	*Staphylococcus* sp.	/	83.83			
R2A-54	light yellow		1241	*Staphylococcus* sp.	/	97.22			
R2A-58	yellow		1232	*Staphylococcus* sp.	/	97.87			
R2A-41	translucent		309	*Staphylococcus agnetis*	HM484980	99.29			
R2A-59	translucent		1185	*Sulfitobacter donghicola*	JAMC01000023	98.98	*Roseobacteraceae*	*Alphaproteobacteria*	*Pseudomonadota*
R2A-32	translucent	+	786	*Cobetia* sp.	/	96.2	*Halomonadaceae*	*Gammaproteobacteria*	
R2A-48	white	+	1206	*Paraglaciecola* sp.	/	97.9	*Alteromonadaceae*		
R2A-49	translucent		1229	*Paraglaciecola* sp.	/	97.22			
R2A-51	white		661	*Paraglaciecola mesophila*	BAEP01000046	98.94			
R2A-60	white		1260	*Paraglaciecola* sp.	/	96.46			
R2A-40	translucent		1271	*Psychrobacter* sp.	AJ313425	98.62	*Moraxellaceae*		
R2A-38	white		1277	*Pseudoalteromonas carragreenovora*	X82136	99.44	*Pseudoalteromonadaceae*		
R2A-39	translucent		530	*Pseudoalteromonas* sp.	/	99.73			
R2A-42	white	+	1250	*Pseudoalteromonas* sp.	JN578478	98.16			
R2A-55	translucent		1245	*Pseudoalteromonas* sp.	/	97.74			
R2A-57	white		1169	*Paraglaciecola* sp.	/	98.01			
R2A-36	white	+	1232	*Vibrio MCVZ*	MCVZ01000110	98.78	*Vibrionaceae*		

319
320
321 Isolates with agarolytic properties are indicated with (+) ; base pairs (bp) refers to the size of the 16S amplicon
322 sequenced and used for the phylogenetic affiliation of isolates, and "WGS" is indicated for the whole genome
323 sequenced.
324
325
326
327

328 R2Ad medium characterized by low nutrients content showed the highest bacterial
329 diversity with 24 isolates gathered in 11 genera: *Microbacterium* sp., *Cellulophaga* spp.,
330 *Postechiella* spp., *Zobellia* spp., *Staphylococcus* spp., *Sulfitobacter* spp., *Cobetia* spp.,
331 *Paraglaciecola* spp., *Psychrobacter* spp., *Pseudoalteromonas* spp. and *Vibrio* spp..
332 MB medium, which is a rich medium enhancing the growth of a broad spectrum of
333 copiotrophic marine bacteria, showed an intermediate level of diversity with 16 isolates
334 gathered in 9 genera with *Brevibacterium* spp., *Polaribacter* spp., *Psychromonas* spp. and
335 *Shewanella* spp. as specific affiliation found in this medium. The lowest bacterial diversity was
336 observed for the VNSS medium, a complex salt-rich medium, with only 5 genera (*Cobetia*
337 spp., *Cellulophaga* spp., *Dokdonia* sp., *Kocuria* sp., *Pseudoalteromonas* spp.), and with a
338 predominance of *Pseudoalteromonas* spp. (25/29 isolates). By using different culture media, a
339 higher diversity of cultivable epiphytic bacteria from *Ulva* sp. was achieved. The use of three
340 media highlighted also the specificity of VNSS medium for the culture of *Pseudoalteromonas*
341 spp.
342
343 Bacteria affiliated to the *Pseudoalteromonas* genus were isolated from all media, but
344 especially from VNSS. Strains were distinguished based on their colony colors, the presence or
345 absence of biochemical enzymes, and activity profiles.
346
347 **2. Evaluation of biological activities**
348
349 **a. Antibacterial activity**
350
351 The antibacterial potential of filtered culture supernatants from 69 isolates obtained
352 from the cultivable epibacterial community associated with *Ulva* sp. was assessed using the
353 agar well diffusion method.

Filtered culture supernatants from 31 isolates (45% of the bacterial total diversity) were found to possess antibacterial activity against the target bacteria. Most of the active isolates were Gram-negative (93.5%) mostly affiliated to *Gammaproteobacteria* (mainly *Pseudoalteromonas)* and showed antibacterial activity against the three target bacteria, which are ubiquitous Gram-negative marine bacteria (**Table 4**).

379 Table 4 : Antibacterial activity of *Ulva* sp. isolates against target-bacteria. The inhibition
380 diameters reported were measured after 48 hours, each measurement corresponding to the
381 average of the triplicates. Isolates with an (*) only showed activity with-concentrated
382 supernatant conditions. The VNSS-42 isolate with an (**) only showed antibacterial activity
383 with *Ulva* sp. homogenate.
384
385

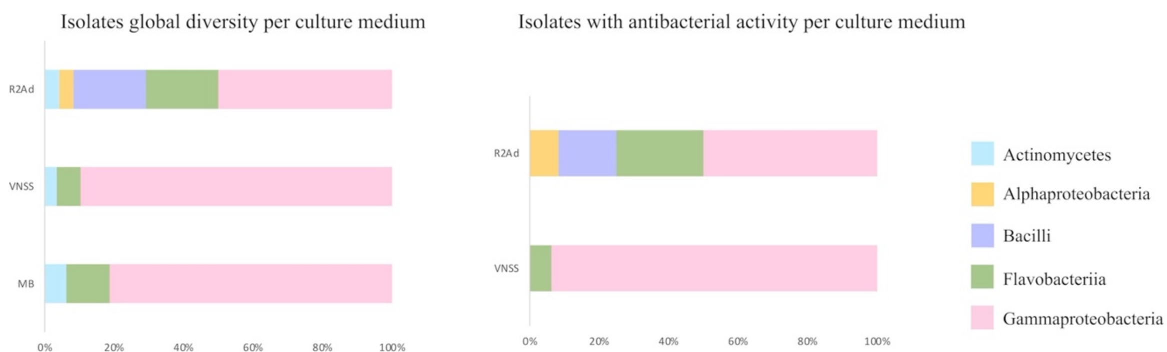
Active isolates	Genus	Inhibitory diameters (mm)		
		Algibacter sp. 1M6	*Paracoccus* sp. 4M6	*Vibrio harveyi* BB120
MB-34	*Atlantibacter*	/	/	12.3 ± 0.6
MB-53	*Pseudoalteromonas*	31.3 ± 8.3	34.5 ± 7.8	37.5 ± 11.0
MB-55	*Pseudoalteromonas*	/	/	25.3 ± 0.6
VNSS-31	*Pseudoalteromonas*	25.0 ± 1.2	25.8 ± 1.7	25.5 ± 1.3
VNSS-32	*Pseudoalteromonas*	25.0 ± 1.2	26.5 ± 1.0	25.5 ± 1.9
VNSS-33*	*Pseudoalteromonas*	22.0 ± 1.8	22.3 ± 5.4	23.0 ± 1.8
VNSS-36	*Pseudoalteromonas*	20.0 ± 0.0	22.3 ± 1.7	21.5 ± 1.3
VNSS-39	*Pseudoalteromonas*	14.0 ± 2.0	14.7 ± 4.2	13.0 ± 2.6
VNSS-40	*Pseudoalteromonas*	18.0 ± 5.7	14.0 ± 0.0	15.5 ± 4.9
VNSS-43	*Pseudoalteromonas*	21.8 ± 1.7	21.8 ± 1.7	24.0 ± 1.6
VNSS-42**	*Dokdonia*	17.0 ± 1.4	16.0 ± 2.8	15.0 ± 4.2
VNSS-44	*Pseudoalteromonas*	15.3 ± 1.0	14.3 ± 4.0	13.8 ± 3.9
VNSS-45	*Pseudoalteromonas*	13.8 ± 4.3	13.5 ± 4.7	12.3 ± 2.1
VNSS-46*	*Pseudoalteromonas*	10.0 ± 0.0	10.0 ± 0.0	10.0 ± 0.0
VNSS-47*	*Pseudoalteromonas*	17.3 ± 1.0	15.8 ± 0.5	14.5 ± 4.7
VNSS-48*	*Pseudoalteromonas*	18.0 ± 0.0	16.0 ± 5.7	16.0 ± 2.8
VNSS-53	*Pseudoalteromonas*	12.5 ± 2.1	13.8 ± 1.7	14.5 ± 3.7
VNSS-55	*Pseudoalteromonas*	14.3 ± 2.6	15.8 ± 0.5	14.8 ± 3.0
VNSS-56*	*Pseudoalteromonas*	16.0 ± 8.5	17.0 ± 2.8	17.5 ± 7.8
R2A-35	*Cellulophaga*	15.0 ± 4.6	14.5 ± 2.1	15.3 ± 6.7
R2A-48	*Paraglaciecola*	28.0 ± 2.7	27.0 ± 5.6	26.3 ± 3.0
R2A-49*	*Paraglaciecola*	15.5 ± 6.4	17.0 ± 0.0	18.5 ± 2.1
R2A-51	*Paraglaciecola*	21.3 ± 8.4	24.5 ± 5.3	24.5 ± 5.4
R2A-60	*Paraglaciecola*	24.5 ± 8.9	27.3 ± 4.9	28.3 ± 2.2
R2A-42	*Pseudoalteromonas*	18.5 ± 1.3	19.5 ± 3.5	23.5 ± 1.3
R2A-46*	*Staphylococcus*	24.8 ± 8.5	26.3 ± 1.5	28.3 ± 2.1
R2A-58	*Staphylococcus*	21.0 ± 7.0	21.8 ± 7.3	20.8 ± 6.1
R2A-59*	*Sulfitobacter*	27.3 ± 1.5	26.3 ± 2.9	28.3 ± 1.2
R2A-36	*Vibrio*	14.0 ± 2.6	14.5 ± 2.4	14.5 ± 3.1
R2A-47*	*Zobellia*	17.8 ± 7.6	17.3 ± 8.7	16.5 ± 7.9
R2A-52*	*Zobellia*	17.3 ± 5.4	17.3 ± 2.2	16.8 ± 5.1
Positive control	*Vibrio harveyi* BB120	12.7 ± 2.08	13.3 ± 0.58	/

386

387 The two Gram positive active isolates were identified as belonging to the
388 *Staphylococcus* genus. Half of the active isolates showed pigmented colonies (yellow, orange).

389 The antibacterial potential from *Ulva* sp. isolates supernatants were independent of a pH effect
390 (pH between 6.5 and 7), but highly dependent on the culture medium and bacterial
391 concentration used to generate the tested supernatants.

392 The antibacterial potential was higher for isolates supernatants from VNSS and R2Ad
393 with 16 active VNSS-isolates supernatants (55% of the total bacteria grown on VNSS medium)
394 mostly belonging to *Gammaproteobacteria* and 12 active R2Ad-isolates supernatants (50% of
395 the total bacteria grown on R2Ad medium) affiliated to *Gammaproteobacteria, Flavobacteriia,*
396 *Bacilli* and *Alphaproteobacteria* (**Figure 2**).



397 Figure 2 : Percentage of epibacterial community isolated from *Ulva* sp. surface and grown on
398 three different culture media (left); percentage of isolates with antibacterial activity per
399 culture medium, only isolates active against the three target bacteria are shown in this graph
400 (right).

401

402 In contrast, only three isolates (*Gammaproteobacteria*) cultured in MB exhibited
403 antibacterial activity, with supernatants from two isolates being active only against *Vibrio*
404 *harveyi* BB120.

405 Moreover, employing *Ulva* sp. homogenate specifically triggered the production of
406 antibacterial compounds by a yellow-pigmented *Dokdonia* sp. from VNSS culture medium
407 (VNSS-42), resulting in inhibitory diameters ranging from 15 mm to 17 mm depending on the
408 targeted bacteria.

409 Antibacterial activity was also dependent on the bacterial concentration. Agar well
410 diffusion assays were performed from 300 µL filter-sterilized supernatants. One third (10/31)
411 of the active isolates only showed antibacterial activity when the 24-hour culture pellets were
412 in contact with a small volume of medium, highlighting the impact of high concentration
413 molecules in supernatants on antibacterial potential. Moreover, most of the isolates had higher
414 inhibitory diameters with this high-bacterial concentration condition.

415

416 **b. Agarolytic capacity evaluation**

417

418 Isolates with agarolytic properties represented 20% of the *Ulva* sp. surface isolates
419 (14/69) and belonged to the *Gammaproteobacteria* (71%) and the *Flavobacteriia* (29%). The
420 majority of the agarolytic isolates supernatants showed antimicrobial activity (86%) or biofilm
421 modulation properties (28%, including four supernatants with a statistically proven inhibitory
422 effect on biofilm formation).

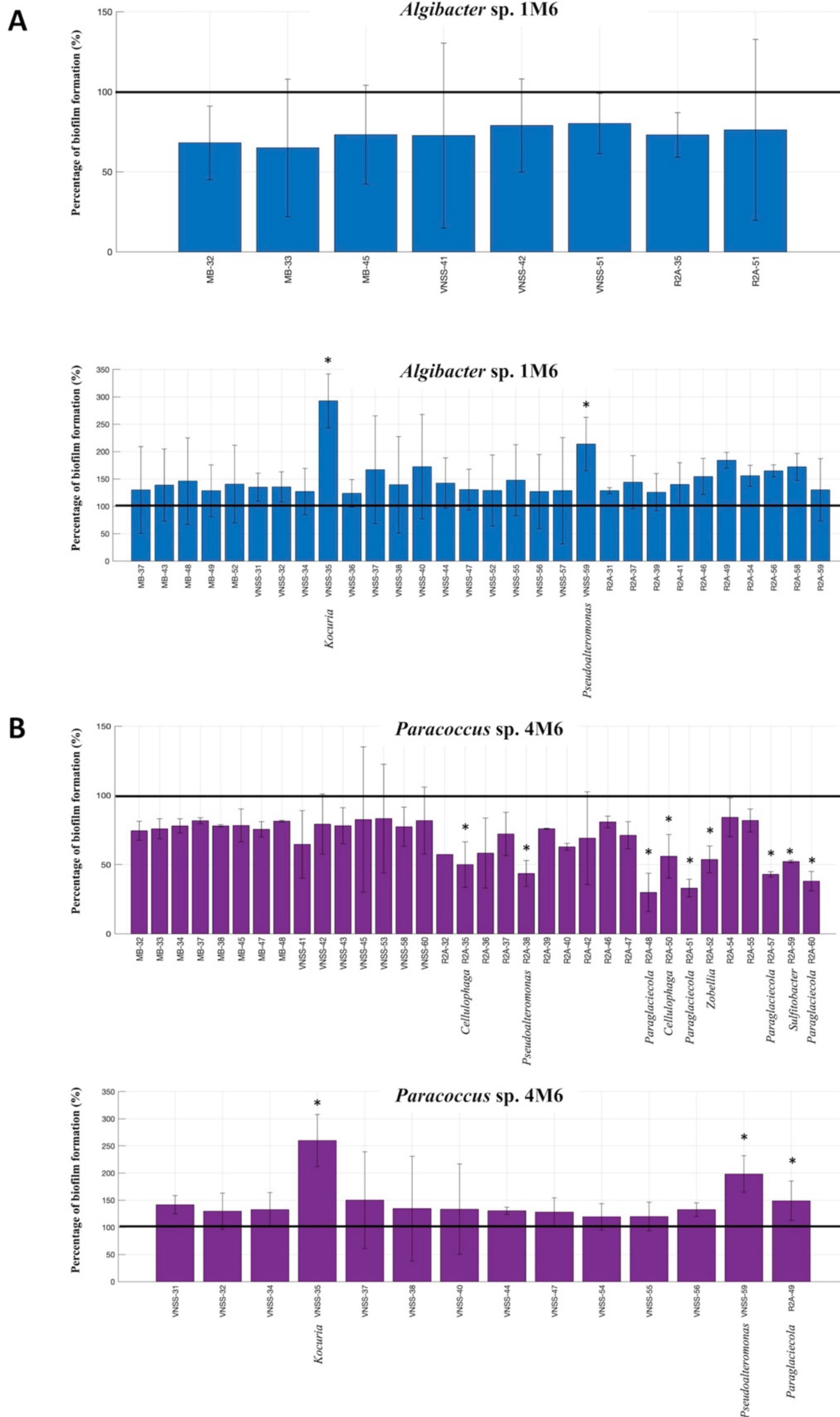
423

424 **c. Biofilm assays**

425

426 The anti- or pro- biofilm effect of 67 supernatants from *Ulva* sp. isolates were assessed
427 against the biofilm formation capacity of *Algibacter* sp. 1M6 and *Paracoccus* sp. 4M6. One
428 fifth of the supernatants (12/67) had a significant effect on biofilm formation (p value < 0.05),
429 either enhancing or reducing its formation (**Figure 3**).

430



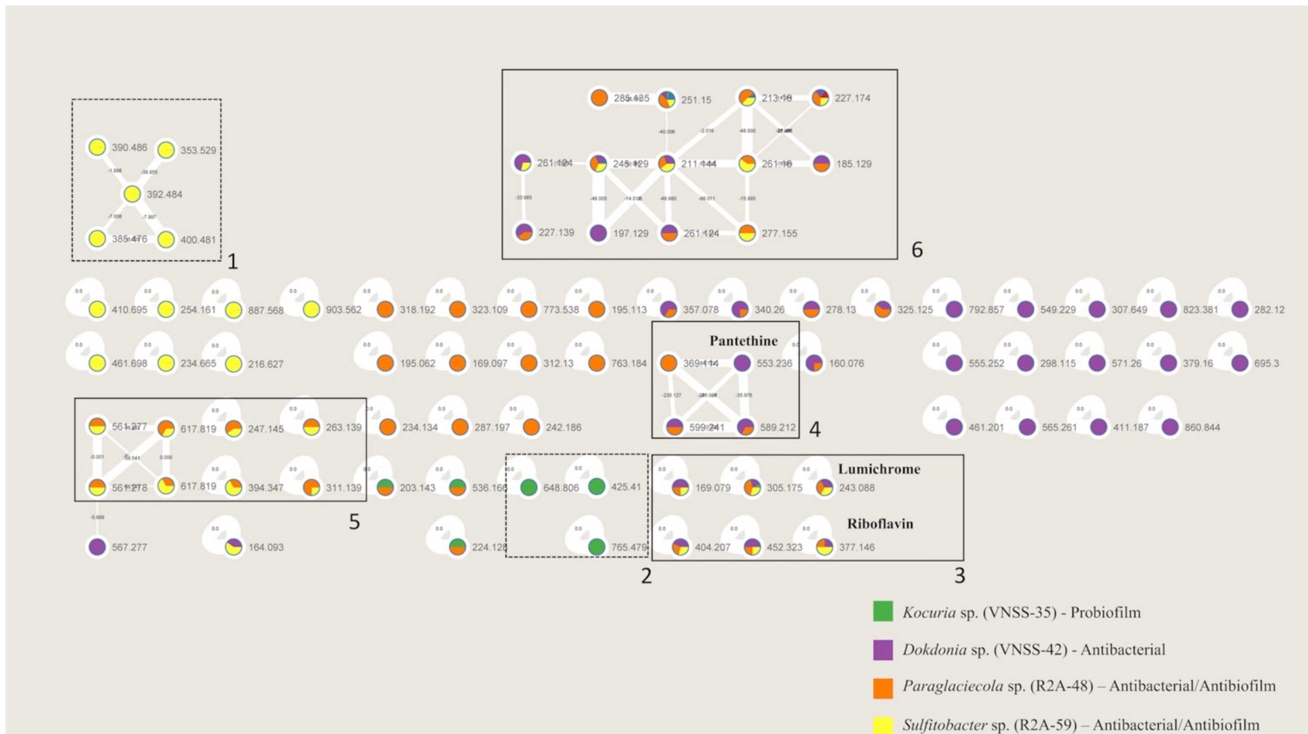
431
432 Figure 3 : Modulation of *Algibacter* sp. 1M6 (A) and *Paracoccus* sp. 4M6 (B) biofilm
433 formation by different isolates supernatants in microtiter plates. Target bacteria were
434 inoculated with bacterial supernatants in 96-well microplates and incubated at 25 °C for 24 h
435 to form biofilms. Bars represent means ± standard error of the mean for three replicates, and
436 the line corresponds to the positive control (target bacteria without addition of supernatant).
437 Only antibiofilm trends showing at least 20% inhibition of biofilm formed and probiofilm
438 trends showing at least 20% additional biofilm formed are represented on this graph. (*)
439 indicates antibiofilm/probiofilm effects proven by Anova and Dunett's post-hoc test.

440 Nine supernatants of the *Ulva* sp. isolates (13%) appeared to contain antibiofilm
441 compounds. Five isolates were affiliated to the *Gammaproteobacteria* (*Paraglaciecola* spp.
442 and *Pseudoalteromonas* spp.), three to *Flavobacteriia* (*Cellulophaga* spp., and *Zobellia* sp.)
443 and one to *Alphaproteobacteria* (*Sulfitobacter* sp.). Six supernatants inhibited more than 50%
444 of the biofilm formed by *Paroccocus* sp. 4M6.The three isolates with probiofilm effects
445 showed less diversity at the class level with two isolates affiliated to *Gammaproteobacteria*
446 (*Paraglaciecola* sp., and *Pseudoalteromonas* sp.) and one to *Actinomycetes* (*Kocuria* sp.).
447 Almost half of the isolates with biofilm modulation properties were pigmented (pink,
448 yellow).
449 Moreover, the biofilm modulating effects of supernatants from *Ulva* sp. surface isolates
450 depended on the target bacteria. This was clearly illustrated by the significant antibiofilm effect
451 displayed by *Ulva* sp. supernatants from 9 isolates that was only observed against the biofilm
452 formed by *Paracoccus* sp. 4M6. The probiofilm properties of some supernatants showed a
453 significant effect for biofilms formed by both target bacteria, with a slight predominance of the
454 effect for biofilms formed by *Paracoccus* sp. 4M6: 2 supernatants from isolates had an effect
455 only on biofilms formed by *Algibacter* sp. 1M6, while 3 supernatants had an effect only on
456 biofilms formed by *Paracoccus* sp. 4M6.
457
458 **a. Molecular networking**
459
460 Organic extracts of the bacterial supernatants were analyzed by LC-ESI-HR MS/MS
461 (positive and negative ion modes) and the fragmentation data obtained were reprocessed using
462 the molecular network approach, via the GNPS online platform. Molecular networking of
463 metabolite mass spectrometry profiles is visualized according to the tested isolates (*Kocuria*
464 sp. VNSS-35; *Dokdonia* sp. VNSS-42; *Paraglaciecola* sp. R2A-48; and *Sulfitobacter* sp. R2A-
465 59), which displayed different biological activities (probiofilm/antibiofilm, antibacterial

466 activities) **(Figure 4).** The node color corresponds to the different strain supernatants.

467 Compounds linked to the culture medium (blue)/to contamination (red) are not included in

468 those produced by the isolates.



469 Figure 4: Molecular networks based on supernatants from four active isolates : probiofilm
470 effect (VNSS-35, *Kocuria* sp.); antibacterial activity after contact with *Ulva* sp. homogenate
471 (VNSS-42, *Dokdonia* sp.); antibacterial activity and antibiofilm effect (R2A-48,
472 *Paraglaciecola* sp. and R2A-59, *Sulfitobacter* sp.)

473

474 *Dokdonia* sp. VNSS-42, whose supernatant only showed antibacterial activity after

475 contact with Ulva sp. homogenate, presented the highest number of specific metabolites (17),

476 followed by *Sulfitobacter* sp. R2A-59 (12), *Paraglaciecola* sp. R2A-48 (12) and *Kocuria* sp.

477 VNSS-35 (3). Most of these specific metabolites remain unknown.

478 The nodes linked together represent a family of molecules, as can be seen for

479 *Sulfitobacter* sp. R2A-59 (yellow nodes) harboring five related halogenated/brominated

480 compounds between m/z 353.529 and 400.481 (box 1). On the other hand, *Kocuria* sp. VNSS-

481 35 (green nodes) was the only isolate whose supernatant displayed a strong probiofilm effect
482 and it showed only three specific compounds with no identification (m/z 425.41, 648.806,
483 765.479) (box 2).

484 Two compounds, riboflavin (m/z 377.146) and lumichrome (m/z 243.088), were
485 identified based on the GNPS metabolites annotation database. They were both shared between
486 three isolates with antibacterial activities (*Dokdonia* sp. VNSS-42, *Paraglaciecola* sp. R2A-
487 48, and *Sulfitobacter* sp. R2A-59), the two later showing in addition antibiofilm effect (box 3).
488 A family of molecules putatively related to pantethine (m/z 553.236) was also found in two
489 isolates, one displaying antibacterial activity (*Dokdonia* sp. VNSS-42) and the second one
490 displaying both antibacterial and antibiofilm activities (*Paraglaciecola* sp. R2A-48) (box 4).
491 Eight unknown compounds (m/z between 247.145 and 617.819), 4 of which forming a family
492 of molecules, were found in *Paraglaciecola* sp. R2A-48 and *Sulfitobacter* sp. R2A-59, both
493 showing antibacterial and antibiofilm activities (box 5). Finally, a family of very low molecular
494 weight peptides (m/z between 185.129 and 285.135) was found in one isolate with antibacterial
495 activity (*Dokdonia* sp. VNSS-42) and two isolates with both antibacterial and antibiofilm
496 activities (*Paraglaciecola* sp. R2A-48 and *Sulfitobacter* sp. R2A-59) (box 6).

497
4. Discussion
498

499 This study reports the isolation and characterization of cultivable epiphytic bacteria
500 from *Ulva* sp.. Cultivable microbiota provides important information about the types of
501 interactions occurring at the surface of the macroalgae between associated bacteria and
502 potential colonizers. In this study, most of the supernatants exhibited antibacterial properties
503 or pro-/anti- biofilm effect against ubiquitous marine bacteria.

504 Very few studies have explored the cultivable diversity of epiphytic bacteria from *Ulva*
505 sp. by using several culture media. Bacteria isolated from the surface of *Ulva* sp. belonged to

506 *Actinomycetes, Alphaproteobacteria, Bacilli, Flavobacteriia* and *Gammaproteobacteria.* The
507 *Gammaproteobacteria* class was the most representative group isolated from *Ulva* sp. surface
508 as it has been described in other studies (Ismail *et al*., 2017, Del Olmo *et al.,* 2018). Some
509 studies, focusing on *Ulva* sp. heterotrophic bacterial communities found a predominance of
510 *Flavobacteriia* class on MA medium (Bolinches *et al*., 1988). Culture media choice constitutes
511 a key factor in the recovering of the cultivable diversity of macroalgae-associated bacteria.
512 MA, VNSS and 20-fold diluted R2A medium have already been used to cultivate macroalgae
513 surface microbiota (Othmani *et al*., 2016; Kleinjan *et al*., 2017; Ismail *et al*., 2017). The
514 variability of isolates depending on culture medium has already been described for brown
515 macroalgae in the early 2000s (Armstrong *et al*., 2000). The relevance of using different
516 bacterial culture media in order to target different bacterial groups from seawater was also
517 underlined by Joint *et al*. (2010). Indeed, they found that *Gammaproteobacteria* represented
518 73% of the bacteria isolated on MA medium, which was confirmed in this study with 81% of
519 the isolates from MA affiliated to *Gammaproteobacteria*. Ismail *et al*. showed that many
520 isolates from *Ulva* sp. are already known to grow on rich media like MA (Ismail *et al*., 2017).
521 However, in our study, a large proportion of isolates were obtained by using poorer medium
522 (VNSS, R2Ad). Higher bacterial levels were obtained using MA as culture growth medium but
523 greater diversity was observed with R2Ad medium (11/18 of the total genera cultivated). VNSS
524 medium seemed to enable the selective growth of *Pseudoalteromonas* genus (25/29 isolates)
525 with distinct phenotypic profiles. Indeed, VNSS medium is commonly used to cultivate
526 *Pseudoalteromonas* (Holström *et al*., 1998, Egan *et al*., 2001).
527
528 The life on the macroalgae surface leads to adaptive behavior from epiphytic
529 associated-bacteria. For instance, some marine heterotrophic bacteria have developed the

530 ability to grow on macroalgae by using algal cell-wall polysaccharides as carbon sources
531 (Wada *et al.*, 2007; Bengtsson *et al*., 2010; Hollants *et al*., 2013).
532 In our study, we highlighted that 20% of the isolates belonging to *Gammaproteobacteria* and
533 *Flavobacteriia* possessed agarolytic properties. Identification of agarolytic bacteria from *Ulva*
534 sp. surface has already been shown by Furusawa *et al*. (2016). Such agarolytic bacteria may
535 cause damage to the macroalgae cell wall, especially on *Rhodophyta* surface (Florez *et al.,*
536 2017).
537 Macroalgae can benefit from the presence of surface-associated bacteria. In fact, some
538 bacterial groups significantly contribute to growth, reproduction and defense of the
539 macroalgae. Members of the *Actinomycetales*, *Alphaproteobacteria*, *Baciliale*, *Cytophaga-*
540 *Flavobacterium-Bacteroides* (CFB) group and *Gammaproteobacteria* are known to be part of
541 the macroalgae-associated core microbiota (Hollants *et al*., 2012). A large proportion of
542 isolates obtained in this study belonged to those groups and may have a role in *Ulva* sp. growth,
543 as it has previously been shown for *Microbacterium* sp., which promotes the development of
544 the thallus and rhizoids of *Ulva intestinalis* (Ghaderiardakani *et al*., 2017). Moreover, *Zobellia*
545 sp., *Shewanella* sp., *Vibrio* sp. and *Pseudoalteromonas* sp. either stimulate or inhibit the
546 zoospore settlement of *Ulva* sp. by the production of specific metabolites (Egan *et al.*, 2001,
547 Patel *et al.*, 2003).
548
549 Antimicrobial and antibiofilm compounds produced by many macroalgae associated-
550 bacteria contribute to the macroalgae defense system against pathogens, herbivores and fouling
551 organisms (Goecke *et al.*, 2010, Hollants *et al*., 2012). Almost half of the isolates obtained
552 from *Ulva* sp. surface (31/69 isolates) presented antibacterial activity against the three Gram-
553 negative ubiquitous marine bacteria targeted. Those bacteria with activity belonged to
554 *Bacillota, Bacteroidota* and *Pseudomonadota* phyla; no antibacterial activity was observed for

555 the *Actinomycetota* phylum. Those results corroborate previous screenings on the antibacterial
556 potential of *Ulva* sp. associated bacteria (Ismail *et al*., 2017). There was a difference in the
557 activity displayed by the isolates depending on the medium: isolates grown on MA showed
558 very little antibacterial potential, while a great proportion of those grown on VNSS and R2Ad
559 had antibacterial effects on *Algibacter* sp. 1M6, *Paracoccus* sp. 4M6 and *Vibrio harveyi*
560 BB120.

561 The very specific relationships that can exist between the macroalga and its bacterial
562 community suggests that some bacteria only express their antibacterial potential when in
563 contact with the macroalga surface. Here, we demonstrated the impact of *Ulva* sp. homogenate
564 on the release of antibacterial compounds by a yellow-pigmented *Dokdonia* sp. grown on
565 VNSS, which did not show activity under standard conditions lacking algal components.

566 Antibiofilm activity was assessed by testing the production of active compounds against
567 two target-bacteria : *Algibacter* sp. 1M6 and *Paracoccus* sp. 4M6, both isolated from the Gulf
568 of Morbihan and known to be pioneers in biofilm formation (Grasland *et al.*, 2003). They
569 display strong and competitive adhesion properties on surfaces and form very thick biofilm,
570 which can enhance the colonization by other bacteria and fouling organisms (Lappin-Scott and
571 Costerton, 1989, Grasland *et al*., 2003, Dhelly *et al*., 2010). Those species are also documented
572 as being part of *Ulva* sp. cultivable microbiota (Nedashkovskaya *et al.,* 2004, Ismail *et al.,*
573 2017). Isolates belonging to *Pseudoalteromonas* genus have been isolated from the surface of
574 *Ulva* sp. and identified as antibiofilm agents' producers (Holmström *et al.*, 1998, Egan *et al*.,
575 2001, Goecke *et al*., 2010). In this study, antibiofilm properties were found in
576 *Gammaproteobacteria* within a diversity of genera (*Pseudoalteromonas, Paraglaciecola*) but
577 also in the *Flavobacteriia* (*Cellulophaga, Zobellia)* and *Alphaproteobacteria (Sulfitobacter)*
578 classes. However, antibiofilm effects were only noticed for biofilm formed by *Paracoccus* sp.
579 4M6. From a phylogenetic perspective, the fact that *Paracoccus* sp. belongs to the

580 *Alphaproteobacteria*, the most abundant and ubiquitous bacterioplankton group in the ocean
581 (Song *et al*., 2009, Brown *et al.,* 2022) may explain the ability of *Ulva* sp. surface microbiota
582 to develop effective defenses to prevent the fouling of its surface.

583 Otherwise, three isolates from *Ulva* sp. surface showed probiofilm effect on both target-
584 bacteria. Those isolates belonged to the *Gammaproteobacteria* (*Paraglaciecola,*
585 *Pseudoalteromonas*) and to the *Actinomycetes* class (*Kocuria* sp.). Isolate affiliated to the
586 *Kocuria* genus showed very strong probiofilm effect and is already known in the literature as
587 marine biofilm bacteria with high production of carbohydrates and proteins (Kavitha and
588 Raghavan, 2018). Isolates from *Ulva australis* surface belonging to *Microbacterium,*
589 *Shewanella, Dokdonia* and *Acinetobacter* genera were previously reported to increase, by their
590 synergistic interactions, biofilm formation at the macroalgae surface, which improved
591 resistance against opportunistic colonizers (Burmolle *et al*., 2006) that can benefit both the
592 macroalga and its associated microbiota. Moreover, probiofilm compounds are even more
593 relevant when produced on a surface as a biofilm matrix can alter mobility of bacterivorous
594 predators (Chan *et al*., 2020). Biofilm modulation as well as antibacterial activity proved to be
595 a competitive advantage for both the macroalga and its associated microbiota and constitute a
596 potential source of new compounds of biotechnological interest.
597

598 As described in several studies, macroalgae, because of their large surface, are in
599 constant interaction with their surrounding environment and constitute a large reservoir of
600 bioactive molecules. Additionally, bioactive compounds from easily cultivable bacteria may
601 offer a more promising and manageable source of natural products for biotechnological
602 applications compared to those derived from macroalgae (Hollants *et al*., 2012). The research

603 of new antibacterial compounds is essential in the context of resistance to antibiotics, which
604 has become a major healthcare problem in the 21th century (D'Costa *et al*., 2006).

605 Molecular network analysis revealed the presence of active molecules in certain
606 bacterial supernatants from isolates showing biological activity. Direct link between the
607 presence of compounds produced by bacterial isolates and bacteria taxonomy/biological
608 activity can only be established through the isolation and purification of these compounds.
609 However, hypotheses may emerge from the concomitant presence of specific compounds in
610 isolate supernatants with similar activities. Although the majority of compounds had no
611 proposed identification, and were possibly new molecules, a few identifications were
612 suggested. For instance, one compound putatively identified as pantethine, was only recovered
613 in *Dokdonia* sp. VNSS-42. This panthetine-like compound was related to three other
614 compounds, two of which were also found in *Paraglaciecola* R2A-48, also exhibiting
615 antibacterial activities. Pantethine (related to B5 vitamin) is the dimeric and oxidized form of
616 pantetheine produced from pantothenic acid (Spry et al., 2008). Although pantethine per se
617 does not possess antibacterial activity, pantetheine and several analogues to pantothenate
618 possess antimicrobial activities against bacteria, fungi and protozoa affiliated to Plasmodium
619 genus causing malaria (Jansen *et al.,* 2019). Moreover, the ability of certain bacteria to use
620 pantetheine/pantethine analogues in place of pantothenic acid in the biosynthesis of Coenzyme
621 A (CoA), an essential growth cofactor, opens up new opportunities in the design of compounds
622 inhibiting the biosynthesis of CoA in pathogenic bacteria, which is particularly relevant in the
623 context of antibacterial resistance (Spry *et al.,* 2008). Several compounds and families of
624 compounds were found in isolates affiliated to *Dokdonia* sp. VNSS-42, *Paraglaciecola* sp.
625 R2A-48 and *Sulfitobacter sp*. R2A-59, all displaying antibacterial and/or antibiofilm activities.
626 Among those compounds, riboflavin and lumichrome have been putatively identified.
627 Riboflavin (vitamin B2) constitutes an essential micronutriment with excellent photosensitive

628 characteristics (Cardoso *et al.,* 2012). Riboflavin has antibacterial photodynamic potential by
629 compromising the oxidation-reduction state of bacteria; it also inhibits bacterial biofilm and is
630 therefore a molecule of interest (Khan *et al.,* 2019; Farah *et al.,* 2022). Moreover, the
631 combination of riboflavin and its derivative lumichrome could act as either quorum sensing
632 signal molecules or as inter-kingdom signal-mimics interfering with quorum sensing in bacteria
633 biofilm (Rajamani *et al.,* 2008). Identifying and characterizing the compounds responsible for
634 this probiofilm effect could be of biotechnological interest. In this context, molecular network
635 analysis revealed the presence of three specific unknown compounds only found in *Kocuria*
636 sp. VNSS-35 supernatant, suggesting their possible involvement in the probiofilm activity
637 displayed by this isolate.
638
639 Moreover, a large proportion of pigmented bacterial isolates (55%) were obtained from
640 the culture of *Ulva* sp. surface microbiota. Pigments derived from marine bacteria rich in
641 secondary metabolites have found applications in the food industry, cosmetic, textile and
642 pharmaceutical industries. Agarwal *et al.* (2023) showed that bacterial pigments are effective
643 against multiple mammalian cancerous cell lines and pathogenic microorganisms. They also
644 have the advantage of being low-cost, biodegradable and easily accessible. The
645 characterization of these strains could be an interesting prospect with a view to adding value to
646 certain potentially antibacterial or antiproliferative molecules. Indeed, long-time known
647 relationship has been established between the production of bacterial pigments and the
648 expression of antibacterial and antifouling phenotypes (Lichstein and Van de Sand, 1945, Egan
649 *et al.*, 2002).
650 Here, we also aimed to optimize the expression of the antibacterial potential of *Ulva* sp.
651 isolates by increasing the compound concentration contained in the culture supernatants tested.
652 Almost half of isolates turned out to possess antibacterial activity, which opens up new

653 opportunities for the screening and identification of bioactivity compounds in bacterial
654 extracts. Controlling the biofilm phenotype of pathogenic bacteria might be a major lever for
655 action as a transition to an attached state could favor resistome evolution (Plakunov *et al*.,
656 2016). In this study, isolates with antibiofilm properties and no antibacterial activity were found
657 and could be of primary interest in inhibiting bacteria without fostering the development of
658 antibiotic resistance. *Cellulophaga* R2A-50, *Paraglaciecola* R2A-57, and *Pseudoalteromonas*
659 R2A-38 lacked antibacterial activity and inhibited *Paracoccus* sp. 4M6 biofilm formation up
660 to 40%. In contrast isolate VNSS-35 affiliated with the *Kocuria* genus, had a probiofilm effect
661 on both *Algibacter* sp. 1M6 and *Paracoccus* sp. 4M6. Studying the interactions of bacterial
662 compounds that can promote biofilm formation by pathogenic bacteria may lead to a better
663 understanding of the factors leading to successful bacterial host invasion (Masters *et al.*, 2019).
664 Controlling the formation of biofilms is increasingly important not only in the medical field
665 but also in the biotechnological and ecological perspectives of biofilm studies. In aquaculture,
666 the use of the ability of *Pseudoalteromonas* to form biofilms to prevent bacterial and fungal
667 infestation of clownfish egg clutches is an example of biofilm formation by specific, non-
668 pathogenic bacteria (Wesseling *et al*., 2015). In the agri-food sector, probiofilm compounds
669 could be used to enhance biofilm formation of bacteria of interest. For instance, strains of the
670 dairy industry thermophilic starter *Streptococcus thermophilus* lose over time their capacity to
671 form biofilms and their ability to attach firmly to abiotic surfaces, compromising their role as
672 starters for dairy fermentation (Couvigny *et al*., 2015).
673
674 *Ulva* model constitutes a widely and globally available resource whose epiphytic
675 microbiota is an inexhaustible source of bioactive compounds. More research is needed to

characterize and isolate these compounds both to better understand their ecological roles and to optimize their use for biotechnological applications.

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Data availability

The dataset supporting the conclusions of this article is available in the NCBI repository SUB13468594

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1047 **Authors' contributions:** S.P., G.L.B. and N.B. wrote the manuscript, and all authors
1048 commented on the final version. Experimental design, data analyses, results interpretation,
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