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1 **Maintaining beneficial alga-associated bacterial communities under heat**
2 **stress: Insights from controlled co-culture experiments using antibiotic-**
3 **resistant bacterial strains**

4
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15 **Keywords:** Algal-bacterial interactions, metabolic networks, systems biology, holobionts

16
17 **One sentence summary:** Inoculation with bacterial helper cultures that complement host
18 metabolism increases the control over host-associated microbiome in the model brown alga
19 *Ectocarpus*.

33 **Abstract**

34

35

36 Brown algae, like many eukaryotes, possess diverse microbial communities. *Ectocarpus* - a
37 model brown alga- relies on these communities for essential processes, such as growth
38 development. Controlled laboratory systems are needed for functional studies of these algal-
39 bacterial interactions. We selected bacterial strains based on their metabolic networks to
40 provide optimal completion of the algal metabolism, rendered them resistant to two
41 antibiotics, and inoculate them to establish controlled co-cultures with *Ectocarpus* under
42 continuous antibiotic treatment. We then monitored the stability of the resulting associations
43 under control conditions and heat stress using 16S metabarcoding. Antibiotics strongly
44 reduced bacterial diversity both in terms of taxonomy and predicted metabolic functions. In
45 the inoculated sample, 63-69% of reads corresponded to the inoculated strains, and the
46 communities remained stable during temperature stress. They also partially restored the
47 predicted metabolic functions of the natural community. Overall, the development of
48 antibiotic-resistant helper cultures offers a promising route to fully controlled laboratory
49 experiments with algae and microbiota and thus represents an important step towards
50 generating experimental evidence for specific host-microbe interactions in the systems
51 studied. Further work will be required to achieve full control and progressively expand our
52 repertoire of helper strains including those currently “unculturable”.

53

54 **Introduction**

55

56 As virtually all eukaryotes, brown algae are colonized by a diverse microbial community,
57 together forming so-called holobionts (Dittami et al. 2021; Egan et al. 2013). The thallus of
58 algae is more than a habitat for microbes; there are numerous dynamic inter-species
59 interactions and metabolic exchanges between both compartments. Technological
60 advancements in sequencing and metagenomics now permit the generation of valuable
61 information about the composition of the host microbiome. Furthermore, computational
62 models of metabolic networks can be used to predict complementarity sets between
63 microbiome species and hosts (Belcour et al. 2020; Frioux et al. 2018; Levy et al. 2015).

64 Brown algae (Phaeophyceae) are important components of the flora on rocky shores in
65 temperate and cold marine environments around the world, establishing critical ecosystem
66 habitats such as kelp forests (Steneck et al. 2002). Many of these kelp forests, especially
67 along the European coast of the North Sea, Eastern Australia, and California, are declining
68 (Krumhansl et al. 2016; Smale 2020) - a phenomenon that is commonly attributed to
69 anthropogenic impacts and global warming, though the precise causes are unknown (Provost
70 et al. 2017).

71 Among brown algae, *Ectocarpus* (a small filamentous alga) has been established as a genetic
72 and genomic model (Cock et al. 2010) and it is known to depend on the bacteria for proper
73 development and for acclimation to low salinities (Dittami et al. 2016; Pedersen 1968; Tapia
74 et al. 2016). According to a recent study, the co-culture of *Ectocarpus* with selected bacteria
75 resulted in increased algal growth, and samples treated by different specific bacteria had
76 diverging metabolite profiles and morphologies (Burgunter-Delamare et al. 2020).

77 Despite advances in our understanding of the diversity of bacterial symbionts and the
78 prospective use of metabolic complementarity to test host-microbe interactions, there are still
79 unanswered questions about symbiont acquisition and host-microbe balance in the face of
80 abiotic stress. Furthermore, it is unresolved if and to what extent imbalances between algae
81 and their associated microbiomes may play a role in ecologically important things like the
82 decline of kelp forests. The establishment of controlled laboratory systems for brown algal-
83 bacterial interaction would constitute a big advance in this field, enabling the mechanistical
84 study of bacterial algal interactions in a changing environment, and the generation of testable
85 hypotheses (van der Loos et al. 2019).

86 The aim of this study was to improve our control of the alga-associated microbiome. Given
87 the strong interdependencies between the algal hosts and their associated microbiome, this
88 challenge was addressed by providing the alga with a defined set of antibiotic-resistant
89 ‘helper bacteria’. These bacteria were selected from a collection of host-derived bacteria,
90 based on their predicted metabolism, to be able to produce the maximum number of
91 compounds that are missing for the predicted metabolism of the host (Dittami et al. 2014). In
92 addition, the algae and bacteria were kept under continuous antibiotic treatment to reduce the
93 proliferation of other non-target strains that might colonize the niche opened up by the
94 antibiotic treatment. The suitability of this approach was tested in the algal host model
95 *Ectocarpus*, and the stability of the microbiome was assessed under both control conditions
96 and under heat stress.

97

Materials & Methods

99 **Beneficial bacterial consortium selection**

100 *In silico* selection of beneficial bacteria was performed in a previous study (Karimi et al.
101 2021a). Briefly, 81 bacterial genomes isolated from *Ectocarpus* (Karimi et al. 2019) were
102 annotated using Prokka v 1.13 (Seemann 2014). Subsequently, bacterial metabolic networks
103 were constructed using Pathway Tools version 23 and the mpwt wrapper implemented in the
104 AuCoMe pipeline (Belcour et al. 2020). The bacterial metabolic networks and the available
105 host metabolic network (Dittami et al. 2020) were submitted to MiSCoTo as SMBL files
106 (Frioux et al. 2018). The latter tool was first used to determine the scope of all algal
107 compounds that, according to the metabolic models, and without limiting metabolite
108 exchange, became producible via exchanges between the host and all 81 bacterial genomes
109 together: a total of 506 compounds listed in Supplementary Table S8 of Karimi et al. (2021a).
110 The same tool was then used to determine the minimal bacterial consortia that enabled the
111 production of these compounds. A total of 18 minimal communities was predicted each
112 comprising 10 bacterial strains (listed in Supplementary Table S1), and the union of all
113 minimal consortia contained 15 bacteria. All of these 15 bacteria and their taxonomic
114 affiliations are listed in Supplementary Table S1, and we attempted to generate antibiotic-
115 resistant strains for each one of them. The host and bacterial networks have also been
116 deposited in the Zenodo repository (Karimi et al. 2021b).

117 **Antibiotic sensitivity testing on algae**

118 Filaments of the brown alga *Ectocarpus subulatus* strain Bft15b were cultured in 10-liter
119 Nalgene™ Round Polycarbonate flasks with Provasoli-enriched autoclaved seawater for 15
120 days. Temperature was maintained constant at 16°C, and constant airflow and a light-dark
121 cycle of 12:12h were applied in controlled culture chambers. The algal filaments were then
122 placed in 90mm sterilized Petri dishes filled with Provasoli-enriched artificial seawater
123 containing the following components: NaCl 450mM, MgSO₄(7H₂O) 16mM, MgCl₂(6H₂O)
124 30 mM, KCl 10mM, CaCl₂ 9mM adjusted to pH 7.8 with 10mM Tris. Culture conditions
125 remained identical, except for the absence of bubbling. To obtain axenic algae, the following
126 antibiotic treatment was selected based on the work of Cottrell and Suttle (1993) and
127 preliminary experiments in our laboratory: three-day exposure to a combination of
128 Chloramphenicol (CHL 9.6 µg/ml) and Neomycin (NEO 24.8µg/ml). This treatment

129 efficiently reduced associated bacteria but caused no visible damage (discoloration, rupture)
130 to the algal cells.

131 **Antibiotic sensitivity tests for bacteria and the generation of antibiotic-resistant strains**

132 Disk diffusion tests were carried out to determine the susceptibility of the selected beneficial
133 bacteria to chloramphenicol (CHL) and neomycin (NEO). Freshly cultured bacterial isolates
134 were spread evenly on the R2A agar plates prepared with natural seawater, and grown in the
135 presence of 30 µg CHL and 30 UI NEO antibiotic discs (Bio-Rad, Nanterre, France
136 (Matuschek et al. 2014) for up to one week at 18°C. Next, we attempted to generate antibiotic
137 resistance in the selected strains. To this means, bacteria were grown in 25 ml of liquid R2A
138 medium in Corning® cell culture flasks at 25°C with agitation (50 rpm) supplemented with
139 0.3 µg/ml of CHL. Once bacterial growth was visible, 1 ml of culture was transferred to a
140 new flask with a higher concentration and this process continued. Based on the “adaptation
141 on demand” hypothesis (Johnsen et al. 2021), the increase in antibiotic concentration leads
142 bacterial population to evolve resistance to the target antibiotic, until growth was observed at
143 the target concentration of CHL (Supplementary Table S2). At this point, the disk diffusion
144 test was repeated to check the obtained resistance (Table 1). Then, the same procedure was
145 carried out starting with 3.93 µg/ml Neomycin (NEO) for the CHL-resistant strains. Finally,
146 the identity of bacterial strains resistant to both antibiotics was confirmed by sequencing the
147 16S rRNA gene with the universal primers 27F and 1492R. After reaching the desired
148 antibiotic resistance, the strains were preserved in 20% (vol/vol) glycerol at -80°C.

149 **Co-cultures of algae and bacteria**

150 For our co-culture experiments, we constructed a set that included all seven bacteria for
151 which resistant strains were obtained. This set corresponds to the predicted minimal
152 community 11 in Supplementary Table S1, but lacks *Frigoribacterium* sp. 9N, *Bacillus*
153 sp. 9J, and *Novosphingobium* sp. 9U. These bacteria were grown in liquid R2A media with
154 both antibiotics at their final target concentration. Once bacterial growth was visible with the
155 naked eye (approximately 5 days), the optical density (OD) of the cultures was determined
156 using Genova plus analyzer UV/Vis life science® spectrophotometer at 600 nm. All cultures
157 were diluted to an OD of 0.1 for the inoculation of the algal cultures. For the co-culture set-
158 up, algal filaments were dispersed in Petri plates (125 ml cultures), prepared with the two
159 liquid antibiotics as described above, and inoculated with 500 µl of each resistant cultured

160 bacterium (OD = 0.1). These cultures, along with a set of controls, were then used in the
161 stress response experiments described below.

162 **Temperature stress experiment**

163 The design of the stress response experiments, as well as the controls included are described
164 in Fig. 1. Briefly, at the start of the experiment (t_0), algal-bacterial co-cultures were
165 inoculated as described above (INOCUL), and in addition, a non-antibiotic treated positive
166 control (NATUR), and an antibiotic-treated non-inoculated alga as a negative control (ATB)
167 were included. For all three of these conditions, we prepared 8 replicate Petri dishes with 4
168 filaments in each (a total of 96 algal filaments). After the acclimation period of 10 days at
169 16°C (t_{10}) in a culture chamber at 16°C , 4 of the 8 replicates for each treatment were placed
170 in a separate but otherwise identical culture chamber and subjected to a temperature increase
171 of 2°C every two days for 6 days (t_{12} , t_{14} , t_{16}). The other 4 replicates were maintained at 16°C .
172 At each of these time points, algal samples (one filament per Petri dish) were collected in
173 Eppendorf tubes and immediately frozen in liquid nitrogen, and later at -80°C for
174 downstream analyses. Furthermore, at all steps of the experiment (Fig. 1), the general algal
175 morphology was observed. Finally, at t_{16} , aliquots (15 ml) of the culture media were taken,
176 centrifuged at 10,000 g for 30 min to pellet the bacterial cells present, and the pellets were
177 stored at -80°C for downstream analysis.

178 **Metabarcoding profiling of co-culture experiments**

179 A metabarcoding approach was implemented to investigate the composition of the bacterial
180 community after the co-culture experiments and temperature disturbances. For each culture
181 condition, frozen algal tissue was freeze-dried and ground (TissueLyserII; Qiagen, Hilden,
182 Germany; 2×60 s, 30 Hz). The powder obtained was then used for DNA extraction (the
183 Macherey-Nagel NucleoSpin 96 Plant II Kit). The DNA extraction for bacterial pellets
184 collected from seawater media was performed using the Wizard® Genomic DNA Purification
185 Kit (Promega, Madison, USA). DNA integrity was verified with gel electrophoresis. Two
186 blank controls were included as negative controls in addition to the samples. Libraries were
187 prepared according to the standard Illumina protocol for metabarcoding MiSeq technology
188 targeting the V3–V4 region (Illumina, 2017). Briefly, the amplicon PCR was performed
189 using NOCHL primer sets (Thomas et al. 2020). The PCR products were then purified using
190 AMPure® XP beads (Agencourt Bioscience, Beverly, MA, USA) followed by index PCR in

191 KAPA HiFi HotStart ReadyMix (KAPA Biosystems, Wilmington, MA, USA) to attach dual
192 indices and Illumina sequencing adapters to each sample using the Nextera XT Index Kit
193 (Epicentre, Madison, WI, USA). A second PCR clean-up was then carried out as described
194 above. The resulting libraries were checked using a bioanalyzer (DNS 1000) to verify
195 fragment size and a Qubit® fluorimeter (Life Technologies, Paisley, UK) for DNA
196 quantification. An equimolar pool of all libraries was subjected to paired-end (2×300 bp)
197 sequencing on a single flow cell of an Illumina MiSeq sequencer using the MiSeq v3 kit
198 (Illumina, San Diego, CA, USA) on Platform GENOMER, FR2424, Roscoff.

199 **Sequence analysis**

200 The resulting raw sequences (10,508,975 read pairs) were processed using DADA2 within
201 the R (v4.0.0) statistical computing environment (Callahan et al. 2016). Reads underwent
202 quality filtering as error rates were calculated and removed from the dereplicated reads. An
203 initial Amplicon Sequence Variant (ASV) table was constructed, and later chimeras were
204 identified using the removeBimeraDenovo function. Finally, taxonomy was assigned using
205 DADA2's native RDP Bayesian classifier against the Silva database version 138 (McLaren
206 2020). Non-bacterial sequences as well as all ASVs with less than ten reads or which were
207 more abundant in the negative control compared to the samples were removed. No
208 rarefaction was conducted but the 'calc_obs_props' function of the 'metacoder' package
209 version 0.3.4 was used to calculate proportions and the abundance of taxa was computed
210 using the 'calc_taxon_abund' function. For diversity analysis, the 'vegan' package was
211 employed using the 'diversity' function. PICRUST2 (v2.4.1) was used to predict functional
212 traits of bacteria based on their marker gene sequences in each treatment with default settings
213 (Douglas et al. 2020).

214 **Statistical analyses**

215 All statistical analyses and graphs were performed within the computing environment R
216 (v4.0.0; R Core Development Team, 2005) using the packages phyloseq (McMurdie and
217 Holmes 2013), phylosmith (Smith 2019), vegan (Oksanen et al. 2020), ggplot2 (Valero-Mora
218 2010), metacoder (Foster et al. 2017), microbiome (Lahti et al. 2017). Statistical tests were
219 considered significant at $\alpha = 0.05$. Differences in community composition (β -diversity) were
220 computed using Bray–Curtis dissimilarity matrices and tested via permutational multivariate

221 analysis of variance (PERMANOVA) and the statistical analysis of the predicted pathway
222 data was performed using STAMP version 2.1.3(Parks et al. 2014).

223

224 **Results**

225 **Antibiotic resistance and sufficient growth obtained for 7 out of 15 bacteria.**

226 To set up a controlled co-culture experiment, we continuously applied antibiotics. Therefore,
227 we needed to generate antibiotic-resistant strains of the bacteria we had selected based on
228 metabolic complementarity. All 15 of the initially bacteria developed resistance to CHL and
229 subsequently to neomycin (NEO) (Supplementary Table S2). For 10 out of these 15 strains
230 the resistance to both antibiotics (Supplementary Figure S1) was durable and 7 strains out of
231 10 exhibited growth rates that were sufficient to prepare co-culture experiments within a
232 timescale of 5 days (Table1)

233 **Metabarcoding survey of algal samples**

234 In total, 71 samples from three algal treatment conditions were considered for the core
235 analysis of the study: bacterial inoculation with ATB treatment (n = 24, labeled as INOCUL);
236 no inoculation and no ATB treatment (n = 23, here known as NATUR), and no bacterial
237 inoculation with ATB treatment (n = 24, labeled as ATB). One sample replicate from the
238 ATB treatment was removed alongside the negative controls because insufficient reads were
239 obtained. Of 10 million raw reads were made up 1 005 455 unique sequences belonging to 1
240 933 different ASVs. The ASVs and taxonomy tables are provided in Supplementary Table
241 S3.

242 **Six inoculated resistant bacteria out of seven persisted in cultures.**

243 Six inoculated bacteria out of seven were detected in metabarcoding data (Fig. 2). Only
244 *Rhizobium* had no reads although this strain had developed resistance to both antibiotics in a
245 timely manner. Overall, the inoculated strains were detected specifically in the conditions
246 they were inoculated in, except for *Maribacter* which was present in all cultures (Fig. 2). In
247 the samples that underwent the INOCUL treatment, reads corresponding inoculated bacteria,

248 amounted to 66% of total reads (69% in control conditions and 63% in stress conditions,
249 Supplementary Figure S2).

250 **Antibiotic treatments reduce bacterial diversity and modify community structure.**

251 The Shannon diversity indices per sample ranged from 3.33 to 4.77 (Fig. 3, See
252 Supplementary Table S4 for additional diversity indices). They differed significantly between
253 ATB-treated, natural, and ATB-treated inoculated samples (two-way ANOVA, $p < 0.001$),
254 but differences between temperature-stressed and control samples were not significant in the
255 same analysis ($p = 0.28$; Fig. 3). Generally, the communities from samples inoculated with
256 the selected bacteria (INOCUL) exhibited lower phylogenetic diversity than control
257 (NATUR) and higher than the antibiotic treated (ABT).

258 Non-metric Multidimensional Scaling (nMDS) visualization of β -diversity using the
259 Bray–Curtis dissimilarity index revealed a clear separation of co-culture conditions (Fig. 4;
260 PERMANOVA, Bray–Curtis method, 999 permutations; $R^2 = 0.75$, $p < 0.001$). However, no
261 clear differences between stress- and control samples were observed for the overall
262 community composition. PERMANOVA analyses carried out separately for ATB, INOCL,
263 and NATUR samples after inculcation (t_{10} - t_{16}) also revealed that the community composition
264 changed slightly but significantly in response to stress for the ATB and INOCL treatments
265 ($p=0.013$ and $p=0.041$, respectively) but not for the NATUR treatment.

266 **Taxonomic composition of bacterial communities**

267 The taxonomic composition of bacterial communities in ATB, INOCUL, and NATUR
268 cultures is illustrated in Fig. 5A, where only the non-inoculated strains are considered
269 (inoculated strains are shown in Fig. 2). In the natural samples, we observed a complex
270 community dominated by sequences corresponding *Flavobacteriales*, *Caulobacterales*,
271 *Oceanospirellales*, and *Rhodobacterales*. ATB treated samples including the INOCUL
272 samples (without the inoculated bacteria), were dominated by sequences belonging to
273 *Flavobacteriales* and more specifically the genus *Winogradskyella*, and only 5-10% of reads
274 corresponded to other groups.

275 The pairwise comparisons in Fig. 5B display the relative abundance of each observed
276 taxonomic group in stress versus control samples. Here, nodes represent taxonomic levels,
277 and each branch represents the phylogenetic relationship between those entities. ASV
278 richness is represented by node size and the relative abundance of reads corresponding to this

279 node by color. For the natural microbiota, this heat tree shows few taxonomic differences in
280 the composition in response to stress, notably an increase in the proportion *Cytophagales*
281 including *Fabibacter*, and a decrease in *Pseudomonadales*. The ATB in INOCL treatment
282 exhibit a few common responses to stress, such as the increase in the part of *Bacillales* in
283 response to stress, and a decrease in *Gammaproteobacteria* and *Rhodobacteriaceae*.
284 Specifically, in the INOCL treatment, we also observed a relative increase in *Micrococcales*
285 and *Rhizobiaceae* in response to stress.

286
287

288 **Taxonomy-based functional profiling of bacterial communities in control and stressed** 289 **samples**

290 In addition to generating the taxonomic profiles, we used Picrust2 to functional profiles
291 based on the metabarcoding data. During this process, 15 ASVs with [Nearest Sequenced](#)
292 [Taxon Index](#) (NSTI) values of more than 2 were eliminated, along with six ASVs with poor
293 alignment (Barbera et al. 2018; Czech et al. 2020; Louca and Doebeli 2017; Mirarab et al.
294 2012). The average of NSTI values for our samples (Supplementary Table S5) was
295 0.047 ± 0.007 (mean + SD), with slightly higher NSTIs (0.053 ± 0.003) for the NATUR
296 samples than the INOCUL samples (0.044 ± 0.009), [indicating the availability of close](#)
297 [reference genomes in the PICRUST2 database](#). Principal component analyses of these
298 profiles revealed that ATB, INOCL, and NATUR samples were clearly associated with
299 distinct functional profiles (Fig. 6A). Using a Kruskal-Wallis test and a Benjamini Hochberg
300 correction, we found that 314 out of 349 predicted metabolic pathways differed significantly
301 ($p_{adj} < 0.05$ in at least one of these conditions; Supplementary Table S5). Interestingly, from a
302 functional point of view, INOCL samples were closer to the NATUR samples than the ATB
303 samples (Fig. 6B). However, none of the predicted metabolic pathways differed significantly
304 in response to stress when analyzing the ATB, INOCL, and NATUR groups separately.

305 **Discussion**

306

307 **The effect of antibiotic treatments on algal cultures**

308 Protocols to obtain truly axenic cultures using antibiotic treatments are available for
309 several microalgal species (e.g., Cirri et al. 2018; von Dassow et al. 2009). For macroalgae
310 axenic cultures are, to the best of our knowledge, available only for *Ulva mutabilis* (Califano
311 and Wichard 2018) and even these do not exhibit normal growth and morphology unless
312 complemented with bacterial morphogens (Spoerner et al. 2012). In most cases, e.g. the
313 filamentous brown alga *Ectocarpus* (Burgunter-Delamare et al. 2020) but also some
314 microalgae (Geng et al. 2016) antibiotic treatments reduced bacterial abundance and led to a
315 shift in bacterial community composition. The example of *Ectoacrpus subulatus* shows that
316 this reduction may be temporary and that the relative bacterial abundance, as estimated by the
317 number of bacterial vs. host reads in metatranscriptomic experiments, may even increase after
318 recovery from strong antibiotic treatments (KleinJan et al. 2023). In our study, the
319 comparison of the NATUR and the ATB treatments confirms a change in the bacterial
320 community. In our case, the community shifted from a diverse assembly of Proteobacteria to
321 a community dominated by Bacteriodetes and notably the genus *Winogradskyella*
322 (Flavobacteria). This is similar to previous results by Burgunter-Delamare et al. (2020) where
323 another, yet undescribed genus of Flavobacteria became dominant after antibiotic treatments.
324 *Winogradskyella* is frequently associated with marine mammals and algae, and uncultivable
325 strains of the genus have been proposed to have specialized in the utilization of the brown
326 algal polysaccharide laminarian while reducing other aspects of their metabolism (Alejandre-
327 Colomo et al. 2021). Our current working hypothesis is that if there is an open niche created
328 by the antibiotic treatment it will be filled by resistant or previously dormant bacteria that are
329 well-adapted to live on macroalgae. We may furthermore hypothesize that the newly
330 established bacteria may, at least in some cases, be less beneficial to the algal host, and in any
331 case, we have no control over the communities that will establish themselves.

332

333 **Inoculation stabilized bacterial communities and partially restores metabolic functions**

334 In this study, we attempted to fill the empty niche created by an antibiotic treatment
335 by inoculation with a controlled and beneficial bacterial community. At the same time, we
336 gave this target community a selective advantage by maintaining an antibiotic treatment.
337 Our data show that this approach worked partially – approximately 63-67% of bacterial reads
338 obtained corresponded to the inoculated bacteria, and only about 31%-37% to others.
339 Although this is still a considerable amount of non-target bacteria, in a comparable study

340 without continuous antibiotic treatment, on average only 11% of the final reads corresponded
341 to the inoculated bacteria (see Burgunter-Delamare et al. (2020), notably Table 4 therein).
342 Furthermore, our Picrust2 analyses underline that the obtained bacterial communities are
343 functionally more similar to the “natural” laboratory communities than the communities after
344 only ATB treatment (Fig. 6). This indicates that the inoculation with selected bacteria was at
345 least to some extent able to restore the functions naturally present in the microbiome.

346 **Bacterial communities had little influence on host temperature stress response or vice** 347 **versa**

348 In *Ectocarpus*, bacterial communities have also been shown to have a significant
349 impact on the holobiont salinity response and vice versa (Dittami et al. 2016; KleinJan et al.
350 2023). We, thus, wanted to test if our approach of using antibiotics and antibiotic-resistant
351 cultures could help stabilize beneficial bacterial communities, and by extension increase
352 holobiont stress tolerance. Unlike in previous experiments with strong changes in salinity, our
353 results on temperature stress exhibited only weak changes in the bacterial community in both
354 the INOCL and in the control (ATB, NATUR), thus making it impossible to conclude on
355 stabilizing effect. Please note, that the absence of an impact of stress on the bacterial
356 communities was observed, despite the strong intensity of the temperature stress. Indeed, at
357 the end of the experiments, we had reached the limit of the thermal tolerance range of our
358 strains: an additional increase in temperature by two degrees led to rapid algal bleaching and
359 death regardless of the treatment (ATB, INOCL, NATUR; data not shown).

360 One possible explanation for the discrepancy between the previous salinity tolerance
361 experiments may be the different effects the stressor had on the host or the microbiome.
362 *Ectocarpus subulatus* is a euryhaline species frequently found in estuaries. It is, as such, well-
363 adapted to strong fluctuations in salinity (Peters et al. 2015) . The associated microbiome of
364 the strain used in Dittami et al (2016), however, might have impoverished and evolved over
365 the 20 years of cultivation in the laboratory, since its original isolation in 1995 (West and
366 Kraft 1996) and marine-freshwater transitions in general, are considered rare among microbes
367 (Logares et al. 2009). Regarding temperature changes, the algal hosts, on the other hand,
368 generally have narrow temperature tolerance windows, and maximum summer temperatures
369 are one of the factors limiting their distribution (Lüning 1984) Regarding bacteria, even
370 bacteria considered psychrophilic usually grow well at 20°C (Morita 1975), and most
371 (mesophilic) bacteria tolerate up to 45°C (Willey et al. 2008) Although this is presently only

372 a hypothesis, is important to keep in mind the possibility of differences in the stress response
373 of the host and the different symbiotic partners. Depending on which component is affected
374 different approaches may be appropriate when attempting to manipulate the system.

375 **Bacterial manipulation with antibiotic-resistant bacteria – a promising but low** 376 **throughput strategy**

377 While our experiments show a clear increase in the control over the *Ectocarpus*-
378 associated bacterial communities, we did not reach complete control over the alga-associated
379 microbiome, and several aspects of these experiments could be improved. First, as illustrated
380 by the metabolic distance between the INOCL and the NATUR communities, we are not yet
381 completely substituting the natural communities in terms of metabolic capacities; This may
382 be related in part to the fact that we were able to co-culture only six of the initially selected
383 16 bacterial strains. At the same time, we are also still limited to cultivable isolates.
384 Increasing our efforts on cultivating the “unculturable” (Zengler et al. 2002) would therefore
385 also help cover a larger portion of the bacterial metabolism in co-culture experiments. In
386 parallel, additional effort, and the use of more or different antibiotics may help increase the
387 control over the remaining alga-associated microbiota. Here, however, a balance needs to be
388 struck, as antibiotic resistance for the bacteria also comes with a cost, and evolving
389 resistances may further alter bacterial physiology (Händel et al. 2013) thus potentially
390 impacting interactions with the host. Another limitation of our approach is the time it takes to
391 prepare these cultures and co-culture experiments. Experiments with one or two bacterial
392 strains of interest are easily feasible, but they are difficult to upscale because each bacterium
393 reacts differently to antibiotics, and sometimes resistances are not stable making repeated
394 treatments necessary. It will thus be more difficult to envision this approach for medium- or
395 high-throughput screening of bacteria in a co-culture context, especially if complex
396 communities are to be tested.

397 Despite these limitations, we believe that the development of a collection of antibiotic-
398 resistant helper cultures for our algae constitutes one promising avenue towards fully
399 controlled laboratory experiments with algae for which there are currently no working
400 axenization protocols. If these cultures can fulfill the metabolic functions of the natural
401 microbiome, their addition will thus enable increased experimental control over the
402 microbiome during experiments, enhancing the reproducibility of microbiome-related work.

403 In the long run we could then progressively aim towards replacing the microbes by the
404 metabolites or metabolic intermediates they provide.

405

406 **Data availability**

407 The Illumina sequence data generated in this study (Raw MiSeq data) have been deposited in
408 the European Nucleotide Archive (ENA) at EMBL-EBI under accession number
409 PRJEB57188.

410 **Acknowledgments**

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412 to antibiotics, Sébastien Colin for help exploring the algal bacterial community by
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415 DADA2 scripts, and Gwenn Tanguy and Erwan Legeay from the GENOMER platform
416 (FR2424, Station Biologique de Roscoff) for access to the sequencing platform and support
417 during library preparation.

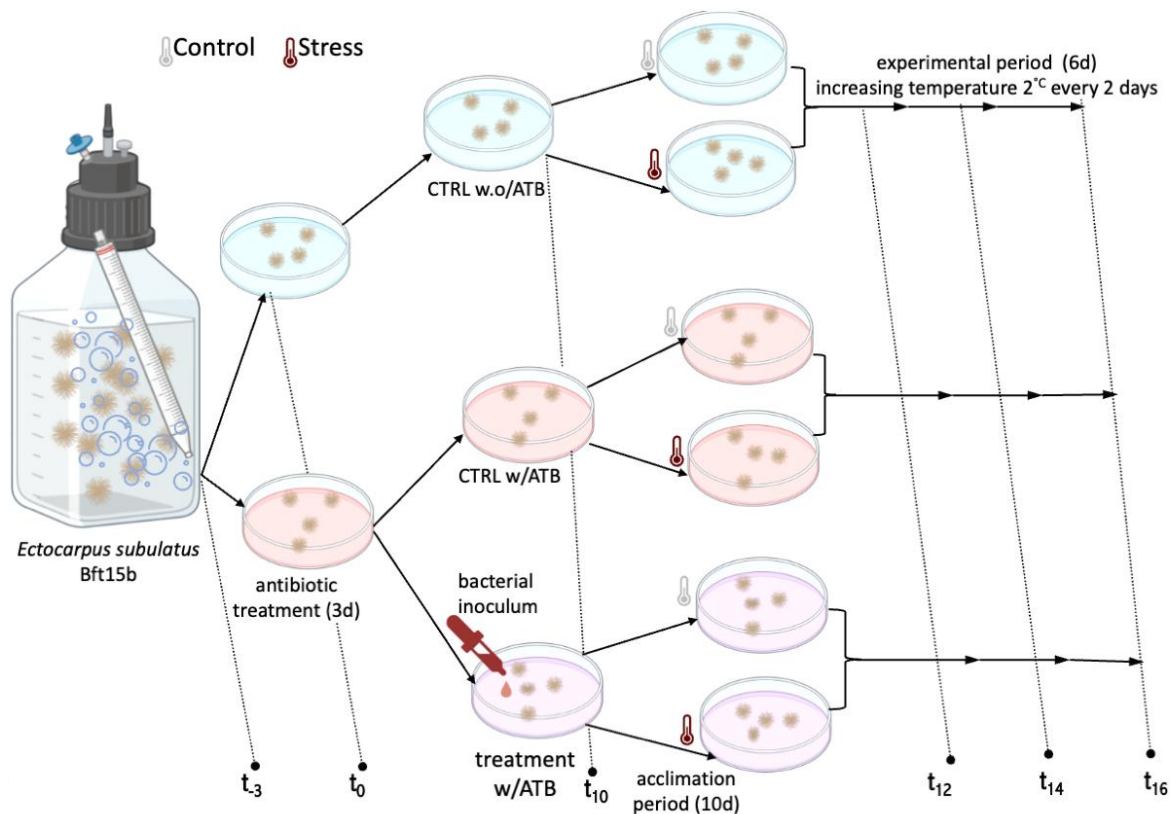
418 **Funding**

419 This work was supported by the Centre national de la recherche scientifique (CNRS)
420 [Momentum call 2017].

421 **Conflict of interest**

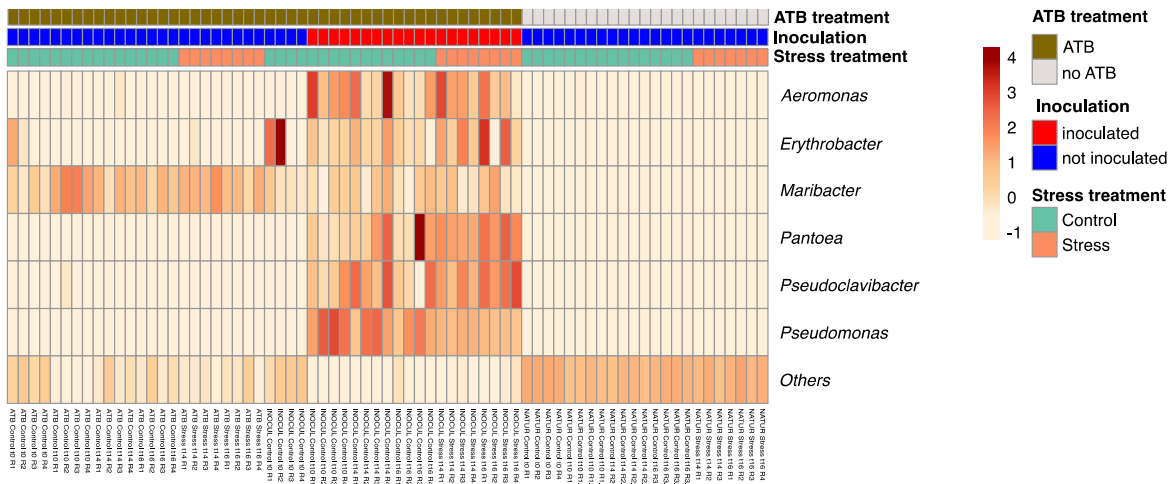
422 The authors declare that they have no conflict of interest.

423 **Figures and Tables**

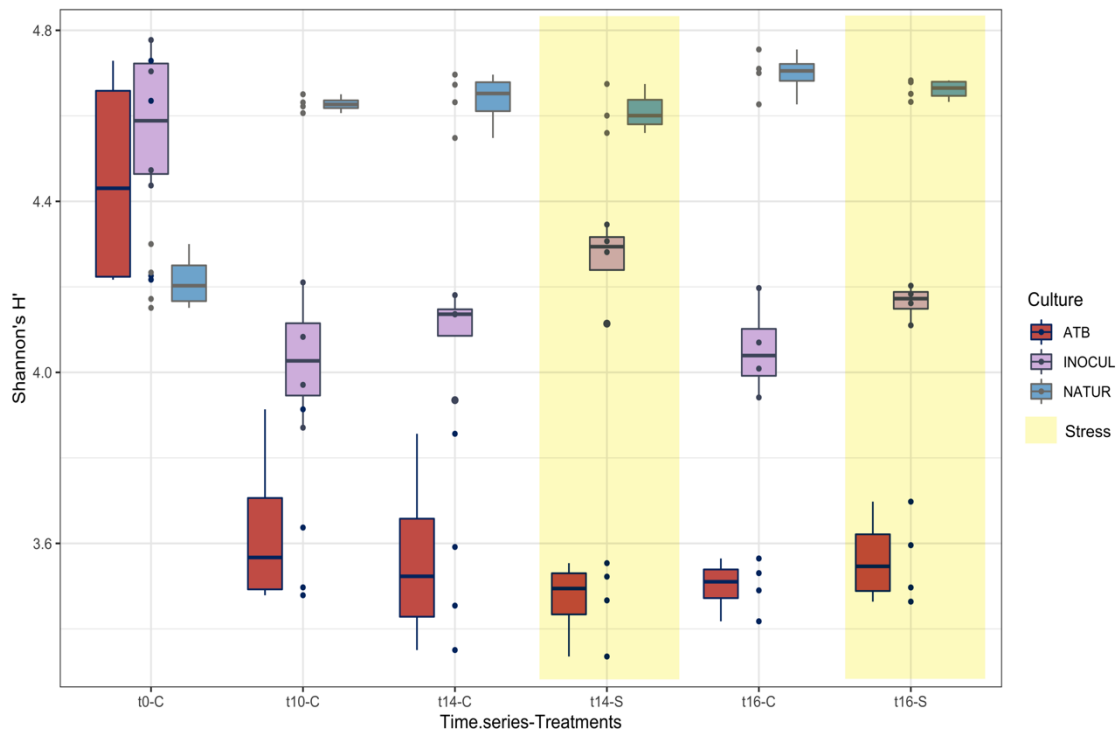


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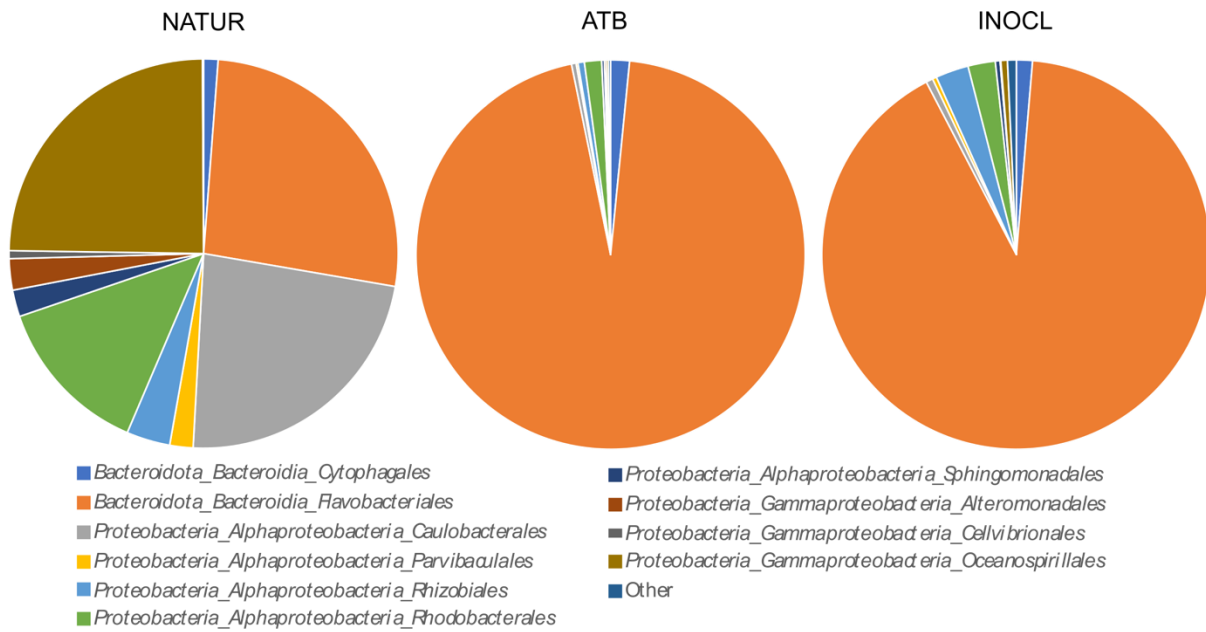
Fig. 1. A schematic illustration of the sampling design and experimental procedure. *Ectocarpus subulatus* was cultivated from stock populations of strain Bft15b. In the climate chamber (16°C) algae were grown for 7 weeks and divided into two series in petri dishes. One of the series was treated for three days with a mixture of two antibiotics (CHL & NEO), after which this group were divided into two series and one series received inoculum of 7 different bacteria. All samples stayed at the same temperature for 10 days. Later, replicates of each condition were moved to higher temperatures where the temperature was increased every 2 days in 2°C. Microbiota samples were taken directly after their separation (t-3), before inoculation (t0), after acclimation(t10), and after 4 and 6 days (t14 and t16). Parts of the figure were created with BioRender.com.



437
 438 **Fig. 2.** Heatmap of relative read abundances corresponding to inoculated bacteria vs. other
 439 bacteria. Rows are centered; unit variance scaling is applied to rows.
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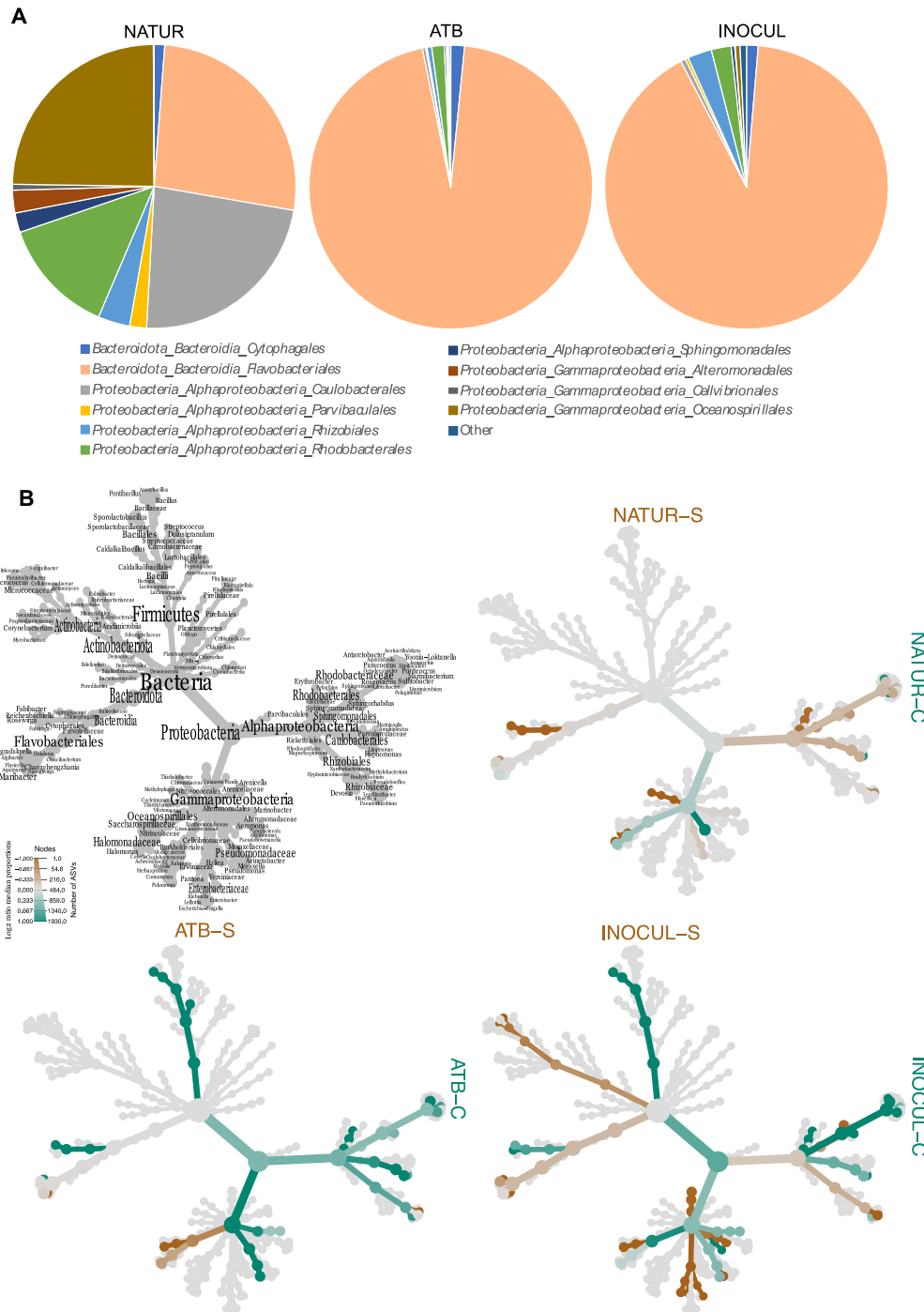


441
 442 **Fig. 3.** Bacterial Alpha diversity. The dot-plot figures show the Shannon diversity of the
 443 bacterial communities in the three culture treatment groups, ATB, INOCUL, and NATUR,
 444 and at different time points in either control (C) or stress conditions (S). Calculations are
 445 based on the relative abundance of observed ASVs.



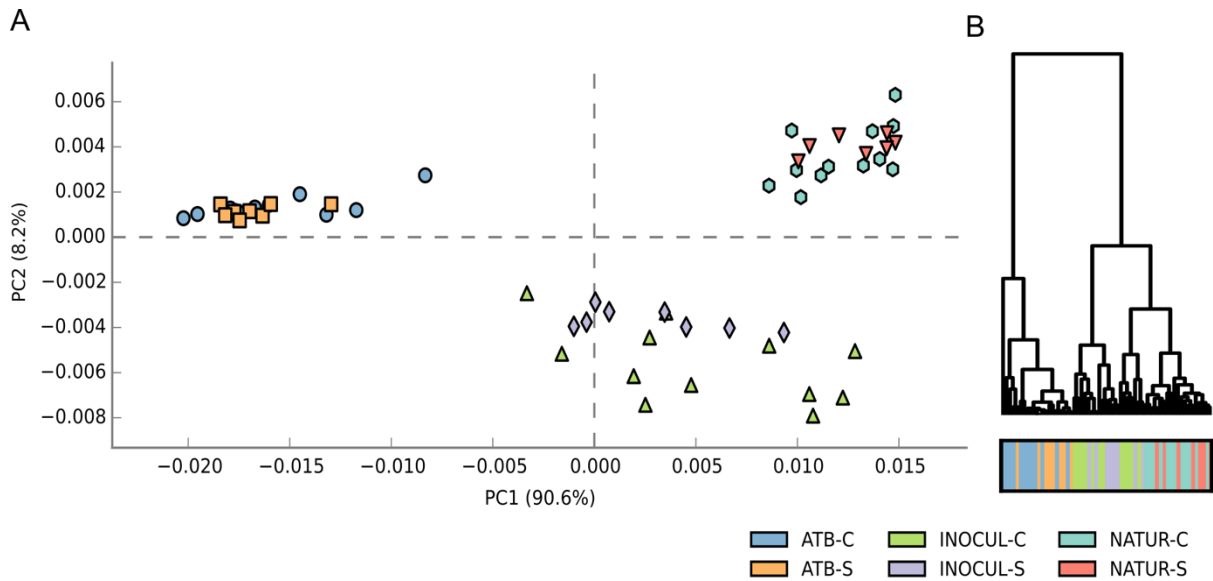
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Fig. 4. Bacterial community composition varies based on algae culture conditions. NMDS visualization of beta diversity of the bacterial communities in culture conditions and in time series in stress and control treatments (C for control and S for stressed), based on Bray–Curtis dissimilarity matrices. Each point is an individual replicate. Based on PERMANOVA testing, temperature changes had no significant effects; PERMANOVA results for the comparison of ATB, INOCL, and NATUR are displayed in the figure.



455
 456 **Fig. 5.** A) Bacterial community in three culture conditions at the order level. Taxon with
 457 abundances more than 0.1% for all three conditions (the remaining were grouped as
 458 “others”). Only non-inoculated taxa were considered for this filter (please refer to Figure 2
 459 for the inoculated taxa), and only samples after T10 (= time of inoculation), to keep the three
 460 conditions comparable. B) Differential heat tree matrix depicting the change in relative read
 461 abundance for the different taxa over different conditions and cultures. The number of taxa
 462 identified at each taxonomic level is represented by the size of the individual nodes in the
 463 gray cladogram. The smaller cladograms show pairwise comparisons between each culture
 464 condition: a brown node indicates that the taxon is more abundant in culture condition labeled
 465 in brown than in the one labeled in green and vice versa (C stands for Control (16 oC) and S
 466 stands for stressed (20 and 22 oC).

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Fig. 6. A) Principal Component Analysis of predicted Metacyc pathways (Picrust2). B) Corresponding average neighbor UPGMA clustering.

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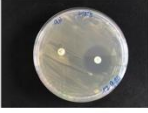


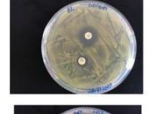
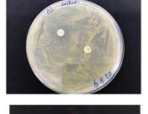


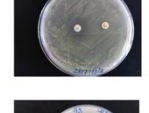
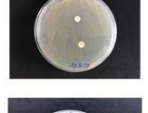


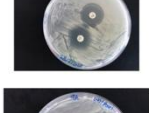
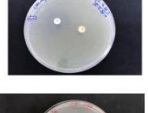


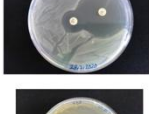
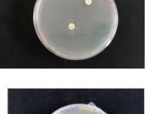


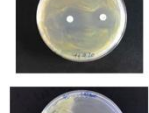
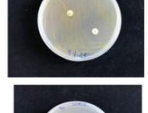
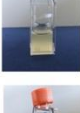

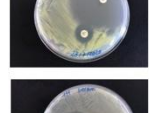
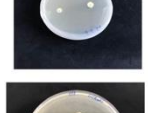


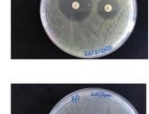
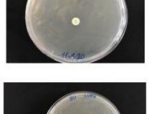


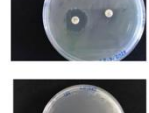
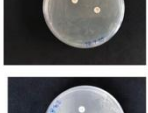


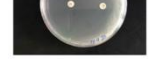



Table 1. The result of disk diffusion test for susceptibility of selected bacteria to CHL & NEO antibiotics before and after resistance. Optical density at 600 nm (OD600) was measured after 5 days of growth in liquid media containing both CHL and NEO antibiotics.

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Selected bacteria consortia	Zone of inhibition (mm)				Growth in liquid media (OD600 after 5 days)
	Before gaining antibiotic-resistance		After gaining antibiotic-resistance		
	CHL	NEO	CHL	NEO	CHL & NEO
<i>Frigoribacterium</i> sp. 9N	61	16	26	0	0.000
<i>Pseudoclavibacter</i> sp. 8L	18	10	0	0	1.186
<i>Maribacter</i> sp. 151	15	0	0	0	0.184
<i>Bacillus</i> sp. 9J	19	22	0	0	0.000
<i>Aeromonas</i> sp. 9A	43	18	0	0	0.671
<i>Erythrobacter</i> sp. EC-HK427	23	21	0	0	0.128
<i>Novosphingobium</i> sp. 9U	51	18	0	0	0.001
<i>Pantoea</i> sp. 111	33	23	0	0	0.432
<i>Pseudomonas</i> sp. 8O	0	22	0	0	0.301
<i>Rhizobium</i> sp. EC-SD404	56	17	0	0	0.145

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Supplementary materials

Selected bacteria consortia	Before ATB resistant	After ATB resistant	Liquid media Inoculation Before ATB resistant	Liquid media Inoculation After ATB resistant
<i>Frigoribacterium</i> sp. 9N				
<i>Pseudoclavibacter</i> sp. 8L				
<i>Maribacter</i> sp. 151				
<i>Bacillus</i> sp. 9J				
<i>Aeromonas</i> sp. 9A				
<i>Erythrobacter</i> sp. EC-HK427				
<i>Novosphingobium</i> sp. 9U				
<i>Pantoea</i> sp. 111				
<i>Pseudomonas</i> sp. 8O				
<i>Rhizobium</i> sp. EC-SD404				

479

480 **Figure S1.** The appearance of normal and antibiotic resistant strains on R2A agar and liquid
481 R2A medium. The antibiotic discs correspond to Neomycine and Chloraphenicolcol,
482 respectively. The liquid ABT treatment corresponded to a mixture of Chloramphenicol (9.6
483 $\mu\text{g/ml}$) and Neomycin (24.8 $\mu\text{g/ml}$). Photos were taken 5 days after inoculation

484

485 **Table S1.** Overview of minimal bacteria consortia predicted by MiSCoTo and of Antibiotic
486 resistance survey.

487

495 **Table S3: Sample metadata**

SampleID	Culture	Replicate	Time series	Temperature	Treatment	CUTim	CuT	CuTimTr
T0.C1.1.1	ATB	R1	t0	16OC	Control	t0-C	ATB-C	ATB-t0-C
T0.C1.2.1	ATB	R2	t0	16OC	Control	t0-C	ATB-C	ATB-t0-C
T0.C1.3.1	ATB	R3	t0	16OC	Control	t0-C	ATB-C	ATB-t0-C
T0.C1.4.1	ATB	R4	t0	16OC	Control	t0-C	ATB-C	ATB-t0-C
T0.C2.1.1	NATUR	R1	t0	16OC	Control	t0-C	NATUR-C	NATUR-t0-C
T0.C2.2.1	NATUR	R2	t0	16OC	Control	t0-C	NATUR-C	NATUR-t0-C
T0.C2.3.1	NATUR	R3	t0	16OC	Control	t0-C	NATUR-C	NATUR-t0-C
T0.C2.4.1	NATUR	R4	t0	16OC	Control	t0-C	NATUR-C	NATUR-t0-C
T0.T1.1	INOCUL	R1	t0	16OC	Control	t0-C	INOCUL-C	INOCUL-t0-C
T0.T2.1	INOCUL	R2	t0	16OC	Control	t0-C	INOCUL-C	INOCUL-t0-C
T0.T3.1	INOCUL	R3	t0	16OC	Control	t0-C	INOCUL-C	INOCUL-t0-C
T0.T4.1	INOCUL	R4	t0	16OC	Control	t0-C	INOCUL-C	INOCUL-t0-C
T10.C1.5.1	ATB	R1	t10	16OC	Control	t10-C	ATB-C	ATB-t10-C
T10.C1.6.1	ATB	R1	t10	16OC	Control	t10-C	ATB-C	ATB-t10-C
T10.C1.7.1	ATB	R1	t10	16OC	Control	t10-C	ATB-C	ATB-t10-C
T10.C1.8.1	ATB	R1	t10	16OC	Control	t10-C	ATB-C	ATB-t10-C
T10.C2.5.1	NATUR	R1	t10	16OC	Control	t10-C	NATUR-C	NATUR-t10-C
T10.C2.6.1	NATUR	R1	t10	16OC	Control	t10-C	NATUR-C	NATUR-t10-C
T10.C2.7.1	NATUR	R1	t10	16OC	Control	t10-C	NATUR-C	NATUR-t10-C
T10.C2.8.1	NATUR	R1	t10	16OC	Control	t10-C	NATUR-C	NATUR-t10-C
T10.T5.1	INOCUL	R1	t10	16OC	Control	t10-C	INOCUL-C	INOCUL-t10-C
T10.T6.1	INOCUL	R1	t10	16OC	Control	t10-C	INOCUL-C	INOCUL-t10-C
T10.T7.1	INOCUL	R1	t10	16OC	Control	t10-C	INOCUL-C	INOCUL-t10-C
T10.T8.1	INOCUL	R1	t10	16OC	Control	t10-C	INOCUL-C	INOCUL-t10-C
T10.C1.5.2	ATB	R2	t14	16OC	Control	t14-C	ATB-C	ATB-t14-C
T10.C1.6.2	ATB	R2	t14	16OC	Control	t14-C	ATB-C	ATB-t14-C
T10.C1.7.2	ATB	R2	t14	16OC	Control	t14-C	ATB-C	ATB-t14-C
T10.C1.8.2	ATB	R2	t14	16OC	Control	t14-C	ATB-C	ATB-t14-C
T10.C2.5.2	NATUR	R2	t14	16OC	Control	t14-C	NATUR-C	NATUR-t14-C
T10.C2.6.2	NATUR	R2	t14	16OC	Control	t14-C	NATUR-C	NATUR-t14-C
T10.C2.7.2	NATUR	R2	t14	16OC	Control	t14-C	NATUR-C	NATUR-t14-C
T10.C2.8.2	NATUR	R2	t14	16OC	Control	t14-C	NATUR-C	NATUR-t14-C
T10.T5.2	INOCUL	R2	t14	16OC	Control	t14-C	INOCUL-C	INOCUL-t14-C
T10.T6.2	INOCUL	R2	t14	16OC	Control	t14-C	INOCUL-C	INOCUL-t14-C
T10.T7.2	INOCUL	R2	t14	16OC	Control	t14-C	INOCUL-C	INOCUL-t14-C
T10.T8.2	INOCUL	R2	t14	16OC	Control	t14-C	INOCUL-C	INOCUL-t14-C
T14.C1.1.2	ATB	R1	t14	20OC	Stress	t14-S	ATB-S	ATB-t14-S
T14.C1.2.2	ATB	R2	t14	20OC	Stress	t14-S	ATB-S	ATB-t14-S
T14.C1.3.2	ATB	R3	t14	20OC	Stress	t14-S	ATB-S	ATB-t14-S
T14.C1.4.2	ATB	R4	t14	20OC	Stress	t14-S	ATB-S	ATB-t14-S
T14.C2.1.2	NATUR	R1	t14	20OC	Stress	t14-S	NATUR-S	NATUR-t14-S
T14.C2.2.2	NATUR	R2	t14	20OC	Stress	t14-S	NATUR-S	NATUR-t14-S
T14.C2.3.2	NATUR	R3	t14	20OC	Stress	t14-S	NATUR-S	NATUR-t14-S
T14.T1.2	INOCUL	R1	t14	20OC	Stress	t14-S	INOCUL-S	INOCUL-t14-S
T14.T2.2	INOCUL	R2	t14	20OC	Stress	t14-S	INOCUL-S	INOCUL-t14-S
T14.T3.2	INOCUL	R3	t14	20OC	Stress	t14-S	INOCUL-S	INOCUL-t14-S
T14.T4.2	INOCUL	R4	t14	20OC	Stress	t14-S	INOCUL-S	INOCUL-t14-S
T10.C1.5.3	ATB	R3	t16	16OC	Control	t16-C	ATB-C	ATB-t16-C
T10.C1.6.3	ATB	R3	t16	16OC	Control	t16-C	ATB-C	ATB-t16-C
T10.C1.7.3	ATB	R3	t16	16OC	Control	t16-C	ATB-C	ATB-t16-C
T10.C1.8.3	ATB	R3	t16	16OC	Control	t16-C	ATB-C	ATB-t16-C
T10.C2.5.3	NATUR	R3	t16	16OC	Control	t16-C	NATUR-C	NATUR-t16-C
T10.C2.6.3	NATUR	R3	t16	16OC	Control	t16-C	NATUR-C	NATUR-t16-C
T10.C2.7.3	NATUR	R3	t16	16OC	Control	t16-C	NATUR-C	NATUR-t16-C
T10.C2.8.3	NATUR	R3	t16	16OC	Control	t16-C	NATUR-C	NATUR-t16-C
T10.T5.3	INOCUL	R3	t16	16OC	Control	t16-C	INOCUL-C	INOCUL-t16-C
T10.T6.3	INOCUL	R3	t16	16OC	Control	t16-C	INOCUL-C	INOCUL-t16-C
T10.T7.3	INOCUL	R3	t16	16OC	Control	t16-C	INOCUL-C	INOCUL-t16-C
T10.T8.3	INOCUL	R3	t16	16OC	Control	t16-C	INOCUL-C	INOCUL-t16-C
T16.C1.1.3	ATB	R1	t16	22OC	Stress	t16-S	ATB-S	ATB-t16-S
T16.C1.2.3	ATB	R2	t16	22OC	Stress	t16-S	ATB-S	ATB-t16-S
T16.C1.3.3	ATB	R3	t16	22OC	Stress	t16-S	ATB-S	ATB-t16-S
T16.C1.4.3	ATB	R4	t16	22OC	Stress	t16-S	ATB-S	ATB-t16-S
T16.C2.1.3	NATUR	R1	t16	22OC	Stress	t16-S	NATUR-S	NATUR-t16-S
T16.C2.2.3	NATUR	R2	t16	22OC	Stress	t16-S	NATUR-S	NATUR-t16-S
T16.C2.3.3	NATUR	R3	t16	22OC	Stress	t16-S	NATUR-S	NATUR-t16-S
T16.C2.4.3	NATUR	R4	t16	22OC	Stress	t16-S	NATUR-S	NATUR-t16-S
T16.T1.3	INOCUL	R1	t16	22OC	Stress	t16-S	INOCUL-S	INOCUL-t16-S
T16.T2.3	INOCUL	R2	t16	22OC	Stress	t16-S	INOCUL-S	INOCUL-t16-S
T16.T3.3	INOCUL	R3	t16	22OC	Stress	t16-S	INOCUL-S	INOCUL-t16-S
T16.T4.3	INOCUL	R4	t16	22OC	Stress	t16-S	INOCUL-S	INOCUL-t16-S

501 **Table S5: NSTI values**

SampleID	Culture	Replicate	Time series	Treatment	NSTI
T0.C1.1.1	ATB	R1	t0	Control	0.05677776
T0.C1.2.1	ATB	R2	t0	Control	0.0480
T0.C1.3.1	ATB	R3	t0	Control	0.0496
T0.C1.4.1	ATB	R4	t0	Control	0.05315927
T0.C2.1.1	NATUR	R1	t0	Control	0.04720949
T0.C2.2.1	NATUR	R2	t0	Control	0.05315927
T0.C2.3.1	NATUR	R3	t0	Control	0.04734416
T0.C2.4.1	NATUR	R4	t0	Control	0.04673616
T0.T1.1	INOCUL	R1	t0	Control	0.0553017
T0.T2.1	INOCUL	R2	t0	Control	0.05546127
T0.T3.1	INOCUL	R3	t0	Control	0.07043085
T0.T4.1	INOCUL	R4	t0	Control	0.06053751
T10.C1.5.1	ATB	R1	t10	Control	0.04864208
T10.C1.5.2	ATB	R2	t14	Control	0.04340053
T10.C1.5.3	ATB	R3	t16	Control	0.04382899
T10.C1.6.1	ATB	R1	t10	Control	0.04341665
T10.C1.6.2	ATB	R2	t14	Control	0.04851226
T10.C1.6.3	ATB	R3	t16	Control	0.04374442
T10.C1.7.1	ATB	R1	t10	Control	0.04311913
T10.C1.7.2	ATB	R2	t14	Control	0.04358509
T10.C1.7.3	ATB	R3	t16	Control	0.04304645
T10.C1.8.1	ATB	R1	t10	Control	0.04264456
T10.C1.8.2	ATB	R2	t14	Control	0.04164816
T10.C1.8.3	ATB	R3	t16	Control	0.04409102
T10.C2.5.1	NATUR	R1	t10	Control	0.05293795
T10.C2.5.2	NATUR	R2	t14	Control	0.05307948
T10.C2.5.3	NATUR	R3	t16	Control	0.05537418
T10.C2.6.1	NATUR	R1	t10	Control	0.05197292
T10.C2.6.2	NATUR	R2	t14	Control	0.05423166
T10.C2.6.3	NATUR	R3	t16	Control	0.05245814
T10.C2.7.1	NATUR	R1	t10	Control	0.05202842
T10.C2.7.2	NATUR	R2	t14	Control	0.05386173
T10.C2.7.3	NATUR	R3	t16	Control	0.05369354
T10.C2.8.1	NATUR	R1	t10	Control	0.05183283
T10.C2.8.2	NATUR	R2	t14	Control	0.05072544
T10.C2.8.3	NATUR	R3	t16	Control	0.05721365
T10.T5.1	INOCUL	R1	t10	Control	0.04151147
T10.T5.2	INOCUL	R2	t14	Control	0.04244061
T10.T5.3	INOCUL	R3	t16	Control	0.04461342
T10.T6.1	INOCUL	R1	t10	Control	0.03788673
T10.T6.2	INOCUL	R2	t14	Control	0.0398021
T10.T6.3	INOCUL	R3	t16	Control	0.03852778
T10.T7.1	INOCUL	R1	t10	Control	0.03395527
T10.T7.2	INOCUL	R2	t14	Control	0.03544289
T10.T7.3	INOCUL	R3	t16	Control	0.03448663
T10.T8.1	INOCUL	R1	t10	Control	0.03623997
T10.T8.2	INOCUL	R2	t14	Control	0.03998218
T10.T8.3	INOCUL	R3	t16	Control	0.03943479
T14.C1.1.2	ATB	R1	t14	Stress	0.04345972
T14.C1.2.2	ATB	R2	t14	Stress	0.04259941
T14.C1.3.2	ATB	R3	t14	Stress	0.04323101
T14.C1.4.2	ATB	R4	t14	Stress	0.04309568
T14.C2.1.2	NATUR	R1	t14	Stress	0.05366224
T14.C2.2.2	NATUR	R2	t14	Stress	0.05228219
T14.C2.3.2	NATUR	R3	t14	Stress	0.05131831
T14.T1.2	INOCUL	R1	t14	Stress	0.04079558
T14.T2.2	INOCUL	R2	t14	Stress	0.03898642
T14.T3.2	INOCUL	R3	t14	Stress	0.04335328
T14.T4.2	INOCUL	R4	t14	Stress	0.04521808
T16.C1.1.3	ATB	R1	t16	Stress	0.04490096
T16.C1.2.3	ATB	R2	t16	Stress	0.04253589
T16.C1.3.3	ATB	R3	t16	Stress	0.04382073
T16.C1.4.3	ATB	R4	t16	Stress	0.04592268
T16.C2.1.3	NATUR	R1	t16	Stress	0.05481751
T16.C2.2.3	NATUR	R2	t16	Stress	0.05781305
T16.C2.3.3	NATUR	R3	t16	Stress	0.05333084
T16.C2.4.3	NATUR	R4	t16	Stress	0.05764996
T16.T1.3	INOCUL	R1	t16	Stress	0.04373309
T16.T2.3	INOCUL	R2	t16	Stress	0.03842605
T16.T3.3	INOCUL	R3	t16	Stress	0.04360146
T16.T4.3	INOCUL	R4	t16	Stress	0.04543375

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