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1 **Alterins, a new family of marine antibacterial cyclolipopeptides.**

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15 **Keywords:** Antimicrobial; chemodiversity; cyclolipopeptides ; *Pseudoalteromonas*

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21 **Abstract**

22 Five strains of *Pseudoalteromonas*, isolated from oyster hemolymph, exhibited antibacterial
23 activity against several Gram-negative bacteria. Bioactive compounds were identified in their
24 cell-free supernatant and characterized as alterins, which are cyclolipopeptides composed of
25 an heptapeptidic ring connected to a fatty acid chain. Using UPLC-HRMS, we describe here
26 37 structural analogs differing from each other by one or more amino acid residue, the
27 length of the fatty acid chain, its hydroxylation and the presence of unsaturation.

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32 **1. Introduction**

33 The genus *Pseudoalteromonas* is one of the main marine bacterial genus gathering
34 many bioactive metabolite-producing species (1). This genus is well-known to be associated
35 with marine organisms (2). One of its ecological significance comes from its ability to
36 produce antimicrobial compounds which may contribute to host defense (3, 4). As filter
37 feeders, marine bivalves are particularly exposed to microorganisms and exhibit high
38 concentrations of cultivable microbiota in hemolymph (5, 6). In this way, marine bivalves can
39 be considered as natural agents of selection. From *Crassostrea gigas* oyster hemolymph, two
40 *Pseudoalteromonas* strains, named hCg-6 and hCg-42, were isolated and exhibited potent
41 antimicrobial activity. This antibacterial activity results from the production of
42 cyclolipopeptides named alterins (7). Alterins contained an heptapeptidic cycle linked to a
43 hydrocarbon tail *via* an amido bond. This cycle is composed of 2 proteinogenic amino acid
44 residues (Leu⁴ and Arg⁵) and 5 exotic ones (Dhb², Dab³ and 3 Dab(β -OH)^{1,6,7}). The latter is
45 quite uncommon since it has only been described so far in only one another antibiotic
46 peptide family, a odilorhabdins (8), which are linear peptides produced by a Non-Ribosomal
47 Peptide Synthetase of the nematode-symbiotic bacterium *Xenorhabdus nematophila* (8). The
48 alterin members differ from each other by the hydrocarbon tail length, the number of
49 unsaturation(s) on the C5 and the hydroxylation or not on the C3. Alterins mechanism of
50 action was elucidated. As a first step, they interact with lipopolysaccharide (LPS) of the
51 target bacteria, then provoke a membrane depolarization and permeabilization leading to
52 the target bacteria lysis (7). To date, the alterin family is made of 8 members, characterized
53 from the strains hCg using purification and NMR analysis. In this paper, we report (i) the
54 production of alterins by three more strains, named *Pseudoalteromonas ostreae* hOe-66^T,
55 hOe-124 and hOe-125 isolated from the flat oyster, *Ostrea edulis* hemolymph (9), (ii) a
56 complete overview of the alterin chemodiversity within *Pseudoalteromonas* strains, using an
57 Ultra-Performance Liquid Chromatography–High-Resolution Mass Spectrometry (UPLC –
58 HRMS) approach.

59

60 **2. Material and methods**

61 *2.1. Crude extract production*

62 The *Pseudoalteromonas* alterin production was investigated from the 5 strains called
63 hCg-6 (JX912482), hCg-42 (JX912481) and hOe-66^T (MT271987), hOe-124 (MT271988) and
64 hOe-125 (MT271989), according to the procedure developed by Desriac *et al.* (2020). Briefly,
65 the five producing strains were grown for 40 hours at 18°C under shaking (150 rpm) in a
66 specific medium (7). The cell-free culture supernatants (100 ml) were subjected to C18 solid-
67 phase extraction column (SPE, C18 2G UPTI-clean cartridges, Interchim, France). Elutions
68 were performed using acetonitrile:water (60:40; v/v). After freeze-drying, the alterin
69 extracts were resuspended in acetonitrile:water (80:20; v/v; 2ml). Alterin concentrations
70 were measured using the BCA method (BCA Protein Assay Kit, ThermoScientific, USA) (Table
71 1).

72

73 2.2. Antibacterial activity of alterin extracts

74 To detect antimicrobial activity from alterin extracts, the well-diffusion method was used
75 (5, 10). Briefly, 15 mL of marine agar were inoculated with an exponential growth culture of
76 the bacterial target (10^6 CFU/mL). When dried, wells (4 mm in diameter) were punch out of
77 the plate and filled with 20 μ L of alterin extracts. Polymyxin B sulphate (1 μ g/ μ L) and
78 acetonitrile:water (80:20) were used as positive and negative controls, respectively. After an
79 overnight incubation at 18°C, antimicrobial activity was detected by an inhibition zone
80 surrounding the well.

81 The minimal inhibitory concentrations (MICs) of crude extracts were determined against
82 the Gram-negative bacteria strains *Escherichia coli* ATCC 25922, *Vibrio harveyi* ORM4, *Vibrio*
83 *pectenicida* CIP105190, *Vibrio tasmaniensis* LGP32 and *Vibrio tapetis* CECT 4600. This assay
84 was conducted into sterile 96-well microliter plates (Nunc-167314) using a method slightly
85 modified from Wiegand *et al.* (2008) (11). Briefly, a two-fold dilution series of the crude
86 extract (100 μ g/mL), solubilized in acetonitrile:water (80:20), was prepared into the
87 appropriate culture medium (100 μ L). Finally, each well was inoculated by the bacterial
88 target (1.10^6 CFU/mL). The MIC was defined as the lowest extract concentration that inhibits
89 the visible growth of the target bacterium after a 48-hour long incubation at the appropriate
90 growth temperature. The antibiotic cyclolipopeptides polymyxine B sulphate was used as
91 positive controls. Acetonitrile:water (80:20) was used as negative control.

92

93 2.3. Liquid chromatography and Mass spectrometry analysis

94 The chemical diversity of alterins was explored using an Acquity UPLC H-Class coupled to
 95 a Xevo G2 S Q-TOF mass spectrometer equipped with an electrospray ionization (ESI) source
 96 (Waters, Manchester, United Kingdom). Alterin abundances were normalized using a
 97 synthetic cyclolipopeptide as internal standard (Heptanoyl-Dab-Leu-Pro-cyclo-(Lys-Ser-Tyr-
 98 Lys-Leu) trifluoroacetate salt, Bachem, Switzerland).

99

100 3. Results

101 3.1. Antibacterial activity from crude extracts

102 At equal bacterial density (*ie* 10⁹ CFU/mL), the alterin concentration was higher in the
 103 alterin extracts resulting from the hCg strain cultures than those resulting the hOe strain
 104 ones (Table 1). Antibacterial activity (radius > 5mm) was detected using the well-diffusion
 105 method for all alterin extracts while no inhibition zone was observed for the solvent used to
 106 solubilize alterin extracts (*i.e.* acetonitrile:water (80:20)). Regarding MIC values, the
 107 antibiotic activity against Gram-negative bacteria was higher using alterin extracts produced
 108 by hCg strains than hOe ones except for *V. tapetis* CECT 4600 which appears not sensitive to
 109 alterins extracts (up to 50 µg/mL).

110

111 Table 1. MIC (µg/mL) of crude extracts obtained from the five *Pseudoalteromonas* supernatants, hCg-6, hCg-42, hOe-66,
 112 hOe-124 and hOe-125. Alterin concentration was obtained using BCA method, and was normalized as concentration
 113 produced by 10⁹ CFU/mL. ND: Not Determined.

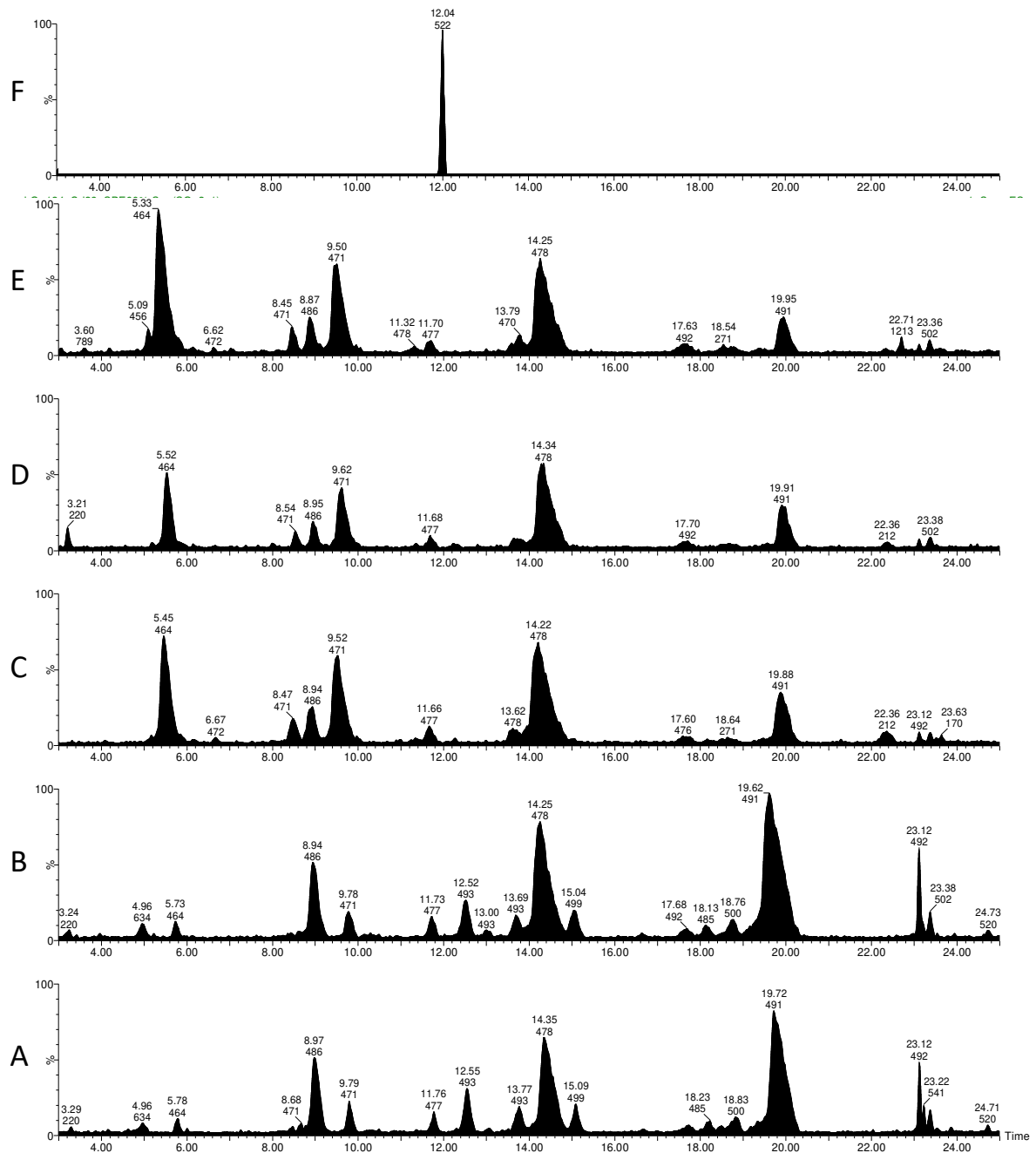
Producing strains	Alterin concentration (µg/mL for 10 ⁹ CFU/mL)	<i>E. coli</i> ATCC 25922	<i>V. harveyi</i> ORM4	<i>V. pectenica</i> CIP 105190	<i>V. tasmaniensis</i> LGP32	<i>V. tapetis</i> CECT 4600
hCg-6	112.4	6.25	1.58	1.58	1.58	>50
hCg-42	276.3	6.25	1.58	1.58	1.58	>50
hOe-66	365.6	25	3.13	3.13	3.13	>50
hOe-124	557.2	25	3.13	6.25	6.25	>50
hOe-125	492.7	25	3.13	6.25	3.13	>50
Polymyxin B	/	1.5	50	100	3	>100

114

115 3.2. *Pseudoalteromonas* hOe strains as alterins producers

116 RP-UPLC-MS analysis allowed us to compare alterin extracts from the five
 117 *Pseudoalteromonas* strains (Figure 1). Similarities were observed between alterin extracts
 118 from hOe and hCg strains, in terms of retention time and m/z ratio. Indeed, m/z were the
 119 same for peaks with the highest intensity (*ie* 464, 486, 471, 478 and 491), between the five

120 chromatograms, as well as for the retention times, revealing that hOe alterin extracts also
 121 contained alterins. Based on liquid chromatography analysis, the resulting extracts were
 122 composed of at least 96% of alterins. Nevertheless, differences in both intensity and
 123 diversity appeared, suggesting that the five strains produced a high diversity of alterin
 124 analogs.



125
 126 **Figure 1.** Base peak intensity chromatogram obtained from crude extracts of the five strains *Pseudoalteromonas* hCg-6 (A),
 127 hCg-42 (B), hOe-66 (C), hOe-124 (D), hOe-125 (E) and the internal standard (F). Time retention and $[M+2H]^{2+}$ were indicated
 128 for most intense peaks (> 5% of intensity).

129

130 **3.3. Structural diversity of alterins**

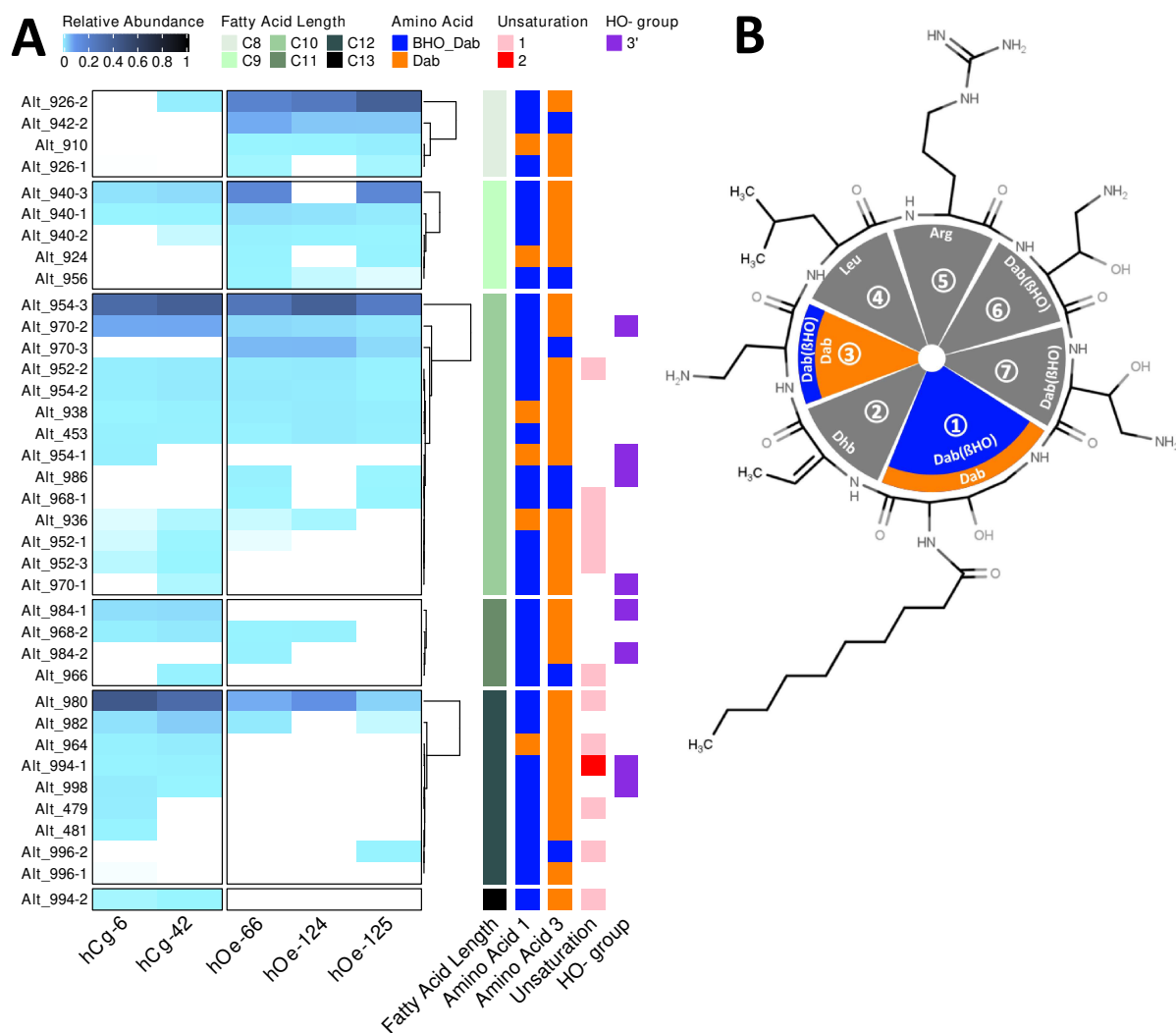
131 From the five alterin extracts, more than 55 alterins or alterin-like structures were
132 identified and among them 37 structures were elucidated by MS/MS (Figure 2A). The alterin
133 members ranged from 453 Da to 998 Da with structural differences detected both on the
134 peptidic cycle and the fatty acid chain. As an example, "Alt_998-1" means Alterin of 998 Da,
135 first isoform.

136 The amino acid residues within the cycle are the same in all alterins, except residues 1 and 3.
137 Most structures carry a Dab(β -OH)¹ and Dab³ (68%) but a few alterins were found, mainly
138 from hOe strains, with the combinations Dab(β -OH)¹-Dab(β -OH)³ or Dab¹-Dab³ (Figure 2B).
139 Regarding the fatty acid chain, the length varied from 8 to 13 carbons (C8 to C13). The most
140 abundant alterin (around 40%) found in hCg strains alterin extract was the Alt_980 with a
141 C12:1 fatty acid chain. By contrast, the most abundant alterin member in the hOe strains
142 alterin extract was Alt_926-2 (around 30%) exhibiting a shorter fatty chain in C8:0. The
143 Alt_954-3, a C10:0 alterin was also produced in high proportions by both hCg and hOe crude
144 extracts (37% and 28%, respectively).

145 The C13 chain was only observed in the Alt_994-2 isoform, exclusively produced by hCg
146 strains. Overall, it appears that the hCg strains mainly produced alterins with fatty acid
147 chains longer or equal to C10 while hOe strains produced alterins with fatty acid chains
148 shorter or equal to C10.

149 Interestingly, 3 lipotriptides were also detected in the crude extracts, the Alt_453, Alt_471
150 and Alt_481. They all carried the Dab(β -OH)¹-Dhb²-Dab³ combination with a fatty acid (C10
151 or C12).

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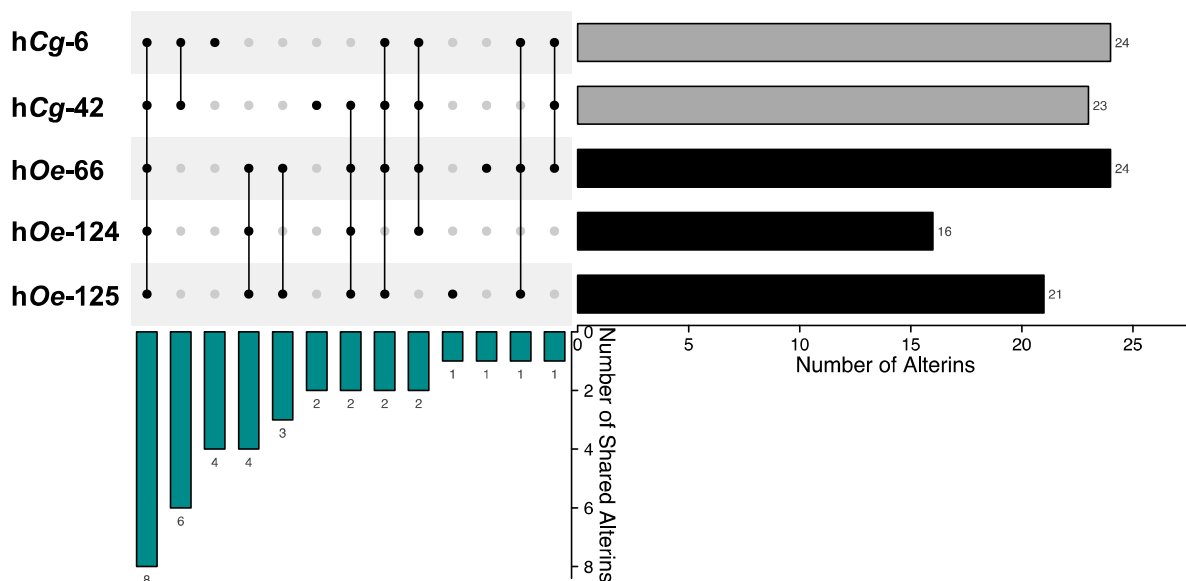
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Figure 2. Representation of the chemodiversity of alterins produced by the five strains *Pseudoalteromonas* hCg-6, hCg-42, hOe-66, hOe-124 and hOe-125. **A.** Heatmap illustrating the relative abundances of alterins identified in the crude extract from each strain, ordered by the fatty acid length (C8, C9, C10, C11, C12 and C13, in green shade), the amino acid 1 and 3 (Dab(βHO), blue; Dab, orange) and the unsaturation (red, with; transparent, without). **B.** General structure of alterins with amino acid residues identifications.

Based on alterin diversity from the 5 alterin extracts, the strains hCg-6 and hOe-66 exhibited the highest diversity, with 24 different alterins (Figure 3), while hOe-124 strain produced only 16 different alterins. A total of eight alterins were common to the five strains, representing from 32% to 89% of the total crude extracts. All 6 hCg-specific alterins carried unsaturation and/or hydroxylations on the fatty acid chain (Alt_994-2, Alt_998, Alt_994-1, Alt_964, Alt_984 and Alt_952-3) while alterins specific to hOe strains had peptidic cycles with either Dab¹-Dab³ or Dab(βHO)¹-Dab(βHO)³ combinations (Alt_942-2, Alt_910, Alt_956 and Alt_970-3).



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Figure 3. Venn representation brought information on the number of alterins produced by each strain and those shared between them.

172

4. Discussion

173 This study revealed the high chemodiversity of the alterin family. In summary, a total of 37
174 alterin peptides were produced and identified from 5 different *Pseudoalteromonas* strains.
175 To date only 8 alterins were reported to be produced by *Pseudoalteromonas* strains (7, 12).
176 Few other major genera are studied for their ability to produce antimicrobial
177 cyclolipopeptides, such as *Bacillus*, *Pseudomonas*, *Streptomyces* or *Paenibacillus*,
178 respectively producing surfactin, pseudofactin, daptomycin and polymyxin (13). To date,
179 cyclolipopeptides from *Pseudoalteromonas* genus is still poorly investigated, with only 2
180 studies reported (7, 13). As genus, *Pseudoalteromonas* is exclusively found in the marine
181 environment (14). Alterins are considered as one of the largest family of antibacterial
182 cyclolipopeptides from marine bacteria. Diversity of alterins in the crude extract seems to be
183 involved in the antibacterial activity since hOe-124 strains produced the lower number of
184 isoforms and exhibited the lower activity. On the other hand, the high production of
185 Alt_940-3 and Alt_982 by all strains except hOe-124 may suggest their major role in
186 antibacterial activity. Nonetheless, the high concentration in alterins seemed to overcome
187 this low diversity. Combining the structure diversity and bioactivity differences of alterin
188 extracts, it seems that the fatty acid chains length is involved in the activity of alterins
189 against Gram-negative bacteria, as described for polymyxins (15). Synergistic effect between
190 the alterin cocktail may contribute to improve the antibacterial activity of alterin extracts, as
191 well as the combination of few particular alterins with saturated fatty acid chains, per

192 example. Overall, structure-activity investigations need to be conducted to identify the key
193 structural features governing antibacterial activity and consider their application, for
194 instance, to treat infectious diseases. Additionally, to the best of our knowledge, none has
195 reported the biosynthesis of the partial cyclolipopetide so far, i.e. the 3 lipotriptides,
196 suggesting that the alterin biosynthesis may result from a sequential biosynthesis
197 mechanism. Moreover the strain SANK 71903 belonging to *Pseudoalteromonas flavipulchra*
198 was also shown to produce alterins (16). Since this strain is phylogenetically distant from the
199 strains isolated from the oyster hemolymph, the prevalence of alterin and its biosynthetic
200 gene cluster in the *Pseudoalteromonas* genus remain to be elucidated as well as their
201 ecological role. The biosynthesis of many similar structures by the bacteria could be the
202 result of an evolutionary strategy, becoming a competitive trait used against other bacteria
203 to colonize the same ecological niche (17).

204

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214 **Ethical Approval:** Not required.

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