

Exploiting the bioactive potential of microbial small molecules for the development of next generation antimicrobials

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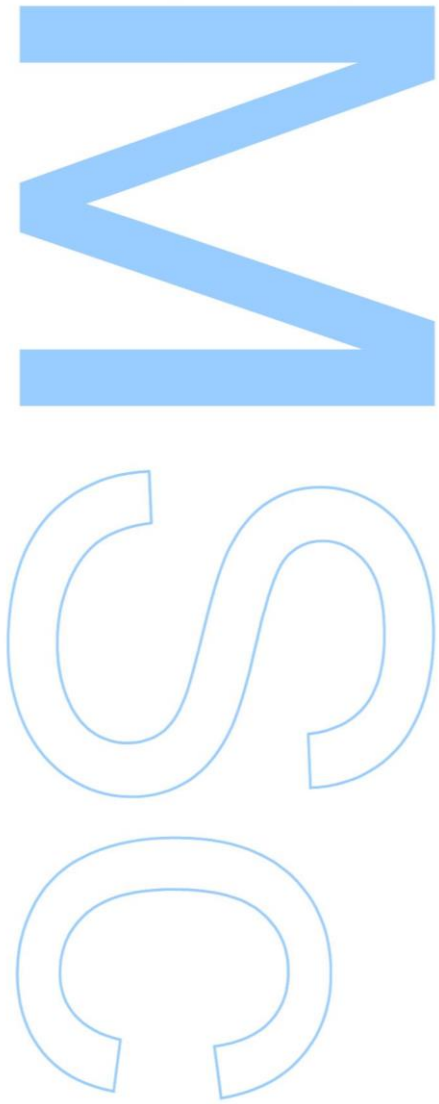
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Todas as correções determinadas pelo júri, e só essas, foram efetuadas.

O Presidente do Júri,

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Resumo

A aquicultura é uma indústria indispensável para satisfazer a procura mundial de peixe. No entanto, devido à intensificação do cultivo, os peixes em aquicultura são alvo de diversos agentes patogénicos. Além das perdas massivas de animais, os surtos bacterianos estão associados ao abuso de antibióticos, levando à disseminação de bactérias resistentes, e deixando resíduos em produtos de aquicultura para consumo humano, ameaças graves para a saúde pública. Por esta razão, são urgentes novas estratégias de biocontrolo que minimizem a ocorrência de doenças em aquicultura.

O Quorum-sensing (QS), um sistema de comunicação entre células bacterianas baseado em moléculas sinalizadoras, que regula fatores de virulência, tem atraído cada vez mais atenção como um potencial alvo para a descoberta e desenvolvimento de novas terapias antimicrobianas. Bactérias probióticas, como *Bacillus*, são fontes potenciais de moléculas inibidoras de QS, num processo denominado de Quorum-Quenching (QQ). Além disso, uma molécula sinalizadora natural, 2-heptyl-4-quinolone - HHQ (percursor do sinal da *Pseudomonas* (PQS)), revelou ter uma dimensão inter-espécies e inter-Reino, que está associada a uma atividade antimicrobiana específica contra determinadas espécies. Assim, o objetivo deste trabalho é explorar o potencial bioativo QQ e capacidade antimicrobiana de moléculas produzidas por probióticos de *Bacillus*, isolados de diferentes espécies de peixes da aquicultura, assim como explorar o potencial bioativo do HHQ e os seus análogos criados sinteticamente. Caracterizar a natureza e a especificidade destas bioatividades, proporcionará ao setor da aquicultura novos compostos naturais com potencial para serem usados como uma nova estratégia de prevenção de doenças.

Abstract

Aquaculture is an indispensable industry to satisfy the world's fish-demand. However, due to intensive farming, aquaculture fish are the target of many bacterial pathogens. Besides massive animal losses, disease outbreaks are associated to the intensive use of antimicrobials, leading to the spread of antimicrobial-resistant bacteria and leaving residues in aquaculture products for human consumption, both serious threats for public health. For this reason, new biocontrol strategies to minimize and prevent disease occurrence in aquaculture are needed.

Quorum-Sensing (QS), a cell-cell communication system based on signalling molecules, that regulates several virulence factors, is attracting increased attention as a potential target for the discovery and development of novel antimicrobial therapies. Probiotic bacteria such as *Bacillus* are potential sources of QS-inhibitors, in a process called Quorum-Quenching (QQ). Also, there is the 2-heptyl-4-quinolone - HHQ (*Pseudomonas* quinolone signal (PQS) precursor), a natural signal compound that has revealed an interspecies and interkingdom dimension which is associated to species specific antimicrobial activity. Thus, the aim of this research was to explore the bioactive QQ and antimicrobial potential of microbial small molecules produced by *Bacillus* probiotics, isolated from different aquaculture fish species as well as explore the bioactive potential of HHQ and their synthetically created analogues. Characterising the nature and species specificity of these important bioactivities could provide the aquaculture sector with new natural compounds with potential to be used as novel disease prevention strategies.

Keywords

Quorum-sensing, Quorum-quenching, Bioactive compounds, Bacillus, 2-Heptyl-4-quinolone, Pseudomonas aeruginosa, Fish Pathogens

Table of Contents

Agradecimentos……I
Resumo/Abstract……II
Table of Contents……..….III
List of tables.....IV
List of figures.....V
List of abbreviations.....VI

1. Introduction
   1.1. Aquaculture importance ..……..……1
      1.1.1. Aquaculture development constrains: Fish diseases & human's health …1
      1.1.2. Vibrionaceae ……..2
         1.1.2.1. *Vibrio harveyi* ……....5
         1.1.2.2. *Vibrio parahaemolyticus* ……..6
         1.1.2.3. *Vibrio vulnificus* ……6
         1.1.2.4. *Vibrio cholerae* ……..6
         1.1.2.5. *Vibrio anguillarum* ……7
         1.1.2.6. *Vibrio fischeri* ……7
         1.1.2.7. *Photobacterium damselae* subsp. *piscicida* ……..7
         1.1.2.8. *Photobacterium damselae* subsp. *damselae* ……...8
      1.1.3. *Aeromonas* ……..……..8
         1.1.3.1. *Aeromonas salmonicida* ……..9
         1.1.3.2. *Aeromonas hydrophila* ……9
   1.2. Antimicrobial drug resistance: facts and solutions ……..9
   1.3. Marine bioactive compounds ……11
      1.3.1. *Bacillus subtilis* ……12
   1.4. Quorum-sensing ……12
   1.5. *Pseudomonas aeruginosa* ……...15
      1.5.1. Quorum sensing systems ……..16
      1.5.2. Quinolone based signalling signal ……17
      1.5.3. Oxygen deprivation, CIO and cytochromes ……19
2. Material and Methods……21
   2.1. Bacterial strains and growth conditions ……..21
   2.2. Synthesis of HHQ analogues ……21

2.3. Quorum-quenching (QQ) screening (anti-QS assay) ……28
2.4. Anti-growth screening ……29
2.5. Anti-Biofilm assay ……...30
2.6. Time-killing curve for *Vibrio scophthalmi* ……..31
2.7. Cytochrome Oxidase testing ……31
2.8. 16S rRNA gene amplification by PCR ……32
2.9. *cydAB* and *cydB* genes amplification by PCR ……32
2.10. *cydAB* and *cydB* cloning ……..……34
2.11. Plasmid pBBR1MCS-2 preparation ……..35
2.12. Comparative Genomics …… 35
2.13. Statistical Analysis ……..36
3. Results……...37
3.1. *Bacillus* isolates from the gut of aquaculture fish produce extracellular molecules active against several fish pathogens ……..37
3.1.1. Fish gut *Bacillus* isolates have antimicrobial activity ……..37
3.1.2. Fish gut *Bacillus* isolates have Quorum-Quenching (QQ) activity ……39
3.2. The *Pseudomonas* Quinolone Signal percursor HHQ inhibits the Vibrionaceae, including aquaculture pathogenic *Vibrio* species ……...41
3.2.1. HHQ inhibits *Vibrio* growth ……41
3.2.2. HHQ inhibits *Vibrio* growth and biofilm formation ……45
3.2.3. Anti-growth and anti-biofilm characteristics of HHQ analogues ……48
3.2.4. *V.scophthalmi* generates tolerant mutants to HHQ ……...52
3.2.5. *cydAB* operon might be responsible for development of HHQ-tolerant mutants in Vibrionaceae ……56
4. Discussion…….. 61
5. Conclusion……...69
6. References……..70

List of tables

Table 1. Summarized virulence mechanisms and respective regulated genes on *P.aeruginosa* (J. Lee & Zhang, 2014; Moradali et al., 2017)……..……**15**
Table 2. List of strains and routine growth conditions used in this study……**22**
Table 3. Molecular structure and carriers of 2-heptyl-4-quinolone (HHQ) and analogues……...**26**
Table 4. Oligonucleotide primers used in this study……**33**
Table 5. Anti-growth diameter of inhibition zones (mm) (Fish isolates against a panel of important fish pathogens)……**39**
Table 6. Growth of different *Vibrio* strains on agar plates containing HHQ……..**43**
Table 7. Growth and biofilm formation of *Vibrio* species in the presence of HHQ at 23ºC and 30º……....**48**
Table 8. Growth of different *Vibrio* species in the presence of HHQ analogues……**52**
Table 9. Comparative genomic analysis (BLAST) for *V.parahaemolyticus* RIMD 2210633 *cydA* (VP1053) and *cydB* (VP1054) on chromosome 1 and for *V.parahaemolyticus* RIMD 2210633 *cydA* (VPA1137) and *cydB* (VPA1138) on chromosome 2 versus other *Vibrio* and *Staphylococcus* strains and *P. aeruginosa,* to understand these genes distribution……..**60**

List of figures

Figure 1. World capture fisheries and aquaculture (FAO, 2018)……..……**1**
Figure 2. Niche specialization of members of Vibrionaceae family. Some members of this family can be found not only on the aquatic environment but can also colonize fish, marine invertebrates, be associated with plankton and algae, and infect humans (Reen, Almagro-Moreno, Ussery, & Boyd, 2006)……..…..….**3**
Figure 3. Strategies for inhibiting bacterial quorum sensing (Chu & Mclean, 2016)……**14**
Figure 4. The four quorum-sensing signalling molecules. From left to right: N-(3-oxododecanoyl)-homoserine lactone (OdDHL); N-butyryl-homoserine lactone (BHL); 2-heptyl-3-hydroxy-4-quinolone (PQS); 2-(2-hydroxyphenyl)-thiazole-4-carbaldehyde (IQS)……**17**
Figure 5. Schematic representation of the AQ biosynthetic pathway. Main elements of the *pqs* QS system (HHQ, PQS, HQNO, PqsE, and PqsR) are in bold. Solid grey arrows represent biosynthesis; dashed grey arrows represent information flow; solid black arrow indicates activation (+); black T-line indicates negative regulation (-) (Rampioni et al., 2016)……..……...**18**
Figure 6. Schematic representation of the *V. parahaemolyticus* RIMD 2210633 cytochrome d oxidase *(cydAB)* operon located on chromosome 2 and the respective PCR products obtained in this study. Operon *cydAB* (VPA1137-VPA1138) and gene *cydB* (VPA1138) were amplified in this study resulting on fragments sizes of 2401bp (*cydAB*) and 1008bp (*cydB*). The primers (Table 4) used are also included in this figure: cydA forward (cydAF), cydB forward (cydBF) and cydB reverse (cyBR)……..……..….**33**
Figure 7. pBBR1MCS-2 plasmid vector used in the study for cloning purposes..…….**35**
Figure 8. Formation of growth inhibition zones for the indicated pathogenic strains (in the left panel) by cell-free supernatant of the spore-forming isolates (on top of the panel), both from NUTRIMU collection. Dilutions of each pathogenic strain grown overnight at the ideal species requirements (Table 2) were spread on the agar plates. Each well was filled with 100 µl cell-free supernatant of the respective spore-forming isolate and the plates were incubated at the ideal pathogenic strain requirements (Table 2). The halos mean a positive result for inhibition. All photos are at the same scale……...**38**

Figure 9. Validation of the reporter systems to optimize final concentrations of signal molecules that completely inhibit the growth of the respective reporter strains (AI1-QQ.1 and AI2-QQ.1). The halos mean a positive result for inhibition. On panel A is represented the growth inhibition of the *E.coli* reporter strains AI1-QQ.1 grown in LB agar plate (supplemented with 100 µg/ml Ampicillin and 30µg/ml Kanamycin, incubated at 37ºC overnight) by applying increasing concentrations of the autoinducers. (1)-(5) represents the 4-hydroxy-5methyl-3-furanone 5000 mM, 500 mM, 250 mM, 100 mM and 50mM concentrations that had no inhibition effect on AI1-QQ.1 (dark shadow is correlated with the orange color of the compound); (6) and (7) represents the N-(Beta-Ketocaproyl)-L-homoserine lactone, 10000 µM and 100 µM concentrations. Both inhibited AI1-QQ.1 reporter strain and 100 µM was choose to following studies. (8) and (9) represents ethyl acetate 100% and 50% ethyl acetate. Only 100% ethyl acetate showed toxic effects on the reporter strain. On panel B is represented the growth inhibition of the *E.coli* reporter strains AI2-QQ.1 grown in LB agar plate (supplemented with 100 µg/ml Ampicillin and 30µg/ml Kanamycin and incubated at 37ºC overnight) by applying increasing concentrations of the autoinducers. (10)-(14) represents 4-hydroxy-5methyl-3-furanone 5000 mM, 500 mM, 250 mM, 100 mM and 50mM concentrations. 100 mM was chosen to following studies. (15) and (16) represents results for N-(Beta-Ketocaproyl)-L-homoserine lactone. The inhibition effect is correlated with ethyl acetate (lactone solvent). (17) and (18) represents ethyl acetate 100% and 50% ethyl acetate. Only 100% showed toxic effects on the reporter strain…..……...**40**

Figure 10. *Bacillus* isolates FI314 and FI376 quorum-quenching (QQ) activity against the respective reporter strains (AI1-QQ.1 and AI2-QQ.1). Cell extracts and culture supernatants (5µL) from *Bacillus* culture supernatants (Sup) and cell extracts (CE) were screened for QS-interfering activities with the respective reporter strains. The test plates were covered with LB soft agar containing 0.8% agar supplemented with 100 µM N-(β-ketocaproyl)-L-homoserine lactone (3oxo-C6-HSL), 100 µg/ml ampicillin, 30µg/ml kanamycin, and 10% (vol/vol) of exponentially growing culture of the reporter strain AI1-QQ.1 or LB soft agar plates containing 0.8%, supplemented with final concentrations of 100 mM 4-hydroxy-5methyl-3-furanone, 100 µg/ml ampicillin, 30 µg/ml kanamycin, and 10% (vol/vol) exponentially growing culture of the reporter strain AI2-QQ.1. The grown colonies show the reestablishment of growth of the reporter strains after 24 hours of incubation at 37°C by interference with the present signal molecule……...**40**

Figure 11. Growth inhibition effect in 6 different *V.fischeri* strains on agar plates containing 10 µM HHQ or not containing (untreated). All the *V.fischeri* strains were tested by streaking in LBS media supplemented with 10 µM HHQ and incubated at 23ºC for 24 hours. All the *V.fischeri* strains were susceptible to HHQ……...**42**

Figure 12. Growth inhibition effect in *V.parahaemolyticus* RIMD 2210633 strain and with different deletions on agar plates containing 10 µM HHQ or not containing (untreated*).* All the *V.parahaemolyticus* were tested by streaking in LBS3 media supplemented with 10 µM HHQ and incubated at 30ºC overnight. None of the deleted genes were responsible for HHQ tolerance on this strain……...**43**

Figure 13. Growth inhibition in *V.gallaecicus* DSM 23502, *V.litoralis* DSM 17657, *Vibrio* 1.19, *V.cholerae* 0395, *V.cholerae* NI6961 strains and *V.cholerae* NI6961 with two different deletions on agar plates containing 10 µM HHQ or not containing (untreated). The strains were tested by streaking in the respective culture media and incubating following the strains requirements (Table 2). Only *V.gallaecicus* DSM 23502 was inhibited by HHQ. *V.cholerae* and *V.anguillarum* experiences less growth with this compound. None of the *V.cholerae* deleted genes were responsible for HHQ tolerance on this strain……**44**

Figure 14. Growth inhibition effect in *V.scophthalmi* DSM 19140 strain on agar plates containing 10 µM HHQ (HHQ-2 days and HHQ-6 days) or not containing (untreated). *V.scophthalmi* strain was tested by streaking in MA media supplemented with 10 µM HHQ and incubated at 23ºC for 2 days, following the strains requirements (Table 2). *V.scophthalmi* DSM19140 was inhibited until 2 days. Tolerant mutant colonies appeared after 6 days……**44**

Figure 15. Growth inhibition effect of *V.scophthalmi* DSM19140 and *V.scophthalmi* DSM19140 mutants on an agar plate containing 10 µM HHQ. *V.scophthalmi* strain and mutants were tested by streaking in MA media supplemented with 10 µM HHQ and incubated at 23ºC for 2 days. (1) Growth inhibition effect of HHQ in *V.scophthalmi* DSM19140 (wildtype). This bacterium showed to be sensitive to HHQ. (2) Growth inhibition effect of HHQ in mutant resistant colonies on agar plates. Both showed tolerance……**45**

Figure 16. Growth (A) and biofilm (B) inhibition effect in 6 *Vibrio* species from UCC collection (*V.parahaemolyticus* RIMD 2210633, *V.fischeri* SR5, *Vibrio* 1.19, V.*litoralis* DSM 17657, *V.scophthalmi* DSM 19140 and *V.gallaecicus* DSM 23502) on 96-well plates containing 10 µM HHQ. Negative controls were included (Untreated, MeOH). All the wells were filled with 100 µl of indicator bacteria (OD_{600} ~0.05). On MeOH

and HHQ wells, 1 µl of 100% MeOH and 10 mM HHQ were respectively added. All the strains were grown on optimal media conditions (Table 2) and incubated at both 23ºC and 30ºC. Data are the average of three independent experiments, each constituting three technical replicates. Error bars represent standard deviation of the means. Significative differences were determined by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. Asterisks represent statistically significant differences of treated *Vibrio* species relative to the control untreated: * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$. (A) Growth of *V.fischeri*, *V.scophthalmi* and *V.gallaecicus* was reduced by HHQ on their ideal temperature conditions (23ºC). *V.parahaemolyticus* showed also significative growth reduction at 23ºC. *V.fischeri* and *V.gallaecicus* were also significative reduced by HHQ at 30ºC. Growth of *V.parahaemolyticus* was not reduced on ideal temperature conditions (30ºC). *Vibrio* 1.19 and *V.litoralis* experiences no inhibition on both 23ºC and 30ºC. (B) Biofilm *of V.scophthalmi* was significative reduced by HHQ both 23ºC and 30ºC.……...**47**

Figure 17. Growth inhibition effect in *V.fischeri* SR5 and *V.parahaemolyticus* RIMD 2210633 on 96-well plates containing 10 µM of HHQ synthetically created analogues. Negative controls were included (Untreated, MeOH, DMSO). HHQ was used as a positive control. All the wells were filled with 100 µl of indicator bacteria (OD_{600} ~0.05). On MeOH, DMSO wells, 1 µl of 100% MeOH and DMSO were respectively added. On HHQ and analogues wells, 1 µl of 10 mM HHQ and 10 mM analogues were respectively added. All the strains were grown on optimal growth conditions (Table 2). Data are the average of three independent experiments, each constituting three technical replicates. Error bars represent standard deviation of the means. Significative differences were determined by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. Asterisks represent statistically significant differences of treated *Vibrio* species relative to the control untreated: **-$p<0.01$, *** - $p<0.001$. HHQ reduces *V.fischeri* growth in all the assays. *V.fischeri* growth was significative reduced by A5, A12, D3, EI and G11. H1 increased *V.fischeri* growth. None of the analogues reduced *V.parahaemolyticus* growth. A12, and H1 analogues and DMSO (on the last graph) significative increased *V.parahaemolyticus* growth. 50 Fig. 15 - Growth inhibition effect of HHQ synthetically created analogues in *V.fischeri* SR5 and *V. parahaemolyticus* on 96 well-plates. *V.fischeri* SR5 *was strongly* inhibited by A12, D3, EI and G11. None of the analogues had inhibition effect in *V.parahaemolyticus*……**49**

Figure 18. Biofilm inhibition effect in *V.fischeri* SR5 and *V.parahaemolyticus* RIMD 2210633 on 96-well plates containing 10 µM of HHQ synthetically created analogues. Negative controls were included (Untreated, MeOH, DMSO). HHQ was used as a positive control. All the wells were filled with 100 µl of indicator bacteria (OD_{600} ~0.05). On MeOH, DMSO wells, 1 µl of 100% MeOH and DMSO were respectively added. On HHQ and analogues wells, 1 µl of 10 mM HHQ and 10 mM analogues were respectively added. All the strains were grown on optimal growth conditions (Table 2). Data are the average of three independent experiments, each constituting three technical replicates. Error bars represent standard deviation of the means. Significative differences were determined by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. Asterisks represent statistically significant differences of treated *Vibrio* species relative to the control untreated: * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$. HHQ reduces *V.fischeri* biofilm in all the assays. *Vibrio fischeri* biofilm was reduced by A4, A5, A12, D3 and G11. H1 increased *V.fischeri* biofilm. The third *V.parahaemolyticus* graph showed that HHQ, C1, C2, C3, C4, D2 and D4 had a significative reduction on biofilm. The last *V.parahaemolyticus* graph showed that MeOH, DMSO, HHQ, EI, G7, G8, G9, G10, G11 and H1 had a significative reduction on biofilm……..**49**

Figure 19. Growth inhibition effect in *Vibrio* species from UCC collection (*V.parahaemolyticus* RIMD 2210633, *V.fischeri* SR5, *Vibrio* 1.19, V.*litoralis* DSM17657, *V.scophthalmi* DSM 19140 and *V.gallaecicus* DSM 23502) on 96-well plates containing 10 µM of the 4-best bioactive (similar to HHQ) HHQ synthetically created analogues. Negative controls were included (Untreated, MeOH, DMSO, A7). HHQ was used as a positive control. All the wells were filled with 100 µl of indicator bacteria (OD_{600} ~0.05). On MeOH, DMSO wells, 1 µl of 100% MeOH and DMSO were respectively added. On HHQ and analogues wells, 1 µl of 10 mM HHQ and 10 mM analogues were respectively added. All the strains were grown on optimal growth conditions (Table 2). Data are the average of three independent experiments, each constituting three technical replicates. Error bars represent standard deviation of the means. Significative differences were determined by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. Asterisks represent statistically significant differences of treated *Vibrio* species relative to the control untreated: * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$. *Vibrio fischeri* growth was significative reduced by A12, D3, EI, G11. *V.litoralis* growth was increased by A12. *V.scophthalmi* growth was reduced by D3 and G11 the results weren't statistically relevant. *V.gallaecicus* growth

was reduced by A12, D3, EI and G11. However, A12 wasn't statistically relevant. HHQ reduced *V.fischeri, V.litoralis, V.scophthalmi* and *V.gallaecicus*. None of the analogues had reduction effect in *V.parahaemolyticus* and *Vibrio* 1.19 growth……**51**

Figure 20. *V.scophthalmi* DSM 19140 (*V.scophthalmi*) and *V.scophthalmi* DSM 19140 m1 (Mutant 1) time kill-curve in absence and in presence of 10 µM of HHQ. (A) OD600 readings as function of time. (B) log OD_{600nm} as function of time. *V.scophthalmi* was grown on MA supplemented with 10 µM HHQ and incubated at 23ºC, 150 rpm. Optical density measures were taken for 52 hours period. Data are the average of three independent experiments. Error bars represent standard deviation of the means. *V.scophthalmi* and Mutant 1 grew exponentially until ~ 6h. With HHQ supplementation *V.scophthalmi* (*V.scophthalmi* + HHQ) and Mutant 1 (Mutant 1 + HHQ) growth were strongly reduced until 24 hours. After 24h and until 52 hours *V.scophthalmi* + HHQ showed slight increasing in the growth. Mutant 1 + HHQ showed a strong increasing in growth, reaching the untreated Mutant 1 optical density after 52 hours. Mutant 1 showed to create tolerance to HHQ……..…..**53**

Figure 21. Amplification of 16S rRNA gene region on *V.scophthalmi* DSM 19140 and *V.scophthalmi* DSM 19140 m1. The amplifications were made by colony PCR. The primers (63F and 1387R) used to the amplifications are represented on Table 4. (1) HyperLadder™ 1kb (BIOLINE, 11-BIO-33053); (2) 16S rRNA *V.scophthalmi* DSM 19140; (3) 16S rRNA *V.scophthalmi* DSM 19140 m1……...**54**

Figure 22. Nucleotide sequence alignment of both sequenced *V.scophthalmi* DSM 19140 (AR1_ADW0022399) and *V.scophthalmi* DSM 19140 mutant 1 (AR2_ADW0022399). Both wildtype mutant colonies showed a 100% similarity between them. No gaps in the pairing sequences were found……**54**

Figure 23. Phylogenetic tree on the basis of 16S rRNA gene sequenced. The tree was constructed using BLAST pairwise alignment after a nucleotide BLAST of the sequenced *V.scophthalmi* DSM 19140 and *V.scophthalmi* DSM 19140 mutant 1. Results showed to be equal for both sequenced regions. The sequences had 100% identity with *V.scophthalmi* LMG 1958 which corresponds to *V.scophthalmi* DSM 19140……...**55**

Figure 24. Amplification of *cydAB* and *cydB* gene region of *V.parahaemolyticus* RIMD 2210633. The amplifications were made by colony PCR. The 2401bp (*cydAB*) and 1008bp (*cydB*) fragments were amplified using the primers (cydA forward, cydB reverse; cydB forward) present on Table 4. (1) HyperLadder™ 1kb (BIOLINE, 11-BIO-33053); (2) – (4) *cydAB* amplified in three different annealing temperatures (53ºC, 55ºC and 58ºC)*;* (5) – (7) *cydB* amplified in three different annealing temperatures (53ºC, 55ºC

and 58ºC)……..……..……..……**57**
Figure 25. Amplification of *cydAB* and *cydB* gene region of *V.parahaemolyticus* RIMD 2210633 using Q5™ High fidelity PCR Kit, for cloning purposes. The amplifications were made by colony PCR. The 2401bp (*cydAB*) and 1008bp (*cydB*) fragments were amplified using the primers (*cydA* forward, *cydB* reverse; *cydB* forward) present on Table 4. (1) HyperLadder™ 1kb (BIOLINE, 11-BIO-33053); (2) *V.parahaemolyticus* RIMD 2210633 *cydAB;* (4) *V.parahaemolyticus* RIMD 2210633 *cydB;* (3) and (5) negative controls……..**58**
Figure 26. Restricted plasmid vector pBBR1MCS-2 on a 1% agarose gel electrophoresis. Plasmid vector pBBR1MCS-2 was previously digested with *Xho*I and *BamH*I restriction enzymes (Roche applied Science). (1) HyperLadder™ 1kb (BIOLINE, 11-BIO-33053); (2) pBBR1MCS-2……..**58**
Figure 27. *V.parahaemolyticus* RIMD 2210633 *cydAB* and *cydB* gene, and plasmid vector pBBR1MCS-2 DNA purified fragments on a 1% agarose gel electrophoresis. (1) HyperLadder™ 1kb (BIOLINE, 11-BIO-33053); (2) *V.parahaemolyticus* RIMD 2210633 *cydAB;* (3) *V.parahaemolyticus* RIMD 2210633 *cydB;* (4) pBBR1MCS-2……**59**
Figure 28. **Amplification of *cydB* from the extracted plasmid after transformation of *E.coli* competent cells.** The 2401bp (*cydAB*) and 1008bp (*cydB*) fragments were amplified using the primers (*cydB* reverse and *cydB* forward) present on Table 4. (1) HyperLadder™ 1kb (BIOLINE, 11-BIO-33053); (2) – (4) 3 different colony PCR of *cydB E.coli* transformants; (5) negative control; (6) positive control……...**59**
Figure 29. Amplification of *cydAB* from the extracted plasmid after transformation of *E.coli* competent cells. The 2401bp (*cydAB*) and 1008bp (*cydB*) fragments were amplified using the primers (*cydA* forward and *cydB* reverse) present on Table 4. (1) HyperLadder™ 1kb (BIOLINE, 11-BIO-33053); (2)1 colony PCR of *cydAB E.coli* transformants; (3) negative control……..**59**

List of abbreviations

QS - Quorum-sensing

QSIs - Quorum-sensing interfering inhibitors

QQ – Quorum-quenching

HHQ - 2-heptyl-4-quinolone

PQS – 2-heptyl-3-hydroxy-4-quinolone / Pseudomonas quinolone signal

PqsR – Pqs Receptor

AHL – Acyl homoserine lactone

AIP – Autoinducing peptide

AI-2 – Autoinducer-2

OdDHL - N-(3-oxododecanoyl)-homoserine lactone

BHL - N-butyryl-homoserine lactone

IQS - 2-(2- hydroxyphenyl)-thiazole-4-carbaldehyde

2-ABA-CoA - 2-aminobenzoylacetyl-coA

CF – Cystic Fibrosis

BCCTs – Betaine/Carnitine/Choline transporters

TRAP – Tripartite ATP independent periplasmic transporters

CPS – Capsular polysaccharide

EPS – Exopolysaccharide

LPS – Lipopolysaccharide

BLIS – Bacteriocin-like inhibitory substance

SBP – Substrate-binding protein

OMPs – Outer membrane proteins

thd – Thermostable haemolysin

PAHs – Polycyclic aromatic hydrocarbons

SGP – Synthetic growth promoters

AGP – Antimicrobial growth promoters

HGT – Horizontal gene transfer

AQNOs - 2-alkyl-4-quinolone N-oxides

HAQs - 4-hydroxy-2-alkylquinolines

HCN – Hydrogen cyanide

CIO – Cyanide-insensitive oxidase

1. Introduction

1.1 Aquaculture importance

Fisheries and Aquaculture are important sectors to the nutrition and employment of millions of people around the world (FAO, 2018). In 2016, total fish production (fisheries and aquaculture included) reached an historical peak of 171 million tonnes (Figure 1), 88% of which were used for direct human consumption, with a record consumption of 20.3 kg per capita (FAO, 2018). This is mainly due to the acknowledgement that fish is an important source of high quality protein containing all essential amino acids, provides essential fats (e.g. long chain omega-3 fatty acids) and represents an important source of micronutrients including vitamins A, B and D and minerals (including calcium, iodine, zinc, iron and selenium) (FAO, 2016). Because fisheries have stagnated (Figure 1), aquaculture (representing 44% of total fish production in 2016) is crucial for providing the amount of fish the world is demanding, while allowing to reduce the percentage of wild fish stocks fished (FAO, 2018). Accordingly, aquaculture is nowadays the fastest growing food producing sector with a global production (including aquatic plants) of 110.2 million tonnes, and an estimated value of USD 243.5 billion (FAO, 2018).

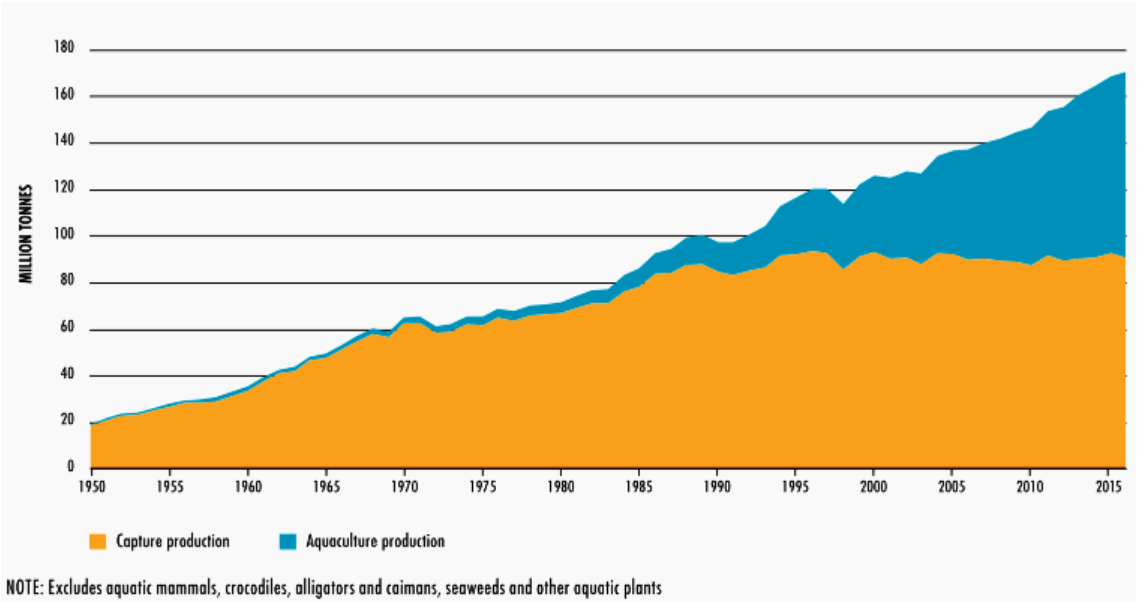


Figure 1. World capture fisheries and aquaculture (FAO, 2018).

1.1.1 Aquaculture development constraints: Fish diseases & human's health

In aquaculture systems, fish are constantly exposed to a series of factors (e.g., handling, water quality deterioration and confinement) that, if not controlled, can lead to

animal stress, cause behavior alteration, less acceptance of food and in the worst panorama, an increasing of fish susceptibility to infections (A.D. Pickering, 1993; Barton A. & Iwama K., 1991; Toranzo, Magariños, & Romalde, 2005). Disease outbreaks are responsible for important economic losses, with a global negative impact of 3 billion US$ per year (FAO, 2018). Bacterial diseases outbreaks are also associated to a misuse of antibiotics, creating selective pressure for emergence of drug resistant bacteria, rendering antibiotic treatments ineffective, and leaving residues in aquaculture products for human consumption, all serious threats for public health (Bruhn et al., 2005; Buschmann et al., 2012; Cabello, Godfrey, Buschmann, & Dölz, 2016; Gauthier, 2015; Hernroth & Baden, 2018). As an example, about 70% of the *Vibrio* strains isolated from aquaculture settings in Mexico are multidrug resistant (Molina-Ajaa, Almudena; Garcia-Gascab, Alejandra; Abreu-Groboisa, Alberto; Bolan-Mejíab, Carmen; Roque, Ana; Gomez-Gilb, 2006; Soumya Haldar, 2012). Also, bacterial virulence has a core importance in causing host diseases and is mainly correlated with the overcome of their defence mechanisms (Dallaire-Dufresne, Tanaka, Trudel, Lafaille, & Charette, 2014). For this reason, novel biocontrol strategies to enhance disease prevention would be essential.

Classic Vibriosis (*Vibrio anguillarum*), Fish Pasteurellosis (*Photobacterium damselae*), Tenacibaculosis (*Tenacibaculum maritimum*) and Furunculosis (*Aeromonas salmonicida*) are among the most common bacterial diseases known to affect important marine fish species, and which are all Gram-negative bacteria (Toranzo et al., 2005). Other bacterial species such as *Vibrio vulnificus*, *Vibrio parahaemolyticus*, *Edwardsiella tarda* and *Aeromonas hydrophila,* can also affect the aquaculture sector. Some of these species (e.g. *Vibrio* spp.) infect fish by penetrating into to the host tissue, and by releasing of extracellular products, such as hemolysins and proteases, which can cause disease symptoms (Soumya Haldar, 2012). Also, bacterial species belonging to the genera *Vibrio* and *Aeromonas* are able to regulate the expression of their virulence genes by using a molecule-based system called Quorum-Sensing (QS) (Chu, Jiang, Yongwang, & Zhu, 2011; de la Fuente et al., 2015). QS is an advantage to bacteria as it allows monitoring the cell population density, and acting as a community by expressing the most benefic phenotypes for the group (Reuter, Steinbach, & Helms, 2016). This mechanism is also interesting to explore alternative targets of combating infections, as its disruption could lead to a repression of virulence genes expression.

1.1.2 Vibrionaceae

The Vibrionaceae family includes seven genera of Gram-negative, facultative anaerobic bacteria that are mostly oxidase-positive, halophilic and motile. These include *Aliivibrio, Vibrio, Salinivibrio, Enterovibrio, Echinimonas, Grimontia* and *Photobacterium,* containing metabolically versatile species (Bier, Schwartz, Guerra, & Strauch, 2015; Garrity, G.M., Bell, J.A., Lilburn, 2005; L. H. Lee & Raghunath, 2018; Sawabe et al., 2013). For example, the *Vibrio* genera includes a total of 142 species of marine origin, a number that is constantly updated by new discoveries (Bier et al., 2015; Garrity, G.M., Bell, J.A., Lilburn, 2005; L. H. Lee & Raghunath, 2018; Sawabe et al., 2013).

Vibrio species inhabit riverine, estuarine and marine aquatic environments and are mainly free living, although some groups can create interactions with eukaryotic hosts and colonize humans, fish and marine invertebrates (Yildiz & Visick, 2009) as exemplified on Figure 2. Because of this, *Vibrio* species have a constant challenge and environmental pressure to adapt, having acquired several survival mechanisms (Reen et al., 2006). These species have a short replication time (less than 9 minutes e.g. *Vibrio natriegens*), contain two chromosomes in which the second one carries more species-specific genes and, carry different virulence factors such as the cholera toxin (CT), the thermostable direct haemolysin (TDH) and the capsular polysaccharide (CPS), all important for their survival, ecology, transmission and/or virulence (Ayiar, Gaal, & Gourse, 2002; Reen et al., 2006).

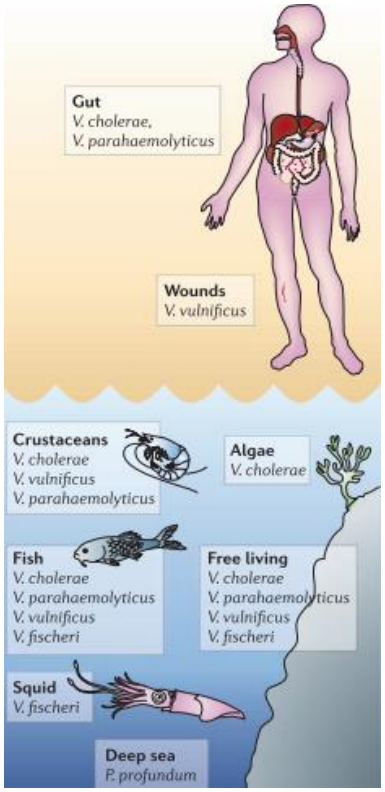


Figure 2. Niche specialization of members of Vibrionaceae family. Some members of this family can be found not only on the aquatic environment but can also colonize fish, marine invertebrates, be associated with plankton and algae, and infect humans (Reen et al., 2006).

Vibriosis, is one of the most prevalent disease in fishes and is widely responsible for mortality in cultured aquaculture systems worldwide. Pathogenic species include *V.harveyi*, *V.parahaemolyticus*, *V.alginolyticus*, *V.anguillarum*, *V.vulnificus*, and *V.splendidus* and can lead to intestinal necrosis, anemia, ascetic fluid, petechial hemorrhages in the muscle wall, liquid in the air bladder in fish and gill and hepatopancreas in the case of shrimps (Lavilla-Pitogo, Leaño, & Paner, 1998; Soumya Haldar, 2012).

V.anguillarum, V.harveyi, V.fischeri, V.parahaemolyticus, *V.vulnificus and V.cholerae* are normally associated with the marine environment. *V.parahaemolyticus, V.cholerae and V.vulnificus* can also be the source of foodborne diseases causing illnesses upon consumption of contaminated seafood or water (L. H. Lee & Raghunath, 2018; Letchumanan, Chan, & Lee, 2014). Human symptoms might vary from gastroenteritis and severe wound infections, to diarrheal disease cholera and fulminant septicaemia (Reen et al., 2006; Yildiz & Visick, 2009). However, there is a difference between the pathogenicity of these three species. *V.parahaemolyticus and V.cholerae* pathogenicity is defined by the presence of virulence factors (TDH and CT), while *V.vulnificus* pathogenicity is defined by the host susceptibility, being considered an opportunistic pathogen (Reen et al., 2006).

Vibrio species have also the capacity to form biofilms, an advantage as they become protected from many stress factors present in the marine environment (e.g. antibiotics, predation) (Chavez-Dozal et al., 2013; Yildiz & Visick, 2009). The capacity to form biofilm plays an important role also in pathogenicity and it is associated not only with flagella but also with extracellular matrix components [capsular polysaccharide (CPS) or exopolysaccharide (EPS), or VPS in *V.cholerae*] that allow bacteria within the biofilm to keep attached to the surface (Yildiz & Visick, 2009). Biofilm formation begins when the bacteria attaches to a surface and it is followed by the formation of microcolonies and three-dimensional (3-D) structures who can be associated with flagella motility (Yildiz & Visick, 2009). It appears that the genetic context of the cell and the type of surface it encounters can substantially influence the usage of a particular type of pili for attachment (Yildiz & Visick, 2009). The capacity of these bacterial species to create biofilms represents a huge problem to aquaculture since the actual antimicrobials have lack of efficacy against biofilm-producing bacterial fish pathogens (Sundell & Wiklund, 2011).

Aquatic bacteria, such as *Vibrio* species are constantly exposed to different environments, in which the osmotic stress is a daily challenge, and many bacteria mediate their response through the synthesis and uptake of compatible solutes (osmolytes) (Reen et al., 2006). Because of this, *V.cholerae*, *V.parahaemolyticus* and *V.fischeri* possess specialized systems previously shown to play an important role on the response to the osmotic stress. Examples include *betAD*, *proVWX*, *ectABC*, and *bcct* that are operons and genes that encode compatible solute systems (synthesis and uptake of compatible solutes) and contribute to protect the cells from changes in turgor pressure or outside pressure (Reen et al., 2006). Another environmental challenge is the scarcity of nutrients and these species (e.g. *V.cholerae)* evolved and acquire mechanisms to overcome this problem, by using Tripartite ATP-independent periplasmic (TRAP) transporters systems from the family of substrate-binding protein (SBP)-dependent secondary transporters that use electrochemical gradients across the membrane to collect low concentrations of nutrients in a more efficient way (Mulligan, Fischer, & Thomas, 2011; Mulligan, Leech, Kelly, & Thomas, 2012).

Although there is a lot of information related with this species, there are still mechanisms that remain poorly understood. Antimicrobials resistance and virulence factors are two of the most important reasons to further studying this species, as a better understanding, will allow to further develop new mechanisms to overcome these diseases with high impact on both human's health and aquaculture sector.

1.1.2.1 *Vibrio harveyi*

Vibrio harveyi, a bioluminescent bacterium is a serious pathogen of marine fish and invertebrates causing diseases such as vasculitis, gastroenteritis and eye lesions (Austin & Zhang, 2006) on different fish species including sole (*Solea senegalensis*) (Zorrilla et al., 2003) and Asian seabass (*Lates calcarifer*) (Ransangan, Lal, & Al-Harbi, 2012). This bacterial species is responsible for luminous vibriosis disease in shrimps (e.g., *Litopenaeus vannamei* and *Penaeus monodon*) leading to high mortalities (>50%) and potential devastation to diverse ranges of marine invertebrates over a wide geographical area infections (Letchumanan et al., 2016; Soumya Haldar, 2012). Also, this pathogen can be the cause of human infections (Letchumanan et al., 2016; Soumya Haldar, 2012). *V.harveyi* pathogenicity is associated to its variety of virulence mechanisms that include proteases, hemolysins, LPS, interaction with bacteriophage, bacteriocin-like inhibitory substance (BLIS), ability to attach and form biofilm and quorum-sensing system (Austin & Zhang, 2006).

1.1.2.2 *Vibrio parahaemolyticus*

Vibrio parahaemolyticus strains are involved in a wide variety of aquatic environments (riverine and marine coastal) and are exposed to fluctuations of several conditions as temperature, salinity, oxygen availability, plankton, tidal flushing and osmotic stress (Depaola et al., 2003; Naughton, Blumerman, Carlberg, & Boyd, 2009; Reen et al., 2006). Interestingly, previous research showed that the strain *V.parahaemolyticus* RIMD2210633 has a growth advantage from other *Vibrio* strains under different conditions e.g. fluctuations on saline environments, showing us an example of adaptive response of this organism (Naughton et al., 2009)

Also, *V.parahaemolyticus* is capable of causing fish diseases and human illness being related with food borne gastroenteritis or diarrhea (Letchumanan et al., 2015; Nair et al., 2007; Naughton et al., 2009). Often, human illness is associated with consumption of raw or undercooked seafood, that have toxigenic genes namely, thermostable direct hemolysin (*tdh*) and/or tdh-related (*trh*) hemolysin genes (Letchumanan et al., 2014; Raghunath, 2014).

1.1.2.3 *Vibrio vulnificus*

Vibrio vulnificus is present in estuarine and coastal waters and it is found especially in oysters and other molluscan shellfish (Oliver, 2015). This bacterium is the cause of the great majority of hospitalizations and deaths by *Vibrio* infections and it is associated with wound infections that occur mainly (95%) on victims that have one or more preexisting risk factors (e.g., immunocompromising conditions or chronic liver diseases resulting in elevated serum iron levels) carrying 50% of fatality rate (Bier et al., 2015; Letchumanan et al., 2016; Oliver, 2015).

1.1.2.4 *Vibrio cholerae*

Vibrio cholerae, a natural inhabitant of aquatic environments is the etiological agent of cholera, and so far, the most widely studied *Vibrio* species, being a well-known human pathogen consisting of more than 200 serogroups (Almagro-Moreno, Pruss, & Taylor, 2015; Lutz, Erken, Noorian, Sun, & McDougald, 2013; Reen et al., 2006). Its capacity to effectively colonize the intestine and the presence and release of virulence factors led to symptoms of severe watery diarrhea, vomiting, and dehydration (Bier et

al., 2015) after being transmitted to humans by consumption of water or food contaminated with virulent strains of *V.cholerae* O1 (Harris, LaRocque, Qadri, Ryan, & Calderwood, 2012).

1.1.2.5 *Vibrio anguillarum*

Vibrio anguillarum is an opportunistic fish pathogen, common to marine and estuarine environments and the cause of classic vibriosis, a deadly haemorrhagic septicaemia disease affecting 50 fresh and salt-water fish species, bivalves and crustaceans and being responsible in both aquaculture and larviculture for severe economic losses worldwide. It affects species of economic importance namely, Salmon (*Salmo salar* L.), rainbow trout (*Oncorhynchus mykiss* (Walbaum)), turbot (*Psettamaxima* L.), sea bass, sea bream (*Sparus aurata* L.); cod, eel, and ayu (*Plecoglossus altivelis)* (Buller, 2004; Frans et al., 2011; Toranzo et al., 2005; Yang & Sun, 2016).

1.1.2.6 *Vibrio fischeri*

Vibrio fischeri is an aquatic free-living bacterium having a symbiotic relationship with the Hawaiian bobtail squid *Euprymna scolopes* and it is an interesting natural model in the study of mutualistic animal-microbe relationships and in physiological and molecular signaling studies (Mandel, Stabb, & Ruby, 2008; Nyholm & Mcfall-ngai, 2004; Varilly & Chandler, 2012). This bacteria is present in the light organ of the squid allowing him to avoid predators by giving light and receiving protection and nutrients in return (Mandel et al., 2008; Nyholm & Mcfall-ngai, 2004). They also have the capacity to form biofilms which protects them from the abiotic factors and predation (Chavez-Dozal et al., 2013). Although, this *Vibrio* species is capable of colonizing the marine environment, it is not able to create human diseases and tough is not a pathogenic concern for human's health.

1.1.2.7 *Photobacterium damselae* subsp. *piscicida*

Photobacterium damselae subsp. *piscicida* is the etiological agent of fish Pasteurellosis, a halophilic bacterium affecting gilthead seabream, seabass and sole (*Solea senegalensis* and *Solea solea*) in the Mediterranean countries of Europe and

hybrid striped bass (*M. saxatilis* x *M. chrysops*) in the USA, causing economic losses in the marine culture (Magariños et al., 2001; Toranzo et al., 2005). This bacterium can create white nodules in the internal viscera, spleen and kidney (Magariños et al., 2001).

1.1.2.8 *Photobacterium damselae* subsp. *damselae*

Photobacterium damselae subsp. *damselae* is considered to be an emerging pathogen of importance in aquaculture by affecting wild fish, molluscs, and crustaceans as well as of fish species of economic importance causing wound infections and hemorrhagic septicemia e.g. turbot, *Psetta maxima*; (Fouz, Larsen, Nielsen, Barja, & Toranzo, 1992) and sea bream, *Sparus aurata*; (Vera, Navas, & Fouz, 1991) and being a concern for humans, as it is capable of causing fatal infections as necrotizing fasciitis (Rivas, Lemos, & Osorio, 2013; Terceti, Ogut, & Osorio, 2016).

1.1.3 *Aeromonas*

Members of the genus *Aeromonas,* belong to the Gammaproteobacteria class and are gram-negative, non-spore-forming, facultative anaerobic and chemoorganotrophic rod-shaped bacteria. *Aeromonas* spp. are present in many natural habitats such as soil or aquatic ecosystems (fresh and brackish water, sewage, and wastewater), healthy or diseased fish, food products, animals and human faeces (Beaz-Hidalgo & Figueras, 2013; Kühn et al., 1997). Some species are responsible for causing septicaemia, ulcerative and hemorrhagic fish diseases who lead to mortality in both wild and aquaculture systems (Beaz-Hidalgo & Figueras, 2013). Most of these bacteria are mesophilic, but there are also psychrophilic *A.salmonicida* strains (Beaz-Hidalgo & Figueras, 2013) .

Aeromonas spp. are opportunistic pathogens that possess specific virulence factors and mechanisms to invade the host (Beaz-Hidalgo & Figueras, 2013); examples of these factors and mechanisms include flagella, fimbriae, lipopolysaccharide (LPS), outer membrane proteins (OMPs), hemolysins, lipases and proteases, iron acquisition systems and quorum sensing (QS) (Beaz-Hidalgo & Figueras, 2013). These bacteria can also cause infections in humans by the exposure to contaminated water leading to wounds infections which can become systemic if not treated (Burr & Frey, 2007; Huddleston, Zak, & Jeter, 2006).

1.1.3.1 *Aeromonas salmonicida*

Aeromonas salmonicida subsp. *salmonicida* (considered the typical strain) is responsible for a fish disease called furunculosis that affects mainly salmonids (Burr & Frey, 2007). However, other marine and freshwater fish species can be affected by other subspecies of *A.salmonicida* such as *A.salmonicida* subsp. *achromogenes, A.salmonicida* subsp. *masoucida, A.salmonicida* subsp. *smithia* and *A.salmonicida* subsp. *pectinolytica,* that are called the atypical strains (Burr & Frey, 2007; T. & I., 1998) and whose disease may be manifested in a different way than the classical furunculosis (B. & D.A, 2007).

A.salmonicida subsp. *salmonicida* is associated with systemic infections (Burr & Frey, 2007) and not only to furunculosis but also other conditions as ulcerative dermatitis and ulcerations (B. & D.A, 2007). In fact, the term furunculosis is associated with the sub-acute or chronic form of the disease and it is recognised by the presence of furuncles or boils, in the skin and musculature (B. & D.A, 2007).

1.1.3.2 *Aeromonas hydrophila*

Aeromonas hydrophila is a ubiquitous bacterium of aquatic environments and a pathogen that causes disease (e.g. motile aeromonad septicemia) in a wide range of homeothermic and poikilothermic hosts due to its multifactorial virulence, namely adhesins (e.g. pili), S-layers, exotoxins such as hemolysins and enterotoxin, and a repertoire of exoenzymes which digest cellular components such as proteases, amylases, and lipases, some that might be regulated by quorum sensing (Cahill, 1990; Chu et al., 2011; Hänninen, Oivanen, & Hirvelä-Koski, 1997). This bacterium can also cause gastrointestinal and extraintestinal infections in humans, including septicemia, wound infections, gastroenteritis and peritonitis (Daskalov, 2006).

1.2 Antimicrobial drug resistance: facts and solutions

The intensive use of antimicrobials that is associated with the spread of antimicrobial-resistant bacteria (Buschmann et al., 2012), has acknowledge antimicrobial drug resistance as a serious global concern for the management of infectious diseases in humans, animals and plants (Roca et al., 2015). In fact, some bacterial species have evolved mechanisms to circumvent all currently known modes of action of antimicrobial

drugs, and there is today an urgent need for the development or discovery of new drugs with novel mechanisms of action (Roca et al., 2015; Sean et al., 2017).

There are some reasons why this phenome occurs and it can be explained with genetic, social and environmental factors. Genetic factors can be associated with the acquisition of specific genes by horizontal gene transfer (HGT) and by plasmids, viruses, integrons and transposons (Bengtsson-Palme, Kristiansson, & Larsson, 2018). As example of a genetic factor we have the appearance of genomic islands (Juhas et al., 2009). Genomic islands are portions of the genome that has evidence of horizontal gene transfer, common on bacterial genomes as they contribute to the diversification and adaptation of microorganisms and are beneficial to make genomes evolve under certain environmental conditions (Juhas et al., 2009). The presence of genomic islands in innumerous bacterial species connects us with the fact that sometimes species share the same environment niche, acquire mechanisms and therefore evolved a resistant phenotype by the existence of a constant environmental pressure to adapt to the presence of certain compounds.

The social factors are associated with the misuse and overuse of antibiotics that could led to antibiotic resistance, by wrong drug administration per example or the use antibiotics to treat infections or health problems that are not associated with bacterial infection such as virus or fungus (Vipin Chandr Kalia, 2015). Also, during several decades, antibiotics were used as antimicrobial growth promoters (AGP) for disease control, as feed additives, or as synthetic growth promoters (SGP) in different animal production sectors, including aquaculture (Gonzalez Ronquillo & Angeles Hernandez, 2017). The overuse of AGP lead to the spread of drug-resistant pathogens being its used banned in the UE (January 1, 2006) - Regulation 1831/2003/EC on additives for use in animal nutrition, replacing Directive 70/524/EEC on additives in feeding-stuffs.

Finally, the environmental factors are associated with the release of hospital, pharmaceutical industries, laboratories, agriculture, aquaculture and domestic residues that contain antibiotics, to the environment (Vipin Chandr Kalia, 2015).

To maintain its intensive productivity, the aquaculture sector uses high inputs of fish protein originating from by-catched fish from the sea that are employed for feeding, together with high levels of water exchange and massive use of antibiotics, which leads to the spread of antibiotic resistance from aquaculture settings to the natural environment (Soumya Haldar, 2012). The sector is constantly defeated by frequent, and sometimes

devastating, bacterial diseases, that lead to a misuse of antibiotics, creating selective pressure for emergence of drug resistant bacteria.

Over the years different solutions have been proposed, as the implementation of bacteriophages (that target specifically the pathogen), the inhibition of chromosome II replication in case of *Vibrio* species, the use of probiotics (e.g. administration of bacterial strains capable of producing inhibitory substances against pathogenic bacteria), and by applying anti-virulence therapies as Quorum-sensing inhibitors (QSIs) (Defoirdt, Sorgeloos, & Bossier, 2011; Vipin Chandr Kalia, 2015).

1.3 Marine bioactive compounds

More than 70% of the earth's surface is covered by oceans. The marine environment is unexploited when it comes to natural products and it can be considered a relevant source of natural compounds for a wide range of areas as human nutrition, animal feed (including aquaculture), biofertilizers, treatment of effluents and bioactive compounds that could be applied in the pharmaceutical and biotechnological areas for the production of next generation antimicrobials and having potential benefits for health (Reen, Romano, Dobson, & O'Gara, 2015; Rocha-Martin, Harrington, Dobson, & O'Gara, 2014).

The marine environment has unique physiological and chemical properties such as pH, pressure, temperature, osmolarity and uncommon functional groups such as isonitrile, dichloroimine, isocyanate, and halogenated functional groups that were previous identified, as also small bioactive compounds with therapeutic potential that are biosynthesized by marine organisms and their symbiotic microorganisms (Rocha-Martin et al., 2014). Also, the fact that marine microorganisms face constant variations and stress in the environment such as nutrient limitation, UV, temperature fluctuations, predation, viral infection and changes in salinity requires them to be equipped with a battery of adaptive responses to meet these challenges (Reen et al., 2006).

The vast repertoire of small molecules encoded in the marine ecosystem has already provided researchers with considerable success in introduction of new classes of therapeutics for clinical medicine, cosmetics and industrial products, being an example the identification of molecules that interfere with QS in pathogenic organisms (Dobretsov, Teplitski, & Paul, 2009; Reen, Romano, et al., 2015). However, the great part and much of what this natural ecosystem has to offer is still undiscovered, and sometimes hidden

from our screening methods, being their study a huge challenge that could led to great discoveries.

1.3.1 *Bacillus subtilis*

Bacillus subtilis is a gram-positive, spore producer bacteria that is generally recognized as safe (GRAS) by the Food and Drug Administration (FDA) and it is an important producer of a great number of secondary metabolites (Khochamit, Siripornadulsil, Sukon, & Siripornadulsil, 2015; Sumi, Yang, Yeo, & Hahm, 2015). Some of these secondary metabolites are antimicrobial peptides (polymyxins and lipopetide surfactin), bacteriocins (subtilosin) and lantibiotics (subtilin) (Phelan et al., 2013; Stein, 2005) and showed activity against *Vibrio* (B. Vaseeharan, 2003), and *Aeromonas* (Kong et al., 2017) strains. *B.subtilis* capacity to form highly resistant endospores, and to grow aerobically and anaerobically, allows this bacterium to survive adequately inside and outside humans, animals, and in aquaculture water systems as spores also facilitate species survival in competitive ecological niches (Olmos, 2014; Phelan et al., 2013).

As it is recognized as safe, the development studies of its secondary compounds would be interesting if their activity were able to interfere with important pathogenic bacteria mechanisms e.g. quorum-sensing systems and virulence factors. Previous studies have shown that *Bacillus* species can interfere with AHLs by producing lactonase enzymes, which inactivate AHLs by opening the lactone ring (Dong, Wang, & Zhang, 2007).

In the aquaculture sector *Bacillus* bacteria have been used as probiotics (Sadat Hoseini Madani, Adorian, Ghafari Farsani, & Hoseinifar, 2018) as they compete for nutrients and thus, inhibit the rapid growth of *Vibrio* and other bacteria, limiting their growth and transfer of resistant genes between bacteria (Hong, Le, & Cutting, 2005; Sadat Hoseini Madani et al., 2018). Some studies even have reported that this bacterium enhances the growth of tilapia (Günther & Jiménez-Montealegre, 2004), the survival and net production of channel catfish (Queiroz & Boyd, 1998) and the immune response of white shrimp (*Litopenaeus vannamei*) (K. Li et al., 2007). All these mechanisms and studies show us the potential of *Bacillus* species on targeting pathogenic species and their versality to be applied in different areas.

1.4 Quorum-sensing

Quorum-sensing (QS) is a molecule-based system that allows microbes to communicate and behave as a community through biosynthesis and perception of small

chemical signals (Marvin et al., 2017). These signalling molecules accumulate in the extracellular environment as the cellular population increases, eventually reaching a threshold (or quorum) and eliciting group multicellular behaviour (Reen, Gutiérrez-Barranquero, Dobson, Adams, & O'Gara, 2015). These signals interact with cognate receptors and induce the transcriptional expression of various target genes including those encoding several bacterial processes (J. Lee & Zhang, 2014). It is also important to note that QS it's not simply induced by high cell densities but it is a combined output of many factors such as diffusion rate and spatial (Cornforth et al., 2014; J. Lee & Zhang, 2014) that allows the population of bacteria to act in a coordinated manner (Vipin Chandr Kalia, 2015).

QS was firstly identified in the luminescent bacteria *V.fischeri* and *V.harveyi* and later found in other microbial organisms (e.g. *Agrobacterium tumefaciens*, *Pseudomonas aeruginosa),* being an important mediator of several physiological processes such as bioluminescence, biofilm formation, antimicrobial resistance, horizontal gene transfer and the production of virulence factors (Eberhard et al., 1981; Fuqua, Winans, & Greenberg, 1994); (Ng & Bassler, 2009); (Tergos & Eleftherios, 2012). As a result, it is not surprising that other bacterial pathogens may have also evolved similar mechanisms, to respond to the several stress and adverse conditions that they face daily in the environment (J. Lee & Zhang, 2014). Because of this, QS is attracting increased attention as a potential target for the discovery and development of novel antimicrobial therapies (Defoirdt, 2018) as of quorum sensing inhibitors (QSIs) (J. Lee & Zhang, 2014). Thus, this QSIs therapies could disarm the bacteria, by blocking the bacterial communication and making the pathogens ''blind'' allowing the infected immune system to clear the infection (Vipin Chandr Kalia, 2015; Y.-H. Li & Tian, 2012).

Three types of QS-systems can be distinguished:

i) The acyl homoserine lactone (AHL) QS-system for Gram-negative bacteria (Tergos & Eleftherios, 2012), often complemented with species specific systems such as the *Pseudomonas* quinolone signal (PQS) in *P.aeruginosa* (Reen, Gutiérrez-Barranquero, et al., 2015);
ii) The autoinducing peptide (AIP) QS-system in Gram-positive bacteria and;
iii) The autoinducer-2 (AI-2) QS-system in both Gram-negative and Gram-positive bacteria (Tergos & Eleftherios, 2012).

AHL–QS signaling is a key component of the biofilm mode of growth in many pathogens (Gutiérrez-Barranquero et al., 2017), and it is found in different and important

aquaculture pathogens such *as A.hydrophila, A.salmonicida, E.tarda, Yersinia ruckeri* or *V.anguillarum* (Natrah, Defoirdt, Sorgeloos, & Bossier, 2011). Therefore, molecules able to inhibit QS and by extension biofilm formation or other related virulence phenotypes, constitute promising weapons to fight these pathogens (Gutiérrez-Barranquero et al., 2017).

QS can be antagonized by i) inhibition of QS signal molecules biosynthesis, ii) mimicry of QS signal molecules using synthetic compounds as analogues, iii) degradation of QS signal molecules, disrupting the signal molecule dissemination (Quorum-Quenching or QQ) and iv) interference with the binding of the signal to the receptor protein (Chu & Mclean, 2016). Some of these strategies are described on Figure 3.

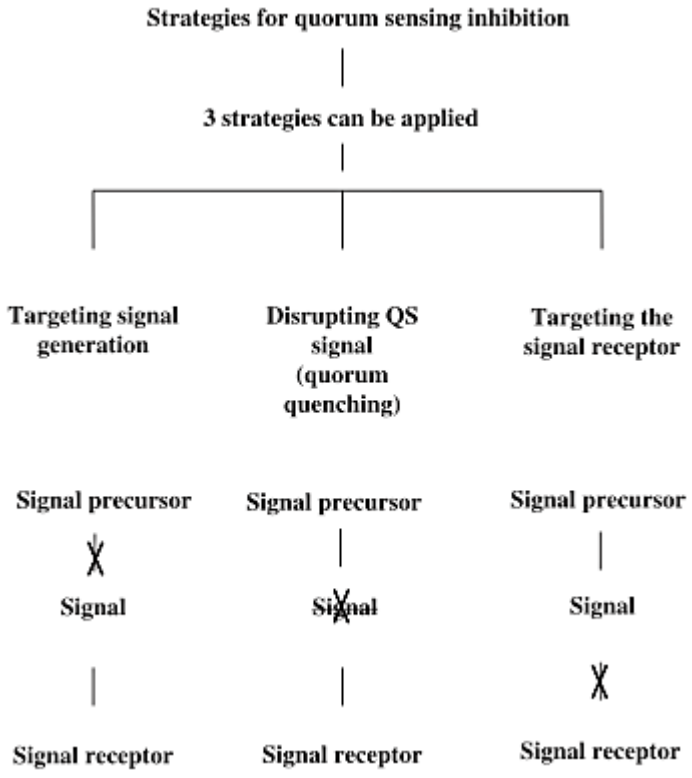


Figure 3. Strategies for inhibiting bacterial quorum-sensing (Chu & Mclean, 2016).

There are two major strategies for the control of bacterial infection, either to kill the organism or to attenuate its virulence such that it fails to adapt to the host environment and is readily cleared by the innate host defenses (Vipin Chandr Kalia, 2015). Thus, new ways to target and combat pathogenic bacteria have been proposed by affecting this QS signaling system.

1.5 *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative bacterium, usually being a commensal on the host and becoming an opportunistic pathogen mainly in immunocompromised immune-systems affecting cystic fibrosis patients, traumatized cornea, burns, Gustilo open fractures, long-term intubated patients and elderly individuals (J. Lee & Zhang, 2014).

This nosocomial bacterium is the main pathogen associated with mortality in Cystic Fibrosis (CF) patients (Lund-Palau et al., 2016; Park et al., 2004). It is a highly adaptable organism that has a full package of virulence mechanism (summarized on Table 1), responsible for their high ability of colonizing and survive in a wide variety of environments and conditions, allowing it to increase its infection and antibiotics resistance capability. In hospital context, this bacterium can resist in disinfected surfaces and in medical equipment and being transmissible from patient-to-patient (Moradali, Ghods, & Rehm, 2017; Russotto, Cortegiani, Raineri, & Giarratano, 2015).

Table 1. Summarized virulence mechanisms and respective regulated genes on *P.aeruginosa* (J. Lee & Zhang, 2014; Moradali et al., 2017).

Virulence mechanisms	Protein or Virulence factors	Regulated genes
Biofilm structure & dynamics	Alginate	Alginate operon (algD, alg8, alg44, algK, algE, algG,algX, algL, algI, algJ, algF, and algA) and algC
	Rhamnosyl-transferases (Rhamnolipids)	*rhlAB*
Immune evasion	Elastase (metalloprotease)	*lasB*
	Alkaline protease	*aprA*
Antibiotic resistance	Efflux pumps	*mexAB-oprM*, *mexXY/oprM* (*oprA*), *mexCD-oprJ*, and *mexEF-oprN*
Motility	Flagella	At least 41 genes clustered in three regions of the genome encode flagellin structural and regulatory components
	Type IV pili	pilM/N/O/P/Q and the fimU-pilVWXY1Y2E operons
Iron scavenging	Protease	*lasA*
	Siderophores (pyoverdine)	Large multimodular enzymes/ non-ribosomal peptide synthetases (NRPSs)
Cytotoxicity	Pyocyanin	*phzABCDEFG, phzM*
	HCN	*hcnABC*
	Exotoxin A	*toxA*

P.aeruginosa has a preference to grow in a microaerobic environment even under aerobic conditions (Arai, 2011; Sabra, Kim, & Zeng, 2002). Specifically during cystic fibrosis infection, *P.aeruginosa* is exposed to this conditions by producing rhamnolipid,

and exopolysaccharide alginate and by being exposed to reactive oxygen species released by host immune cells, that blocks the oxygen transfer and restricts the oxygen diffusion (Arai, 2011). Hydrogen cyanide, an important virulence factor and a respiratory chain inhibitor is produced at 300 mM concentrations at high cell densities and in biofilms which are characterized for being environments with less oxygen solved conditions (Arai, 2011; Williams, Zlosnik, & Ryall, 2007). Not only cyanide but also elastase (protease and exotoxin with tissue-damaging proteolytic activity) and pyocyanin (respiratory inhibitor) are enhanced by microaerobic conditions (Kon et al., 1999; Sabra et al., 2002; Voggu et al., 2006) showing us the impact of the creation of this environment by *P.aeruginosa* in the production of virulence factors. In fact, there are many environment conditions and stresses that the bacterium faces every day, being tough the type of virulence pathways activated dependent on the environment conditions.

Because of its mechanisms for adaptation, survival (Moradali et al., 2017) and the fact that very few antibiotics can inhibit this pathogen (Tergos & Eleftherios, 2012) the infection is hard to eradicate and thus there is today an urgent need to a more in-depth understanding and study of its pathways and regulatory mechanisms, that may hold the key to develop new mechanisms of action to control and prevent the bacterial infections.

1.5.1 Quorum-sensing systems

Previous research showed that quorum-sensing (QS), a cell-cell communication system and a concept that begun based on the prototype *luxI-luxR* system in *V.fischeri*, is correlated with the control of the expression of virulence factors in many species, being *P.aeruginosa* one of them (Fugère et al., 2014; J. Lee & Zhang, 2014). Thus, QS systems that regulate more than 10% of *P.aeruginosa* genes (Moradali et al., 2017) are responsible for the synthesis of extracellular molecules and other compounds.

In *P.aeruginosa* there are four QS systems, controlled by transcriptional regulators: LasR, RhlR, MvfR (PqsR) and IqsR (Dulcey et al., 2013; J. Lee & Zhang, 2014) with the respective QS signals OdDH, BHL, PQS and IQS (Figure 4).

Briefly, each signaling molecule accumulates in the extracellular environment at high population density, binds to its corresponding regulator, which activates the transcription of various downstream targets leading to a complex circuit of production of virulence factors (Dulcey et al., 2013; J. Lee & Zhang, 2014).

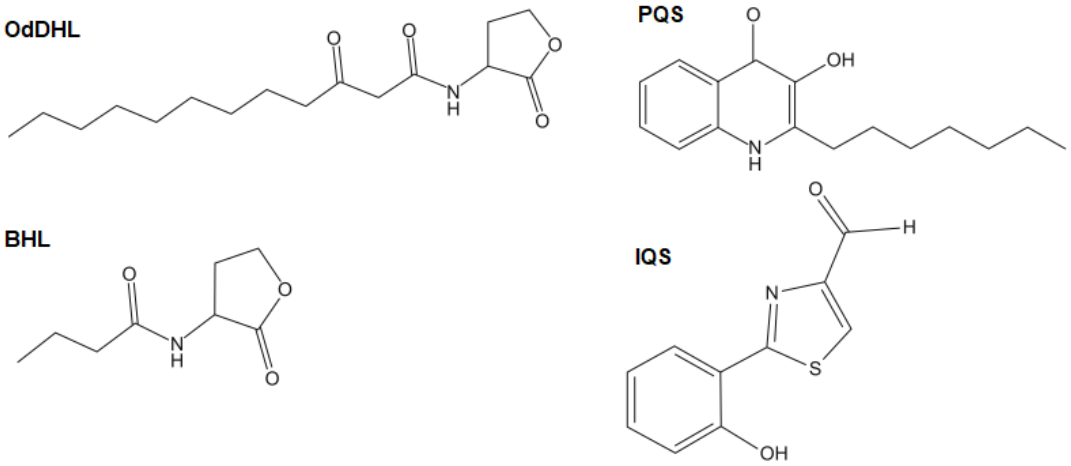


Figure 4. The four Quorum-sensing signalling molecules. From left to right: N-(3-oxododecanoyl)-homoserine lactone (OdDHL); N-butyryl-homoserine lactone (BHL); 2-heptyl-3-hydroxy-4-quinolone (PQS); 2-(2- hydroxyphenyl)-thiazole-4-carbaldehyde (IQS).

Although existing and interdependent relationship, there is also an important hierarchy that supports the function of these four QS systems. The *las* system is hierarchically on the top (J. Lee & Zhang, 2014) controlling, via a positive feedback loop, the transcription of *rhlR, rhlI, lasI* and virulence genes induced by LasR-OdDHL complex (Pesci et al., 1997). It also positively regulates PqsR (Xiao et al., 2006), controls IQS production (J. Lee et al., 2013) and controls the production of PQS that also positively influences BHL production by enhancing the transcription of *rhl* (Pesci et al., 1999). However, this sequence it's not always linear and exceptions may occur due to dependence on the environment and stress conditions that might trigger other responses.

The relevance of these QS signaling mechanisms in cystic fibrosis is clear, as PQS, HHQ, and the derivative HQNO, which are part of PqsR synthetized molecules, are often found in the sputum, bronchoalveolar fluid and mucopurulent fluid of patients of this disease (Collier et al., 2002).

1.5.2 Quinolone-based signaling

P.aeruginosa produces over 56 alkyl-quinolone derivatives, however mostly of their biological activities remains unknown (Szamosvári & Böttcher, 2017). From this 56 derivates, 16 of which belong to the class of 2-alkyl-4-quinolone N-oxides (AQNOs) and have various chain lengths and degrees of saturation. These are related with the 4-hydroxy-2-alkylquinolines (HAQs) quinolone quorum sensing signals, including 4-

hydroxy-2-heptylquinoline (HHQ) and 3,4-dihydroxy-2-heptylquinoline (PQS) used to regulate its virulence mechanisms (Dulcey et al., 2013; Szamosvári & Böttcher, 2017). Previous researchers find out that HAQs biosynthesis, requires the *pqsABCDE* (PA0996-PA1000) operon which is induced by MvfR (PqsR transcriptional regulator) after PQS or HHQ binding (Dulcey et al., 2013; Rampioni et al., 2016), although HHQ induces ~ 100-fold less than PQS (Xiao et al., 2006). The 2-heptyl-3-hydroxy-4-quinolone (PQS) signalling molecule, was discovered in 1999 by Pesci and co-workers and its chemically unique from the AHL previously described (Pesci et al., 1999).

PqsA (anthranilate-coenzyme A ligase) activates anthranilate to form anthraniloyl-coenzyme A (Coleman et al., 2008), then anthraniloyl-CoA and malonyl-CoA are used to the synthesis of 2-aminobenzoylacetyl-coA (2-ABA-CoA) by PqsD mediator, then 2-aminobenzoylacetate (2-ABA) is produced mediated by the thioesterase activity of PqsE (Dulcey et al., 2013; Rampioni et al., 2016). Finally, the condensation of octanoyl-coA and 2-ABA via the PqsBC heterodimer produces HHQ, the direct precursor of PQS (Dulcey et al., 2013; Rampioni et al., 2016). The *pqsH* (PA2587), a putative flavin-dependent monooxygenase and *pqsL* (PA4190) equally a monooxygenase are also required on this *pqs* system, the first one that oxidizes HHQ, in the presence of O_2 and NADH, producing PQS, and the second one for the synthesis of HQNO and related N-oxides (J. Lee & Zhang, 2014; Rampioni et al., 2016; Schertzer, Brown, & Whiteley, 2010). This AQ pathway is summarized on Figure 5.

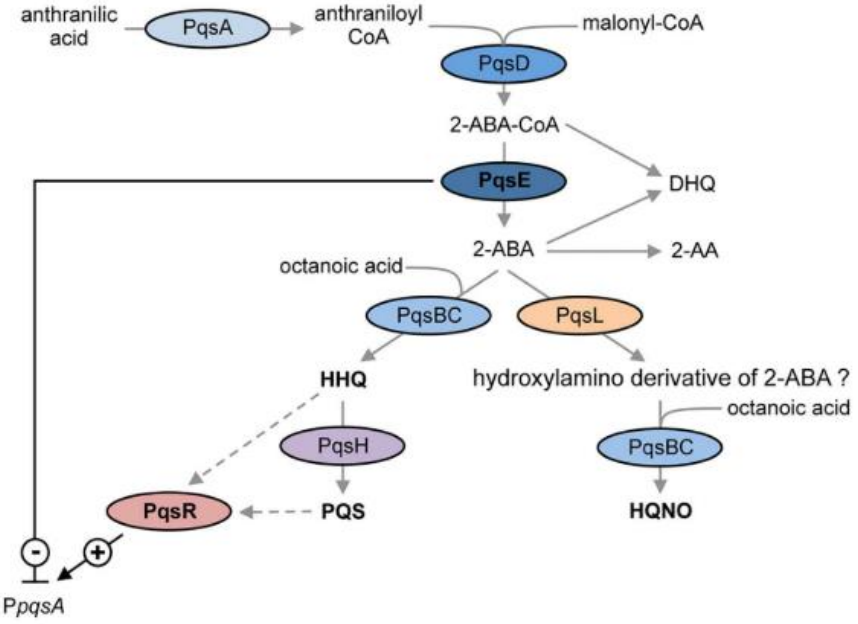


Figure 5. Schematic representation of the AQ biosynthetic pathway. Main elements of the *pqs* QS system (HHQ, PQS, HQNO, PqsE, and PqsR) are in bold. Solid grey arrows represent biosynthesis; dashed grey arrows represent information flow; solid black arrow indicates activation (+); black T-line indicates negative regulation (-) (Rampioni et al., 2016).

1.5.3 Oxygen deprivation, CIO and cytochromes

As previous stated *P.aeruginosa* has a preference to grow in microaerobic environments with less oxygen solved conditions (Arai, 2011; Sabra et al., 2002), being this tension a key factor on the biosynthesis of numerous virulence factors, as hydrogen cyanide (HCN), a respiratory chain inhibitor that leads to lethality of host organisms (Arai, 2011; J. Lee & Zhang, 2014; Ryall, Davies, Wilson, Shoemark, & Williams, 2008).

Additionally, the increase of these released compounds is connected not only with the creation of low oxygen environments but also with increased cell densities that elevates the expression of *hcnABC*, the regulator genes of HCN synthesis as shown on table 1 (Castric, 1994). As a matter of fact, the transition from exponential phase to stationary phase plays a major role in *P. aeruginosa*, as previously demonstrated in recent research on other species, namely on *E.coli,* which showed that low O_2 concentrations (stationary phase) increases the presence of compounds as cyanide and also leads to increased expression of specific membrane content (cytochrome bd) (Edalat, 2012), suggesting a connection between QS systems and oxygen deprivation.

ANR is the transcriptional regulator associated with bacterial anaerobic growth which is activated in low oxygen environments and is the key controller that acts in parallel with the QS systems (LasR-OdDHL or RhlR-BHL) for the expression of hydrogen cyanide biosynthesis genes *(hcnABC*) (J. Lee & Zhang, 2014; Pessi & Haas, 2000) being a fundamental co-regulator in anaerobic environments (Schuster & Greenberg, 2006).

As *P.aeruginosa* is exposed to some environments with scarcity of O_2, the aerobic terminal oxidases that have different levels of oxygen affinities are very important so this bacterium can deal with the low oxygen availability (e.g. Growth and biofilms).

The main terminal oxidases of this bacterium are the *aa3* oxidase (*aa3*), the *cbb3* oxidase 1 (*cbb3-1*), and *cbb3* oxidase 2 (*cbb3-2*) that use cytochromes as electron donors and *bo3* oxidase (*Cyo*) and cyanide-insensitive oxidase (CIO) that use ubiquinol as electron donor (quinol oxidases) (Arai, 2011; Cunningham & Williams, 1995; Jo, Price-Whelan, & Dietrich, 2014). To not be affected by the HCN respiratory chain inhibitor, *P. aeruginosa* has this CIO genes that protect the bacteria from this virulence factor. *CioA* and *CioB* genes (CIO) are homologous to subunits I and II of cytochrome bd-I from *E. coli* and it was proposed in previous researches that the slight differences in sequence and structure of the *CydB* subunit are responsible for cyanide resistance (Edalat, 2012).

Also, other quinolone based compounds produces by *P.aeruginosa* were demonstrated to inhibit other species as 2-heptyl-4-quinolone N-oxide (HQNO) and, 2-nonyl-4-quinolone N-oxide (NQNO), that from all AQNOs are the best well studied, demonstrated to moderately inhibit the growth of *S.aureus* and select for metabolically impaired small-colony variants on agar plates by blocking the electron transport chain through cytochrome b inhibition (Fugère et al., 2014; Hoffman et al., 2006; Szamosvári & Böttcher, 2017).

2. Material and Methods

2.1 Bacterial strains and growth conditions

Strains, media and growth conditions are described in Table 2. *Bacillus* strains, *Shigella sonnei and Edwardsiella tarda,* were cultured on Luria-Bertani (LB) medium [Tryptone 1% (w/v), yeast extract 0.5% (w/v), NaCl 0.5% (w/v), agar 1.5% (w/v)]. *Vibrio fischeri* was cultured on Luria-Bertani high-salt agar (LBS) [Tryptone 1% (w/v), yeast extract 0.5% (w/v), NaCl 2% (w/v), agar 1.5% (w/v) and 20mM Tris-HCl (pH 7.5)]. *Vibrio parahaemolyticus* strains from UCC collection were cultured on Luria-Bertani with 3% NaCl (LBS3) [Tryptone 1% (w/v), yeast extract 0.5% (w/v), NaCl 3% (w/v), agar 1.5% (w/v)]. Trypticase soy agar (TSA) [Casein peptone (pancreatic) 1.5% (w/v), NaCl 0.5% (w/v), soya peptone (papainic) 0.5% (w/v), agar 1.5% (w/v)] was routinely used to culture *Aeromonas hydrophila*, *Aeromonas veronii* and *Vibrio* 1.19. *Vibrio vulnificus*, *Vibrio anguillarum*, *V.parahaemolyticus* LMG 2850, *Vibrio harveyi, Vibrio litoralis, Vibrio scophthalmi, Vibrio cholerae* and *Photobacterium damselae* subsp. *damselae* were cultured on Marine media (MA) [Peptone 0.5% (w/v), yeast extract 0.1% (w/v), Ferric Citrate 0.01% (w/v), NaCl 1.945% (w/v), magnesium chloride 0.59% (w/v), magnesium sulphate 0.324% (w/v), calcium chloride 0.18% (w/v), potassium chloride 0.055% (w/v), sodium bicarbonate 0.016% (w/v), potassium bromide 0.008% (w/v), strontium chloride 0.0034% (w/v), boric acid 0.0022% (w/v), sodium silicate 0.0004% (w/v), sodium fluoride 0.00024% (w/v), ammonium nitrate 0.00016% (w/v), disodium phosphate 0.0008% (w/v) and agar 1.5% (w/v)]. *Vibrio gallaecicus* was cultured on Nutrient Agar with NaCl (NA 101) [Peptone 0.5% (w/v), Meat extract 0,3% (w/v), NaCl 3% (w/v), and agar 1.5% (w/v)]. *E.coli* biosensors AI1-QQ.1 and *E.coli* AI2-QQ.1 were cultured in LB, supplemented with 100 µg/ml Ampicillin and 30µg/ml Kanamycin.

2.2 Synthesis of HHQ analogues

HHQ synthetically created analogues were synthesized by incorporating main modifications on C-3, C-5, C-6, C-7 and C-8 positions on the anthranilate ring and on the alkyl chain length at position C-2. The synthesis of HHQ and other HHQ-based analogs (Reen et al., 2012, 2016; Reen, Shanahan, et al., 2015) was carried out via previously described methods. The molecular structure and carriers of the synthetically created analogues are represented on Table 3.

Table 2. List of strains and routine growth conditions used in this study.

Strain[1]	Description	Media[2]	Temperature (ºC)	Source
Aeromonas veronii	-	TSA	25	NUTRIMU Collection
Aeromonas hydrophila subsp. *hydrophila* LMG 2844	-	TSA	25	NUTRIMU Collection
Shigella sonnei LMG 10473	-	LB	37	NUTRIMU Collection
Edwadsiella tarda LMG 2793	-	LB	37	NUTRIMU Collection
AI1-QQ.1; *E. coli* DH5α/prS488	reporter strain to identify AHL-QQ compounds	LB	37	(Weiland-Brãuer, Pinnow, & Schmitz, 2015)
AI2-QQ.1; *E. coli* DH5α/pRS489	reporter strain to identify AI-2-QQ compounds	LB	37	(Weiland-Brãuer et al., 2015)
Vibrio parahaemolyticus				
LMG 2850	-	MA	25	NUTRIMU Collection
VP2	-	LBS3	30	UCC Collection
KEIO464	-	LBS3	30	UCC Collection
RIMB 210623	-	LBS3	30	UCC Collection
RIMD 2210633	-	LBS3	30	UCC Collection
RIMD 2210633 bet AD DAP MUTANT	mutant	LBS3	30	UCC Collection
Δest ABC double	mutant	LBS3	30	UCC Collection
ect AD double crossover	mutant	LBS3	30	UCC Collection
bact double crossover	mutant	LBS3	30	UCC Collection
ΔBCCT double crossover	mutant	LBS3	30	UCC Collection
Δprovwx	mutant	LBS3	30	UCC Collection
Δprovwx double crossover	mutant	LBS3	30	UCC Collection
pro- double crossover 1	mutant	LBS3	30	UCC Collection
Δect ABC	mutant	LBS3	30	UCC Collection
Vibrio cholerae				
O395	-	MA	30	UCC Collection
N16961	-	MA	30	UCC Collection
ΔTRAP N16961 2	mutant	MA	30	UCC Collection
ΔTRAP VC1777-VC1779 N16961 3	mutant	MA	30	UCC Collection
Vibrio fischeri				
WH1	-	LBS	23	UCC Collection
H905	-	LBS	23	UCC Collection
CG101	-	LBS	23	UCC Collection
ET101	-	LBS	23	UCC Collection
SR5	-	LBS	23	UCC Collection
SA1	-	LBS	23	UCC Collection
Vibrio 1.19	-	TSA	23	UCC Collection
Vibrio gallaecicus DSM 23502	-	NA 101	23	UCC Collection
Vibrio litoralis DSM 17657	-	MA	23	UCC Collection
Vibrio scophthalmi				
DSM 19140	-	MA	23	UCC Collection
DSM 19140 m1	Tolerant mutant	MA	23	This study
DSM 19140 m3	Tolerant mutant	MA	23	This study
DSM 19140 m4	Tolerant mutant	MA	23	This study

Table 2. List of strains and routine growth conditions used in this study (continuation).

Strain[1]	Description	Media[2]	Temperature (ºC)	Source
Vibrio anguillarum DSM 21597	-	MA	25	NUTRIMU Collection
Vibrio harveyi	-	MA	25	NUTRIMU Collection
Vibrio vulnificus LMG 13545	-	MA	25	NUTRIMU Collection
Photobacterium damselae subsp. *damselae* LMG 7892	-	MA	25	NUTRIMU Collection
Bacillus sp.				
FI87	Fish isolate	LB	37	NUTRIMU Collection
FI89	Fish isolate	LB	37	NUTRIMU Collection
FI92	Fish isolate	LB	37	NUTRIMU Collection
FI94	Fish isolate	LB	37	NUTRIMU Collection
FI99	Fish isolate	LB	37	NUTRIMU Collection
FI101	Fish isolate	LB	37	NUTRIMU Collection
FI105	Fish isolate	LB	37	NUTRIMU Collection
FI108	Fish isolate	LB	37	NUTRIMU Collection
FI109	Fish isolate	LB	37	NUTRIMU Collection
FI114	Fish isolate	LB	37	NUTRIMU Collection
FI120	Fish isolate	LB	37	NUTRIMU Collection
FI123	Fish isolate	LB	37	NUTRIMU Collection
FI132	Fish isolate	LB	37	NUTRIMU Collection
FI135	Fish isolate	LB	37	NUTRIMU Collection
FI136	Fish isolate	LB	37	NUTRIMU Collection
FI139	Fish isolate	LB	37	NUTRIMU Collection
FI140	Fish isolate	LB	37	NUTRIMU Collection
FI141	Fish isolate	LB	37	NUTRIMU Collection
FI142	Fish isolate	LB	37	NUTRIMU Collection
FI144	Fish isolate	LB	37	NUTRIMU Collection
FI152	Fish isolate	LB	37	NUTRIMU Collection
FI153	Fish isolate	LB	37	NUTRIMU Collection
FI157	Fish isolate	LB	37	NUTRIMU Collection
FI162	Fish isolate	LB	37	NUTRIMU Collection
FI164	Fish isolate	LB	37	NUTRIMU Collection
FI168	Fish isolate	LB	37	NUTRIMU Collection
FI187	Fish isolate	LB	37	NUTRIMU Collection
FI191	Fish isolate	LB	37	NUTRIMU Collection
FI226	Fish isolate	LB	37	NUTRIMU Collection
FI242	Fish isolate	LB	37	NUTRIMU Collection
FI268	Fish isolate	LB	37	NUTRIMU Collection
FI276	Fish isolate	LB	37	NUTRIMU Collection
FI300	Fish isolate	LB	37	NUTRIMU Collection
FI302	Fish isolate	LB	37	NUTRIMU Collection
FI304	Fish isolate	LB	37	NUTRIMU Collection
FI307	Fish isolate	LB	37	NUTRIMU Collection
FI314	Fish isolate	LB	37	NUTRIMU Collection

Table 2. List of strains and routine growth conditions used in this study (continuation).

Strain[1]	Description	Media[2]	Temperature (ºC)	Source
Bacillus sp.				
FI321	Fish isolate	LB	37	NUTRIMU Collection
FI324	Fish isolate	LB	37	NUTRIMU Collection
FI326	Fish isolate	LB	37	NUTRIMU Collection
FI330	Fish isolate	LB	37	NUTRIMU Collection
FI333	Fish isolate	LB	37	NUTRIMU Collection
FI335	Fish isolate	LB	37	NUTRIMU Collection
FI338	Fish isolate	LB	37	NUTRIMU Collection
FI342	Fish isolate	LB	37	NUTRIMU Collection
FI347	Fish isolate	LB	37	NUTRIMU Collection
FI348	Fish isolate	LB	37	NUTRIMU Collection
FI353	Fish isolate	LB	37	NUTRIMU Collection
FI354	Fish isolate	LB	37	NUTRIMU Collection
FI355	Fish isolate	LB	37	NUTRIMU Collection
FI359	Fish isolate	LB	37	NUTRIMU Collection
FI361	Fish isolate	LB	37	NUTRIMU Collection
FI367	Fish isolate	LB	37	NUTRIMU Collection
FI368	Fish isolate	LB	37	NUTRIMU Collection
FI373	Fish isolate	LB	37	NUTRIMU Collection
FI375	Fish isolate	LB	37	NUTRIMU Collection
FI376	Fish isolate	LB	37	NUTRIMU Collection
FI377	Fish isolate	LB	37	NUTRIMU Collection
FI378	Fish isolate	LB	37	NUTRIMU Collection
FI387	Fish isolate	LB	37	NUTRIMU Collection
FI390	Fish isolate	LB	37	NUTRIMU Collection
FI401	Fish isolate	LB	37	NUTRIMU Collection
FI424	Fish isolate	LB	37	NUTRIMU Collection
FI429	Fish isolate	LB	37	NUTRIMU Collection
FI436	Fish isolate	LB	37	NUTRIMU Collection
FI442	Fish isolate	LB	37	NUTRIMU Collection
FI480	Fish isolate	LB	37	NUTRIMU Collection

[1] LMG – Strains available from the Belgian Coordinated Collections of Microorganisms (BCCM); RIMD – Research Institute of Microbial Diseases; DSM – German Collection of Microorganisms and cell cultures.

[2] TSA - Trypticase soy agar; LB - Luria-Bertani medium; MA – Marine media; LBS3 - Luria-Bertani 3% NaCl; LBS - Luria-Bertani high-salt agar; NA 101 - Nutrient Agar with NaCl.

Table 3. Molecular structure and carriers of 2-heptyl-4-quinolone (HHQ) and analogues.

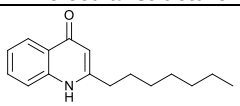
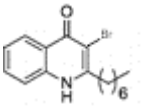
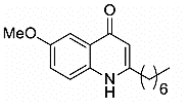
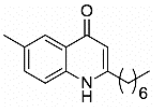
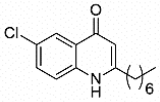
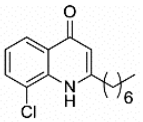
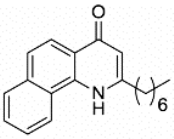
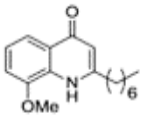
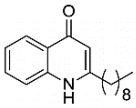
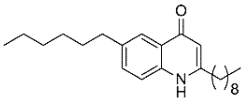
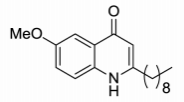
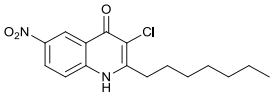
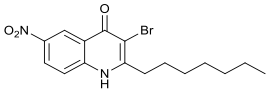
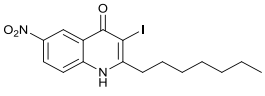
Compound	Molecular structure	Carriers
HHQ or *2-hptyl-4-quinolone*		MeOH
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A4		MeOH
A5		MeOH
A7		MeOH
A8		MeOH
A9		MeOH
A11		MeOH
A12		MeOH
A14		MeOH
A15		MeOH
B1		MeOH
B2		MeOH
B3		MeOH

Table 3. Molecular structure and carriers of 2-heptyl-4-quinolone (HHQ) and analogues (continuation).

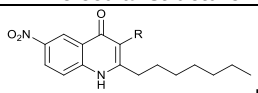
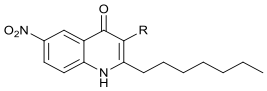
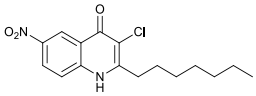
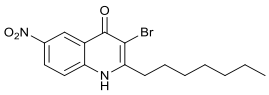
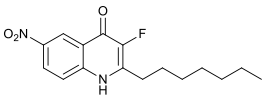
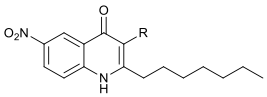
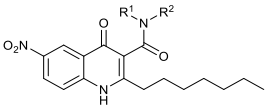
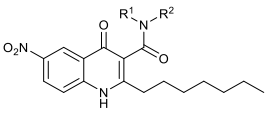
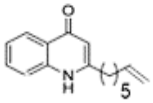
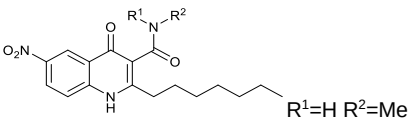
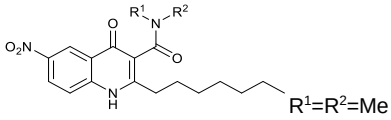
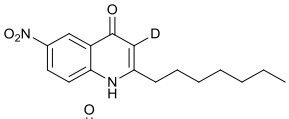
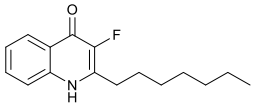
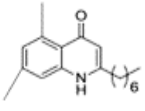
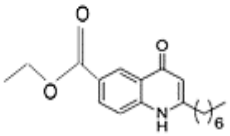
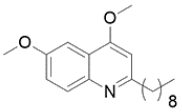
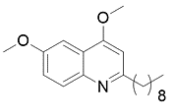
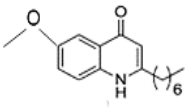
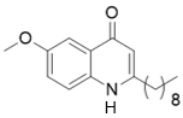
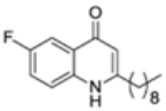
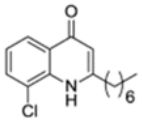
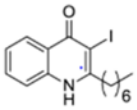
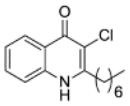
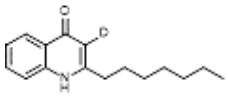
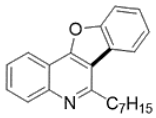
Compound	Molecular structure	Carriers
B4	 R=H	MeOH
B5	 R=Me	MeOH
C1		DMSO
C2		DMSO
C3		DMSO
C4	 R=Me	DMSO
D1	 R^1=R^2=H	MeOH
D2	 R^1=R^2=H	DMSO
D3		DMSO
D4	**Unccharacterized**	DMSO
D5	 R^1=H R^2=Me	DMSO
D6	 R^1=R^2=Me	DMSO
EI		MeOH
F1		DMSO
G1		MeOH

Table 3. Molecular structure and carriers of 2-heptyl-4-quinolone (HHQ) and analogues (continuation).

Compound	Molecular structure	Carriers
G2		MeOH
G3		MeOH
G4		MeOH
G5		MeOH
G6		MeOH
G7		MeOH
G8		MeOH
G9		MeOH
G10		MeOH
G11		MeOH
H1		MeOH

2.3 Quorum-quenching (QQ) screening (anti-QS assay)

The quorum-quenching (QQ) activity was screened in 67 *Bacillus* isolates (Table 2) from the bacterial collection of NUTRIMU lab that were previously identified and characterized as putative probiotics (PRO). Both cell-extracts and culture supernatants were screened to establish the intracellular or extracellular localization of the QQ compounds. Screening was achieved by using two *E.coli* biosensors (Weiland-Brãuer et al., 2015) previously established to identify novel biomolecules interfering with QS. One reporter strain, (AI2-QQ.1; *E.coli* DH5α/pRS489) detects autoinducer-2 (AI-2)-interfering activities, while the other (AI1-QQ.1; *E.coli* DH5α/pRS488) responds to interference with acyl-homoserine lactones (AHL) (Weiland-Brãuer et al., 2015), thus covering both QS systems of Gram-negative pathogens affecting aquaculture fish.

First, a validation test was done accordingly to Weiland-Brãuer et al., 2015. A pre-inoculum of AI1 and AI2 was prepared in falcons with 5mL LB medium supplemented with 100 µg/ml Ampicillin and 30µg/ml Kanamycin and incubated at 37°C overnight while shaking at 150 rpm. Next day, each indicator bacterial culture was diluted in fresh medium (OD_{600}~1.0), and spread with a cotton swab on a LB agar plate (supplemented with 100 µg/ml Ampicillin and 30µg/ml Kanamycin) in three different directions. Increasing concentrations of the autoinducers (100 µM and 10000 µM N-(Beta-Ketocaproyl)-L-homoserine lactone and 50mM, 100 mM, 250 mM, 500 mM and 5000 mM of 4-hydroxy-5methyl-3-furanone) were then applied in order to optimize final concentrations of signal molecules that completely inhibit the growth of the respective reporter strains (AI1-QQ.1 and AI2-QQ.1) (Figure 9). The solvents (ethyl acetate 50% and 100%) were also included to discard possible toxic effects on the reporter strains as it was used to prepare N-(Beta-Ketocaproyl)-L-homoserine lactone.

The cell extract and the culture supernatants were prepared following Weiland-Brãuer et al., 2015 with some modifications. Isolated spore formers were grown in 5ml LB overnight at 37ºC at 120 rpm. Cells were harvested by centrifugation at 7,000 g and the culture supernatant were subsequently filtered using 0.2µm sterile syringe filters (Whatman®). The filtered supernatants were stored at 4ºC. The cell extract was prepared from the cell pellet using a Bead Bug microtube homogenizer (Benchmark Scientific, Inc.). The cell pellet was suspended on 1000 µl [50 mM Tris-HCL (pH 8.00)] with addition of beads 425µm - 600µm (Sigma-Aldrich) for cellular disruption (30 seconds, 4,000 strokes/min) at room temperature (RT). After centrifugation 10,000 g and 4ºC for 45

minutes, the extracts were filtered using 0.2µm filter units. Supernatant and cell extracts were stored until 1 week at 4ºC.

The quorum-quenching assay was made in LB soft agar plates containing 0.8% agar supplemented with 100 µM N-(β-ketocaproyl)-L-homoserine lactone (3oxo-C6-HSL) (Sigma-Aldrich, Munich, Germany), 100 µg/ml ampicillin, 30µg/ml kanamycin, and 10% (vol/vol) of exponentially growing culture of the reporter strain AI1-QQ.1. LB agar plates were coated with the agar mixture. AI-2 quorum-quenching plates were prepared similarly, but the agar was supplemented with final concentrations of 100 mM 4-hydroxy-5methyl-3-furanone (Sigma-Aldrich, Munich, Germany), 100 µg/ml ampicillin, 30 µg/ml kanamycin, and 10% (vol/vol) exponentially growing culture of the reporter strain AI2-QQ.1. After 10 minutes 5 µl of the test substances (cell extracts/culture supernatants) were added followed by a 1-hour incubation at room temperature and overnight incubation at 37ºC. A positive result to QQ is visualized by growth of the respective reporter strain.

2.4 Anti-growth screening

All the *Bacillus* supernatants were tested for the anti-growth capacity against a panel of 9 important fish pathogens including *A.veronii*, *A.hydrophila*, *S.sonnei*, *E.tarda*, *V.anguillarum, V.vulnificus, V.harveyi*, *V.parahaemolyticus* and *P.damselae subsp.damselae*. Briefly, dilutions (OD_{600}~0.1) of each indicator bacterial culture, grown overnight at the ideal species requirements (Table 2), were spread with a cotton swab on the agar plate in three different directions. MA was used to grow *V.anguillarum, V.vulnificus, V.harveyi, V.parahaemolyticus* and *P.damselae subsp damselae*, LB medium to *S.sonnei* and *E.tarda*, and TSA medium to *A.veronii* and *A.hydrophila*. After the swab, 9 mm diameter wells were punched in the agar and filled with 100 µL of each culture supernatant previously prepared. The plates were incubated in an inverted position, at 25ºC to MA and TSA plates and 37ºC to LB plates. Zones of growth inhibition around the wells were considered as positive result.

The HHQ and its synthetic analogues were tested for their capacity to inhibit growth of several species of Vibrionaceae family. First, each bacterial strain from UCC collection (that include strains belonging to *V.parahaemolyticus, V.fischeri*, *V.litoralis, V.scophthalmi, V.gallaecicus, V.cholerae, V.* 1.19 and *V.anguillarum species;* Table 2) was tested by streaking in the respective culture media supplemented with 10µM HHQ at the ideal temperature conditions. The positive control was considered the bacteria

cultured on normal media without HHQ supplementation. All the assays were done in duplicate. Prior to the assay, the ideal incubation time was established for each bacterial strain (due to their different growth rates). Thus, in each assay, *V.parahaemolyticus*, *V.gallaecicus* and *V.cholerae* were incubated overnight, *V.fischeri*, *V.litoralis* and *V.anguillarum* were incubated for 24 hours and *V.scophthalmi* and *Vibrio* 1.19 were incubated for 48 hours. This assay was done by using fresh grown cultures and by picking a single and isolated colony to each testing plate.

Adding to the previous assay, a second inhibition test of HHQ and its synthetic analogues was done in 96-well flat-bottomed polystyrene plates. Overnight (~16 hours) pre-inoculum liquid cultures of each bacterial culture (*V.parahaemolyticus, V.fischeri*, *V.litoralis, V.scophthalmi, V.gallaecicus* and *V.* 1.19*)*, were made in 5ml of the respective media by inoculating a single and isolated colony from fresh grown cultures followed by incubation at the ideal temperature conditions (Table 2) at 150 rpm, overnight. The wells were filled with 100 µl total volume of indicator bacteria (OD_{600} ~0.05) with 1 µl HHQ with a final concentration of 10 µM HHQ. There were two positive controls, 100 µl of indicator bacteria (OD_{600} ~0.05) and 100 µl of indicator bacteria (OD_{600} ~0.05) with 1 µl addition of MeOH or DMSO (HHQ and analogues carriers) making a dilution of 1/100 and a final percentage of 1%. The negative control was only filled with 100µl of the respective media. The plates were incubated overnight at the ideal temperature for maximum bacterial growth according to species requirements (Table 2) without shaking. Optical density was measured at Absorbance of 600nm in a Multitaskan™ GO Microplate Spectrophotometer (ThermoFischer Scientific, Inc.) to establish the antimicrobial activity in the presence of HHQ and its analogues. To test the HHQ synthetically created analogues the same procedure was applied, changing the supplementation of HHQ by the respective compound. All the assays were done in triplicate (technical and biological replicates).

2.5 Anti-Biofilm assay

Quantification of biofilm formation in the presence or absence of HHQ and its analogues, was made according to Han, Mizan, Jahid, & Ha, 2017 with some modifications. The 96 well plates tested in the growth assay were washed once with deionized water (by gently pipetting up and down with a micropipette) to clean planktonic cells, after the bacterial cultures content had been discarded. The plates were dried and stained with 125 µl of 0.1% (w/v) crystal violet dye (CV; Sigma-Aldrich, St. Louis, MO,

USA) for 45 minutes at room temperature, followed by washing by submersion in water and then air-dried again for 1 hour. The adherent CV was solubilized using 125 µl of 95% (v/v) ethanol at room temperature for 10 minutes and the absorbance was read at 595nm using a Multitaskan™ GO Microplate Spectrophotometer (ThermoFischer Scientific, Inc.). All the assays were done in triplicate (technical and biological replicates).

2.6 Time killing curve for *Vibrio scophthalmi*

Overnight (~16 hours) pre-inoculum liquid cultures of *V.scophthalmi* DSM 19140 and *V.scophthalmi* DSM 19140 m1 (Table 2), were made in 20 ml of MA Broth by inoculating a single and isolated colony from fresh grown cultures followed by incubation at 23 ºC and 150 rpm overnight. Both overnight liquid cultures were subsequently diluted (OD_{600} ~0.05) in MA Broth supplemented with 10 µM HHQ on a final volume of 75 ml. The cultures were incubated at 23ºC while shaking at 150 rpm. Optical density measures at Absorbance of 600nm were taken for 52 hours period on a Multitaskan™ GO Microplate Spectrophotometer (Thermo Fischer Scientific, Inc.). The positive control was considered the bacteria cultured without HHQ supplementation. The assay was done in duplicate (biological replicates).

2.7 Cytochrome Oxidase testing

The cytochrome oxidase activity test was used as a standard assay and as control to detect oxidase negative bacterial laboratory contaminations, as all the members of the *Vibrionaceae* family are oxidase positive. This is a quick test that reveals the presence of the enzyme cytochrome oxidase, when the N, N-dimethyl-p-phenylenediamine and alpha-naphthol saturated in the paper react to indophenol blue. The test was done by picking colonies with a sterile loop followed by smear in the diagnostic paper, with results observed within 60 seconds (Sigma-Aldrich, 2013). The development of dark blue or black colour was considered a positive result while a negative result was represented by the absence of colour (Sigma-Aldrich, 2013).

2.8 16S rRNA gene amplification by PCR

Oligonucleotide primers 63F and the 1387R were used to amplify 16 rRNA gene (Table 4). These primers are specific for amplification of 16S rRNA genes from bacteria and provide gene amplification from a wider range of bacteria than other 16S rRNA primers (Marchesi et al., 1998). PCR reaction mixture contained 25 µl GoTaq Green Master Mix (GoTaq DNA Polymerase, 2X Green GoTaq Reaction Buffer, dATP, dGTP, dCTP, dTTP and MgCl), 2 µl of each primer (10pmol), 1 µl DNA Template and ddH_2O on a total of 50 µl. PCR amplification was performed under the following conditions: 5 minutes initial denaturation at 94ºC, 45 seconds denaturation step at 94ºC, 45 seconds in the annealing step at 55ºC, 90 seconds extension at 72ºC followed by 10 minutes final extension step at 72ºC. The resulting PCR product was extended for 30 cycles. A negative control with no template DNA was also included. PCR products were analysed by electrophoresis on a 1% agarose gel (Figure 21), purified using the QIAquick PCR purification kit (QIAGEN) and sent for sequencing, for later nucleotide sequence alignment and phylogenetic analysis.

Table 4. Oligonucleotide primers used in this study.

Primers	**Primer sequence (5'-3') ***	**Reference (s)**
cydAF - *cydA* forward (*Xho*I)	CCGCTCGAGCGGTATAGTCACTGTCTGTAC	This study
CydBF - *cydB* forward (*Xho*I)	CCGCTCGAGCGGGCATCTAGAATTTGTCAC	This study
CydBR - *cydB* reverse (*BamH*I)	CGGGATCCCGCGTTAATGGTACGCTTCG	This study
63F	CAGGCCTAACACATGCAAGTC	(Marchesi et al., 1998)
1387R	GGGCGGWGTGTACAAGGC	(Marchesi et al., 1998)

*Underlined are the sites for XhoI and BamHI restriction enzymes.

2.9 *cydAB* and *cydB* genes amplification by PCR

cydAB operon and *cydB* gene alone, were amplified using oligonucleotide primers described in Table 4 and illustrated on Figure 6. All primers (*cydA* forward, *cydB* reverse; cydB forward) were designed based on the nucleotide sequence data of *cydAB* (size 2401bp) and *cydB* (size 1008bp) obtained from Gene Bank (https://www.ncbi.nlm.nih.gov/genbank/).

For *cydAB* and *cydB* detection, the PCR reaction mixture contained 10 µl GoTaq Green Master Mix 2x (GoTaq DNA Polymerase, 2X Green GoTaq Reaction Buffer, dATP, dGTP, dCTP, dTTP and MgCl)1 µl of each primer, 1 µl DNA Template and ddH_2O on a total of 20 µl. PCR conditions were as follows: 10 minutes initial denaturation at

96ºC, 30 seconds denaturation step at 95ºC, 30 seconds in three different annealing temperatures (53ºC, 55ºC and 58ºC to determine the ideal annealing temperature for both *cydB* and *cydAB*), 1 minute extension to *cydB* and 2 minutes and 30 seconds to *cydAB,* both at 72ºC, followed by 10 minutes final extension step at 72ºC. The resulting PCR product was extended for 30 cycles. A negative control with no template DNA was also included. The PCR products were analysed by electrophoresis on a 1% agarose gel (Figure 24).

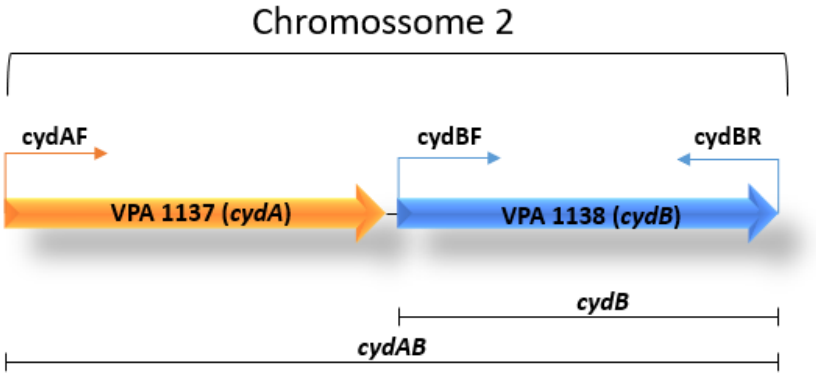


Figure 6. Schematic representation of the *V.parahaemolyticus* RIMD 2210633 cytochrome d oxidase *(cydAB)* operon located on chromosome 2 and the respective PCR products obtained in this study. Operon *cydAB* (VPA1137-VPA1138) and gene *cydB* (VPA1138) were amplified in this study resulting on fragments sizes of 2401bp (*cydAB*) and 1008bp (*cydB*). The primers (Table 4) used are also included in this figure: *cydA* forward (cydAF), cydB forward (cydBF) and *cydB* reverse (cyBR).

For *cydAB* and *cydB* cloning, the Q5™ High fidelity PCR Kit (New England BioLabs) was used to a more precise and robust performance with less error rates on DNA amplification, as it has an error rate > 50-fold lower than Taq DNA Polymerase. The PCR reaction mixture contained 10 µl 5x Q5 reaction Buffer, 1 µl dNTPs (10mM), 2.5 µl of each primer (10 pmol), 2.5 µl DNA Template, 0.5 µl Q5 high-fidelity DNA polymerase and ddH_2O on a total of 50 µl. PCR amplification was performed under the following conditions: 30 seconds initial denaturation at 98ºC, 10 seconds denaturation step at 98ºC, 30 seconds in the annealing step at 53ºC to *cydB* and 50ºC to *cydAB*, 1.30 minutes extension step to *cydB* and 2 minutes extension step to *cydAB* both 72ºC followed by 2 minutes final extension step at 72ºC. The resulting PCR product was extended for 30 cycles. A negative control with no template DNA was also included. PCR products were resolved by electrophoresis (Figure 25) on a 1% agarose gel and purified using the QIAquick PCR purification kit (QIAGEN) and stored at -20ºC.

2.10 *cydAB* and *cydB* cloning

A volume of 28 µl of *cydAB* and *cydB* PCR fragments, previously amplified with Q5™ High fidelity enzyme, were digested at 37ºC overnight, with 1 µl of *Xho*I and *BamH*I restriction enzymes (Roche applied Science), in the presence of 5 µl 1X Buffer B (Thermo Scientific™) and 15 µl Nuclease Free water (Invitrogen™) in a total of 50 µl. The digested DNA fragments were run on a 1% gel to check the size and digestion and, later purified using the QIAquick PCR purification kit (QIAGEN).

Simultaneously, 43 µl of plasmid vector pBBR1MCS-2 (Figure 7) was also digested with the same enzymes (1µl of each), in the presence of 5µl 1X Buffer B (Thermo Scientific™) in a total of 50 µl. The plasmid was physically separated by a 1% agarose gel electrophoresis (Figure 26), and cut under UV light followed by extraction from gel slices using the QIAquick gel extraction kit (QIAGEN). All the final DNA purified fragments were checked on a 1% agarose gel (Figure 27).

Ligation of *cydAB* and *cydB* fragments to pBBR1MCS-2 was performed with T4 DNA ligase (New England BioLabs). To *cydAB* a 12 µl mixture of 0.5 µl of T4 DNA ligase (New England BioLabs), 1.2 µl 10x ligase Buffer (New England BioLabs), 6.3 µl plasmid and 4 µl *cydAB*) was incubated at 16ºC, overnight. To *cydB* a 12 µl mixture of 0.5 µl of T4 DNA ligase (New England BioLabs), 1.2 µl 10x ligase Buffer (New England BioLabs), 9.3 µl plasmid and 1 µl *cydB* was incubated at 16ºC, overnight. A vector self-ligation control was also prepared in which the ligation reaction didn't contain *cydAB* and *cydB* fragments. The ligated fragments were subsequently used to transform *E.coli* CH3-Blue competent cells (BIOLINE). Briefly, 4 µl of both ligated and non-ligated plasmids were incubated for 20 minutes on ice with 50 µl of competent cells. The commercial plasmid vector pUC19 (New England Biolabs, Beverly, MA, USA) was used as an internal positive control of transformation efficiency. Plasmid-carrying colonies were isolated on selective media (LB supplemented with 50µg/ml Kanamycin) by incubation at 37ºC, overnight.

All colonies obtained were submitted to a colony screening PCR (Figure 28 and 29), using taq DNA polymerase and the same PCR conditions previously described for *cydAB* and *cydB* detection, to check for correct *cydAB* and *cydB* cloning. A positive transformant was used for plasmid extraction using QIAprep Spin Miniprep kit (QIAGEN). New plasmid was named pAR1 and visualized on a 1% agarose gel by electrophoresis.

2.11 Plasmid pBBR1MCS-2 preparation

For cloning purposes, the *Escherichia coli* DH5α containing plasmid vector pBBR1MCS-2 (Figure 7) was cultured overnight at 37ºC, in 5 ml of LB supplemented with 50µg/ml Kanamycin, with shaking at 150 rpm. Inoculum was prepared from a single fresh colony previously grown in agar medium under the same conditions. QIAprep Spin Miniprep kit (QIAGEN) was used for plasmid extraction. Plasmid DNA purity was determined to be ~1.8 (260/280 ratio measured on NanoDrop Spectrophotometer) and stored at -20ºC.

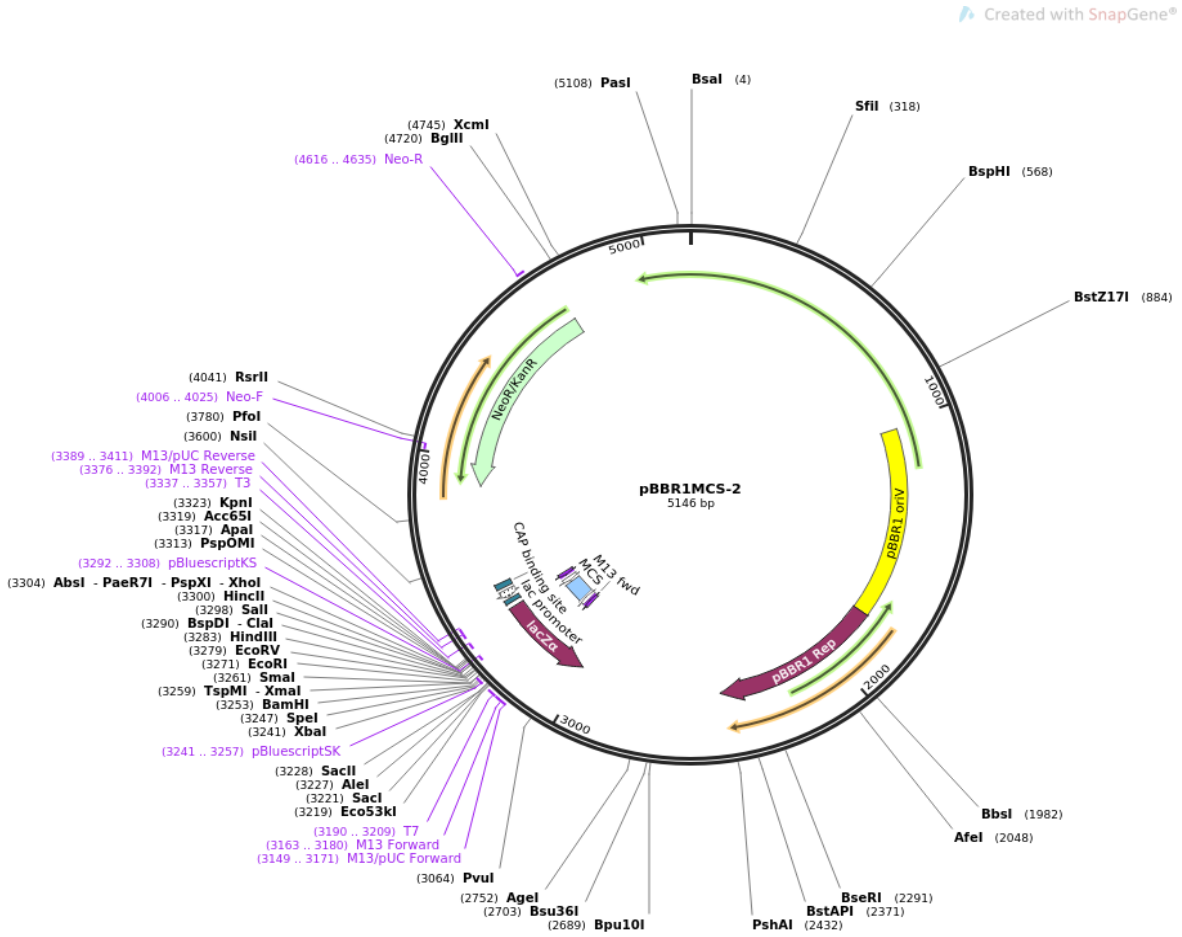


Figure 7. pBBR1MCS-2 plasmid vector used in the study for cloning purposes.

2.12 Comparative genomics

A BLAST analysis at the NCBI website (https://www.ncbi.nlm.nih.gov/) was done to identify sequence homology of *cydA* and *cydB* genes present in *V.parahaemolyticus* RIMD 2210633 with other *Vibrio* species (from UCC collection), with *P.aeruginosa* and

three nonpathogenic *Staphylococci* species (*Staphylococcus carnosus, S.piscifermentans*, and *S.gallinarum*). BLAST analysis was also used to understand the amplitude of genes absent on other *Vibrio* species that are only present in *V.parahaemolyticus* RIMD 2210633 chromosome 2.

2.13 Statistical Analysis

Data presentation was performed using GraphPad Prism 6 and Excel. All values were expressed as the mean ± standard deviation (SD) The results were statistically analysed with one-way analysis of variance (ANOVA) followed by Dunnett's multiple comparison test with the SPSS software (version 25.0). Differences between experimental groups were considered significant at $P < 0.05$ significance level.

3. Results

3.1 *Bacillus* isolates from the gut of aquaculture fish produce extracellular molecules active against several fish pathogens

3.1.1 Fish gut *Bacillus* isolates have antimicrobial activity

The cell-free supernatants of 67 fish-gut *Bacillus* isolates from the NUTRIMU collection of putative *Bacillus* probiotics, isolated from the gut of several aquaculture species were tested for their antimicrobial activity. The test was done against a panel of the 9 most important aquaculture fish pathogens (including *V.harveyi*, *V.anguillarum* DSM 21597*, V.vulnificus* LMG 13545*, V.parahaemolyticus* LMG 2850*, P.damselae* subsp. *damselae* LMG 7892*, A.veronii, A.hydrophila* subsp. *hydrophila* LMG 2844*, S.sonnei* LMG 10473*, E.tarda* LMG 2793, Table 2) to understand their capacity to inhibit these species and thus provide new strategies of fighting fish diseases.

As shown on Figure 8, members of Vibrionaceae family were the most affected by *Bacillus* cell-free supernatants. The supernatant of the strain FI314 showed antimicrobial activity against six species, five of them members of Vibrionaceae*,* including *V.harveyi, V.anguillarum, V.vulnificus, V.parahaemolyticus, P.damselae* subsp. *damselae* and a member of Enterobacteriaceae family (*S.sonnei*)*.* Strain FI330 also showed antimicrobial activity against the five members of Vibrionaceae but had no effect against *S.sonnei*. Strain FI442 showed antimicrobial activity against members of Vibrionaceae, namely *V.anguillarum, V.vulnificus,* and *P.damselae* subsp. *damselae.* Strain FI333 showed antimicrobial activity against *V.anguillarum* and *E.tarda.* Other *Bacillus* strains showed antimicrobial activity against just one pathogen, namely: FI135 and FI368 against *V.parahaemolyticus,* FI162 against *V.harveyi,* FI321 and FI354 against *V.anguillarum,* FI335 against *E.tarda*, and FI376 against *P.damselae* subsp. *damselae*. None of the *Aeromonas* (*A.veronii* and *A.hydrophila*) strains tested were inhibited by the *Bacillus* supernatants.

The diameter (mm) of each inhibition zone is presented in Table 5. As the diameter of the wells were 9 mm, only inhibition zones equal or superior to 10 mm values were considered positive. FI314, FI330 and FI442 showed the higher inhibition zones targeting members of the Vibrionaceae family (Table 5).

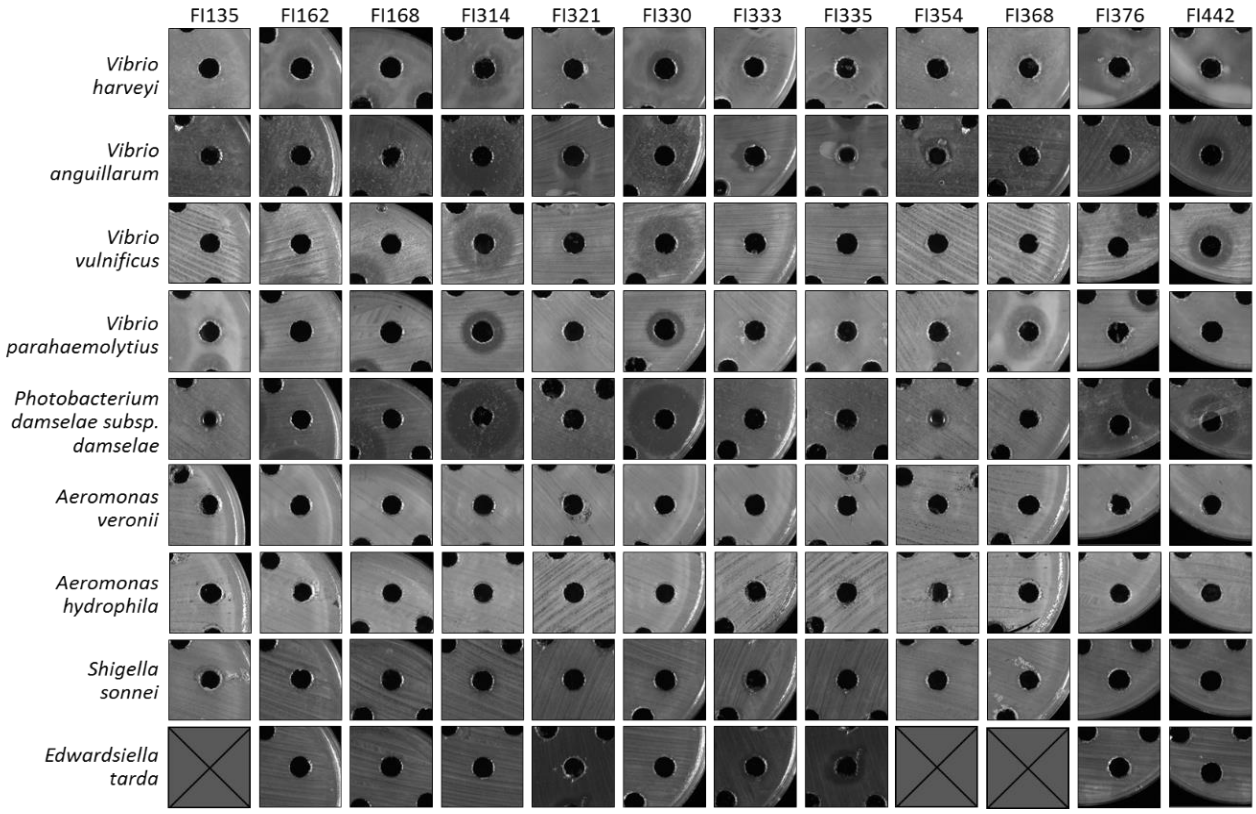


Figure 8. Formation of growth inhibition zones for the indicated pathogenic strains (in the left panel) by cell-free supernatant of the spore-forming isolates (on top of the panel), both from NUTRIMU collection. Dilutions of each pathogenic strain grown overnight at the ideal species requirements (Table 2) were spread on the agar plates. Each well was filled with 100 µl cell-free supernatant of the respective spore-forming isolate and the plates were incubated at the ideal pathogenic strain requirements (Table 2). The halos mean a positive result for inhibition. All photos are at the same scale.

Table 5. Anti-growth diameter of inhibition zones (mm) (Fish isolates against a panel of important fish pathogens).

Pathogenic	**Fish isolates**										
strains	**FI135**	**FI162**	**FI314**	**FI321**	**FI330**	**FI333**	**FI335**	**FI354**	**FI368**	**FI376**	**FI442**
V. harveyi	-	19	16	-	16	-	-	-	-	-	-
V. anguillarum	-	-	30	/	26	/	-	/	-	-	17
V. vulnificus	-	-	25	-	24	-	-	-	-	-	18
V. parahaemolyticus	12	-	18	-	16	-	-	-	/	-	-
P. damselae* subsp. *damselae	-	-	31	-	30	-	-	-	-	16	22
A. veronii	-	-	-	-	-	-	-	-	-	-	-
A. hydrophila	-	-	-	-	-	-	-	-	-	-	-
S. sonnei	-	-	/	-	-	-	-	-	-	-	-
E. tarda	nt	-	-	-	-	10	13	nt	nt	-	-

- No inhibition effect; **/** inhibited and unmeasured; nt – not tested

3.1.2 Fish gut *Bacillus* isolates have Quorum-Quenching (QQ) activity

QQ potential of *Bacillus* isolates was tested using 2 *E.coli* reporter strains AI1-QQ.1 and AI2-QQ.1 (Table 2) (Weiland-Brãuer et al., 2015). These strains contain a gene encoding a lethal protein under the control of an inducible promoter responsive to the respective QS signal molecules. Thus, AI1-QQ1 has a *luxI* promoter expressing the lethal gene *ccdB* (pRS488), and AI2-QQ1 contains a AI-2-inducible *lsrA* promoter also expressing the same gene (pRS489) (Weiland-Brãuer et al., 2015). Because of this, both strains are unable to grow in the presence of the respective signal molecules unless nontoxic interfering molecules are present (Weiland-Brãuer et al., 2015).

First, a validation assay of the individual concentrations of the signal molecules [N-(Beta-Ketocaproyl)-L-homoserine lactone and 4-hydroxy-5-methyl-3-furanone] to be used for complete growth inhibition of the reporter strains AI1-QQ.1 and AI2-QQ.1 (Table 2) respectively, were determined on agar plates supplemented with growing concentrations of the auto-inducers, as depicted in Figure 9. Growth inhibition of AI1-QQ.1 was achieved with 100 µM N-(Beta-Ketocaproyl)-L-homoserine lactone, while inhibition of AI2-QQ.1 required significantly higher concentrations of its signal molecule, with the application of at least 100 mM 4-hydroxy-5-methyl-3-furanone. Ethyl acetate 100% showed toxicity against both reporter strains, but at 50% concentration didn't show a significant effect (Figure 9A on 8 and 9; Figure 9B on 17 and 18), being an adequate solvent concentration to perform such assays.

Once established the ideal concentrations of the signal molecules N-(Beta-Ketocaproyl)-L-homoserine lactone and 4-hydroxy-5-methyl-3-furanone, the same 67 *Bacillus* isolates from the NUTRIMU collection of putative *Bacillus* probiotics (Table 2), were tested for their QQ activity. The cell-free supernatants of isolates FI314 and FI376, showed quorum-quenching activity when using the AI1-QQ.1 reporter strain (Figure 10), as shown by the appearance of bacterial colonies.

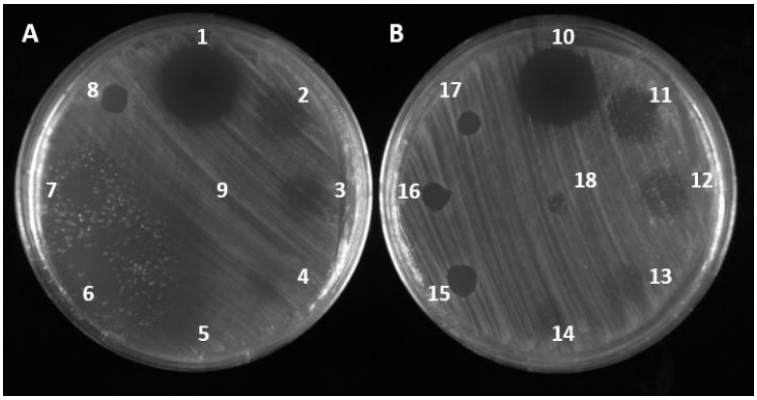


Figure 9. Validation of the reporter systems to optimize final concentrations of signal molecules that completely inhibit the growth of the respective reporter strains (AI1-QQ.1 and AI2-QQ.1). The halos mean a positive result for inhibition. On panel A is represented the growth inhibition of the *E.coli* reporter strains AI1-QQ.1 grown in LB agar plate (supplemented with 100 µg/ml Ampicillin and 30µg/ml Kanamycin, incubated at 37ºC overnight) by applying increasing concentrations of the autoinducers. (1)-(5) represents the 4-hydroxy-5methyl-3-furanone 5000 mM, 500 mM, 250 mM, 100 mM and 50mM concentrations that had no inhibition effect on AI1-QQ.1 (dark shadow is correlated with the orange color of the compound); (6) and (7) represents the N-(Beta-Ketocaproyl)-L-homoserine lactone, 10000 µM and 100 µM concentrations. Both inhibited AI1-QQ.1 reporter strain and 100 µM was choose to following studies. (8) and (9) represents ethyl acetate 100% and 50% ethyl acetate. Only 100% ethyl acetate showed toxic effects on the reporter strain. On panel B is represented the growth inhibition of the *E.coli* reporter strains AI2-QQ.1 grown in LB agar plate (supplemented with 100 µg/ml Ampicillin and 30µg/ml Kanamycin and incubated at 37ºC overnight) by applying increasing concentrations of the autoinducers. (10)-(14) represents 4-hydroxy-5methyl-3-furanone 5000 mM, 500 mM, 250 mM, 100 mM and 50mM concentrations. 100 mM was chosen to following studies. (15) and (16) represents results for N-(Beta-Ketocaproyl)-L-homoserine lactone. The inhibition effect is correlated with ethyl acetate (lactone solvent). (17) and (18) represents ethyl acetate 100% and 50% ethyl acetate. Only 100% showed toxic effects on the reporter strain.

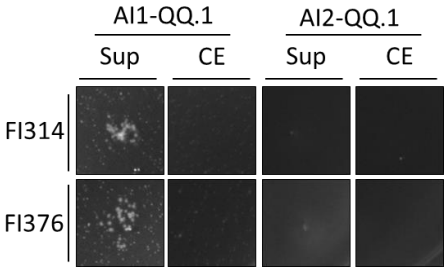


Figure 10. *Bacillus* isolates FI314 and FI376 quorum-quenching (QQ) activity against the respective reporter strains (AI1-QQ.1 and AI2-QQ.1). Cell extracts and culture supernatants (5µL) from *Bacillus* culture supernatants (Sup) and cell extracts (CE) were screened for QS-interfering activities with the respective reporter strains. The test plates were covered with LB soft agar containing 0.8% agar supplemented with 100 µM N-(β-ketocaproyl)-L-homoserine lactone (3oxo-C6-HSL), 100 µg/ml ampicillin, 30µg/ml kanamycin, and 10% (vol/vol) of exponentially growing culture of the reporter strain AI1-QQ.1 or LB soft agar plates containing 0.8%, supplemented with final concentrations of 100 mM 4-hydroxy-5methyl-3-furanone, 100 µg/ml ampicillin, 30 µg/ml kanamycin, and 10% (vol/vol) exponentially growing culture of the reporter strain AI2-QQ.1. The grown colonies show the reestablishment of growth of the reporter strains after 24 hours of incubation at 37°C by interference with the present signal molecule.

3.2 The *Pseudomonas* Quinolone Signal precursor HHQ inhibits the Vibrionaceae, including aquaculture pathogenic *Vibrio* species

The PQS precursor (HHQ) antimicrobial and antibiofilm activity was tested against different members of the Vibrionaceae family, to understand if this molecule has species-specific ways of targeting *Vibrio* species and to further characterise the nature of that bioactivity.

3.2.1 HHQ inhibits *Vibrio* growth

The antimicrobial activity of HHQ was tested against different members of the Vibrionaceae family, from UCC collection (Table 2), by plating each pathogen in HHQ-supplemented agar medium. As shown in Table 6, different *Vibrio* species showed different sensibilities to HHQ.

There were sensitive species, such as *V. fischeri.* shown in Figure 11, where all *V.fischeri* strains tested, including *V.fischeri* WH1, *V.fischeri* H905, *V.fischeri* CG101, *V.fischeri* ET101, *V.fischeri* SR5 and *V.fischeri* SA1, were unable to grow in HHQ-supplemented medium.

There were resistant species, such as *V.parahaemolyticus,* whose growth was not affected by the presence of HHQ (Table 6, Figure 12). To further understand HHQ resistance, different *V.parahaemolyticus* mutants*,* created by deleting from the wildtype strain RIMD 2210633 (Table 2) several genes (*bet AD DAP MUTANT; ect AD double crossover; Δect ABC; Δest ABC double; bact double crossover; ΔBCCT double crossover; Δprovwx; Δprovwx double; pro- double crossover 1*) (Table 2). *betAD*, *proVWX*, *ectABC*, and *bcct* regions represent operons that encode compatible solute systems and contribute to protecting the cells from changes in turgor pressure or outside pressure (Reen et al., 2006). Mutations on these operons were tested to understand if these genes are associated with the mechanisms of *V.parahaemolyticus* resistance to HHQ. As shown in Figure 12, *V.parahaemolyticus* strains and all the tested mutants grew in the presence and in the absence of HHQ, which means that none of the genes tested were responsible for HHQ resistance.

Table 6. Growth of different *Vibrio* strains on agar plates containing HHQ.

Species	Strain	HHQ GROWTH*
Vibrio parahaemolyticus	29.vp2.	+
	RIMD 2210633	+
	RIMD 2210633 *bet AD DAP MUTANT*	+
	Δest ABC double	+
	ect AD double crossover	+
	bact double crossover	+
	ΔBCCT double crossover	+
	Δprovwx	+
	Δprovwx double crossover	+
	pro- double crossover 1	+
	11.KEIO464	+
	RIMB 210623	+
	Δect ABC	+
Vibrio cholerae	O395	+/-
	NI6061	+/-
	ΔTRAP NI6961 2	+/-
	ΔTRAP VC1777-VC1779 NI6961 3	+/-
Vibrio anguillarum		+/-
Vibrio fischeri	WH1	-
	H905	-
	CG101	-
	ET101	-
	SR5	-
	SA1	-
Vibrio	1.19	+
Vibrio litoralis	DSM17657	+
Vibrio gallaecicus	DSM23502	-
Vibrio scophthalmi	DSM19140	-
	DSM19140 m1	+
	DSM19140 m3	+
	DSM19140 m4	+

*(+) HHQ had no effect on bacterial growth; (+/-) HHQ reduced the bacterial growth; (-) HHQ Inhibits the bacterial growth.

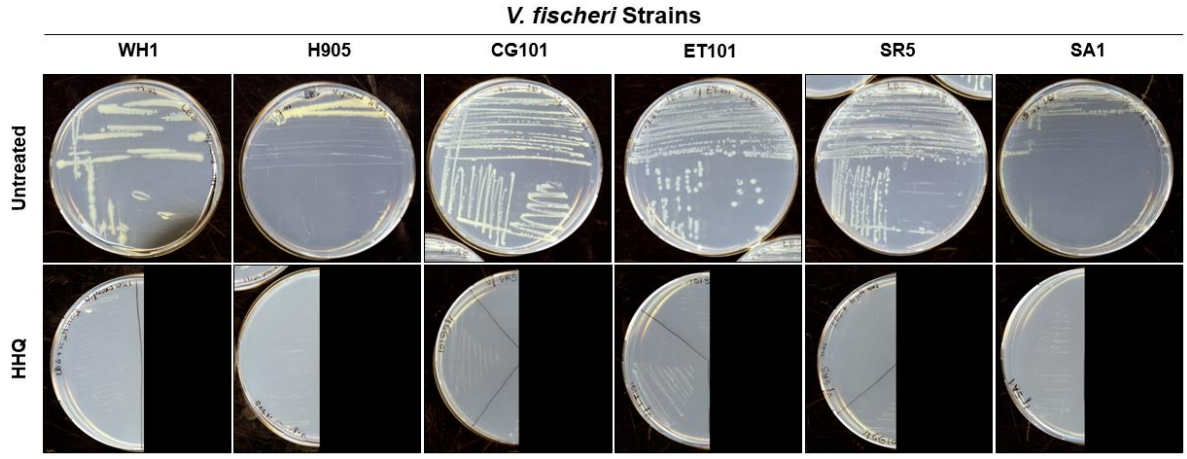


Figure 11. Growth inhibition effect in 6 different *V.fischeri* strains on agar plates containing 10 μM HHQ or not containing (untreated). All the *V.fischeri* strains were tested by streaking in LBS media supplemented with 10 μM HHQ and incubated at 23ºC for 24 hours. All the *V.fischeri* strains were susceptible to HHQ.

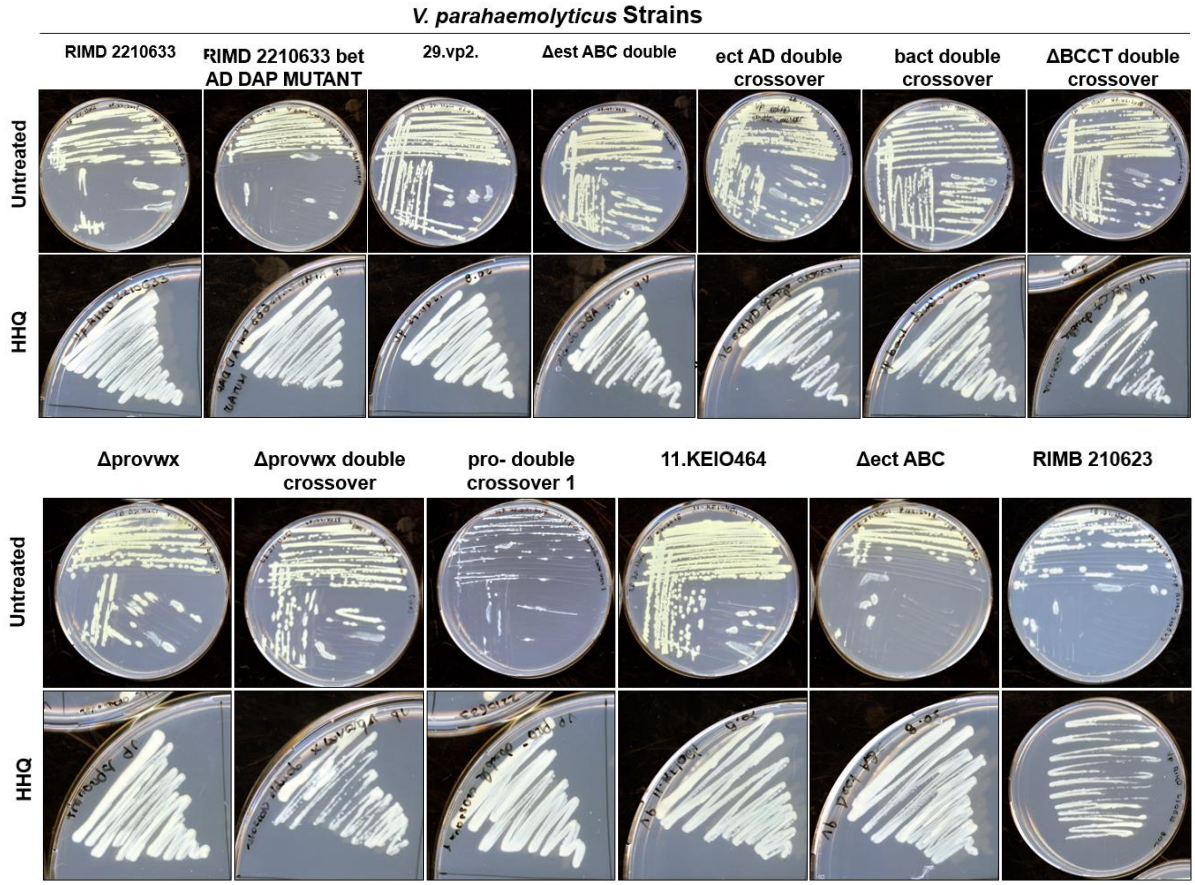


Figure 12. Growth inhibition effect in *V. parahaemolyticus* RIMD 2210633 strain and with different deletions on agar plates containing 10 µM HHQ or not containing (untreated*).* All the *V. parahaemolyticus* were tested by streaking in LBS3 media supplemented with 10 µM HHQ and incubated at 30ºC overnight. None of the deleted genes were responsible for HHQ tolerance on this strain.

The effect of HHQ was also explored and tested on other different *Vibrio* species including *V.gallaecicus* DSM 23502, *V.litoralis* DSM 17657, *Vibrio* 1.19, *V.cholerae* O395*, V.cholerae* N16961, *V.anguillarum* and also *V.cholerae* N16961 with mutations on the TRAP (Table 2) transporter system which is used by *Vibrio* strains to transport sugars. As shown in Figure 13, of those strains, only *V.gallaecicus* DSM 23502 was susceptible to HHQ. *V.litoralis* DSM 17657 *and Vibrio* 1.19 grew in the presence and in the absence of HHQ meaning that they are tolerant to this compound. Finally, *V.cholerae* and *V.anguillarum* experienced less growth with this compound, being partially inhibited by it. The *V.cholerae* mutants grew in the presence and in the absence of HHQ, which means that none of the mutations were responsible for the observed HHQ-partial resistance.

The most unexpected result was observed when the antimicrobial effect of HHQ was tested on *V.scophthalmi* DSM 19140. Curiously, the growth of the strain was

inhibited during 2 days, but after 6 days on agar plates, resistant colonies started to appear (Figure 14).

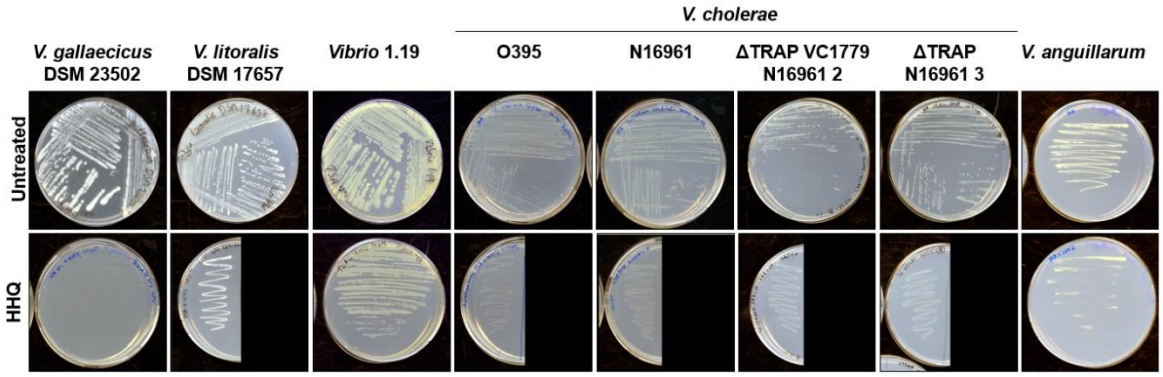


Figure 13. Growth inhibition in *V.gallaecicus* DSM 23502, *V.litoralis* DSM 17657, *Vibrio* 1.19, *V.cholerae* 0395, *V.cholerae* NI6961 strains and *V.cholerae* NI6961 with two different deletions on agar plates containing 10 µM HHQ or not containing (untreated). The strains were tested by streaking in the respective culture media and incubating following the strains requirements (Table 2). Only *V.gallaecicus* DSM 23502 was inhibited by HHQ. *V.cholerae* and *V.anguillarum* experiences less growth with this compound. None of the *V.cholerae* deleted genes were responsible for HHQ tolerance on this strain.

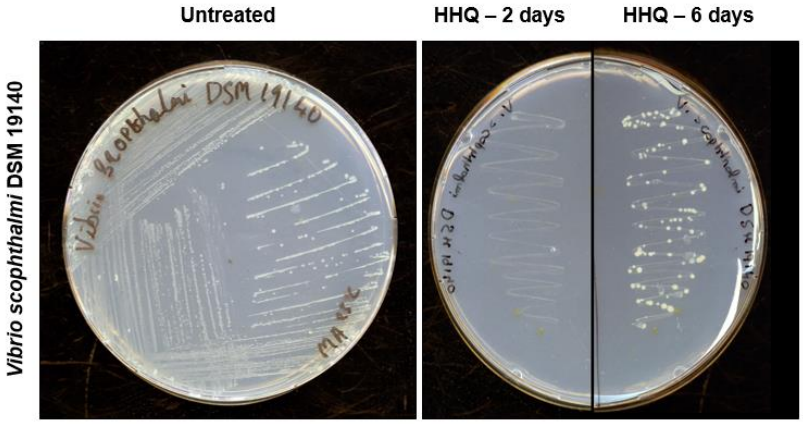


Figure 14. Growth inhibition effect in *V.scophthalmi* DSM 19140 strain on agar plates containing 10 µM HHQ (HHQ-2 days and HHQ-6 days) or not containing (untreated). *V.scophthalmi* strain was tested by streaking in MA media supplemented with 10 µM HHQ and incubated at 23ºC for 2 days, following the strains requirements (Table 2). *V.scophthalmi* DSM19140 was inhibited until 2 days. Tolerant mutant colonies appeared after 6 days.

Novel assays to confirm the observed apparent gain of tolerance, were done with these mutant colonies (Figure 14) on new agar plates supplemented with HHQ. As shown in Figure 15, after 2 days the wildtype *V.scophthalmi* DSM 19140 showed to be sensitive as previously demonstrated on Figure 14. On contrary, the two mutant colonies (Figure 15: (2)) picked from the previous assay maintained their tolerance.

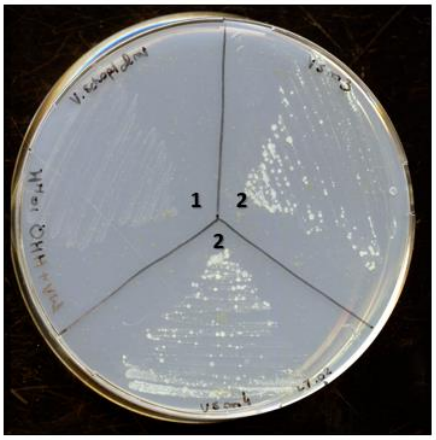


Figure 15. Growth inhibition effect of *V.scophthalmi* DSM19140 and *V.scophthalmi* DSM19140 mutants on an agar plate containing 10 µM HHQ. *V.scophthalmi* strain and mutants were tested by streaking in MA media supplemented with 10 µM HHQ and incubated at 23ºC for 2 days. (1) Growth inhibition effect of HHQ in *V.scophthalmi* DSM19140 (wildtype). This bacterium showed to be sensitive to HHQ. (2) Growth inhibition effect of HHQ in mutant resistant colonies on agar plates. Both showed tolerance.

Three different groups of bacterial strains were obtained from the antimicrobial assays: i) a tolerant group composed of *V.parahaemolyticus, V.litoralis* and *V. 1.19,* which can grow in the presence of HHQ*;* ii) a sensitive group including *V.fischeri* and *V.gallaecicus*, which are completely inhibited in the presence of HHQ; iii) and a third group composed by *V.cholerae* and *V.anguillarum,* that experience less growth in the presence of HHQ, and *V.scophthalmi* characterized by creating tolerant mutants (Table 6).

3.2.2 HHQ inhibits *Vibrio* growth and the biofilm formation

To further characterize the effect of HHQ on *Vibrio* spp., its effect was also tested on liquid medium. Besides evaluating if the observed effects on solid medium were maintained on liquid cultures, these assays also analysed the biofilm inhibition effect of HHQ, since *Vibrio* species form biofilms and in the case of pathogens, biofilm formation might promote virulence. These tests were done for the 6 *Vibrio* species (*V.parahaemolyticus* RIMD 2210633, *V.fischeri* SR5, *Vibrio* 1.19, V.*litoralis* DSM 17657, *V.scophthalmi* DSM 19140 and *V.gallaecicus* DSM 23502) selected from the initial panel of UCC collection strains (Table 2) at both 23ºC and 30ºC on 96 well-plates, to understand the HHQ impact differences between ideal and not ideal strains temperature

conditions. Because methanol was used as the solvent to HHQ it was included in the tests to rule out any possible influence on bacterial growth and biofilm formation.

As shown in Figure 16, *V.parahaemolyticus* and *V.scophthalmi* are the strains of this panel of 6 *Vibrio* species with higher capacity to form biofilms. *V.parahaemolyticus* showed higher optical densities on growth and on biofilm at 23ºC (calculated mean: OD600~0.750, OD595~0.870) than at 30ºC (calculated mean: OD600~0.718, OD595~0.643) although these differences aren't significative. Other species as *V.fischeri, Vibrio* 1.19 and *V.gallaecicus* clearly grow better at 23ºC even though there's not significative differences in the biofilm formation at both temperatures. *Vibrio* 1.19 almost had no growth at 30ºC. *V.litoralis* had similar growth results at both temperatures, however higher optical densities on biofilm formation at 23ºC. In *V.scophthalmi,* growth and biofilm results are very similar at both temperatures.

Regarding HHQ antimicrobial and antibiofilm effect on *Vibrio* species, as shown in Figure 16, both growth and biofilm formation of *V.fischeri*, *V.scophthalmi* and *V.gallaecicus* were strongly inhibited by HHQ, at both temperatures, confirming the previous results obtained on the agar plates assays. On growth results at 23ºC, HHQ showed to significantly inhibit ($0.001<p<0.05$) *V.parahaemolyticus*, *V.fischeri*, *V.scophthalmi* and *V.gallaecicus. V.fischeri*, and *V.gallaecicus* growth were also significative reduced by HHQ at 30ºC ($0.001<p<0.05$). Biofilm of *V.scophthalmi* was significative reduced ($p<0.05$) at both temperatures. Accordingly, on ideal temperature conditions (Table 2), *V.parahaemolyticus, Vibrio* 1.19 and *V.litoralis* experienced no inhibition by HHQ, maintaining their tolerance, as showed previously on the agar plates assay. Interestingly, HHQ inhibits biofilm formation of *Vibrio* 1.19 but has no significant effect on its growth, although the results were not statistically relevant. These results are summarized on Table 7.

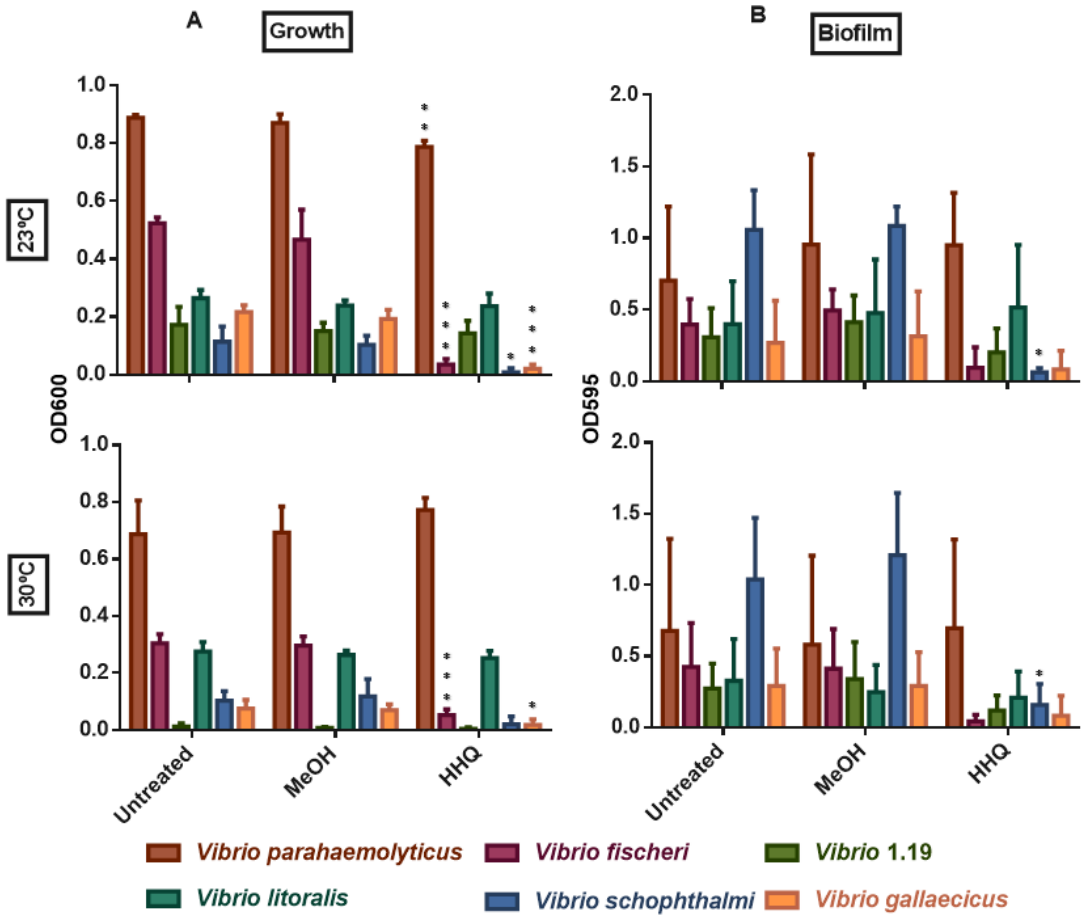


Figure 16. Growth (A) and biofilm (B) inhibition effect in 6 *Vibrio* species from UCC collection (*V.parahaemolyticus* RIMD 2210633, *V. fischeri* SR5, *Vibrio* 1.19, V. *litoralis* DSM 17657, *V. scophthalmi* DSM 19140 and *V. gallaecicus* DSM 23502) on 96-well plates containing 10 µM HHQ. Negative controls were included (Untreated, MeOH). All the wells were filled with 100 µl of indicator bacteria (OD_{600} ~0.05). On MeOH and HHQ wells, 1 µl of 100% MeOH and 10 mM HHQ were respectively added. All the strains were grown on optimal media conditions (Table 2) and incubated at both 23ºC and 30ºC. Data are the average of three independent experiments, each constituting three technical replicates. Error bars represent standard deviation of the means. Significative differences were determined by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. Asterisks represent statistically significant differences of treated *Vibrio* species relative to the control untreated: * - p<0.05, ** - p<0.01, *** - p<0.001. (A) Growth of *V.fischeri*, *V.scophthalmi* and *V.gallaecicus* was reduced by HHQ on their ideal temperature conditions (23ºC). *V.parahaemolyticus* showed also significative growth reduction at 23ºC. *V.fischeri* and *V.gallaecicus* were also significative reduced by HHQ at 30ºC. Growth of *V.parahaemolyticus* was not reduced on ideal temperature conditions (30ºC). *Vibrio* 1.19 and *V.litoralis* experiences no inhibition on both 23ºC and 30ºC. (B) Biofilm *of V.scophthalmi* was significative reduced by HHQ both 23ºC and 30ºC.

Table 7. Growth and biofilm formation of *Vibrio* species in the presence of HHQ at 23ºC and 30ºC.

Species	Strain	HHQ – 23ºC		HHQ – 30ºC	
		GROWTH*	BIOFILM*	GROWTH*	BIOFILM*
Vibrio parahaemolyticus	RIMD 2210633	-**	+	+	+
Vibrio fischeri	SR5	-**	-	-**	-
Vibrio	1.19	+	+	+	+
Vibrio litoralis	DSM 17657	+	+	+	+
Vibrio scophthalmi	DSM 19140	-**	-**	-	-**
Vibrio gallaecicus	DSM 23502	-**	-	-**	-

*(+) HHQ had no effect on bacterial growth or biofilm; (-) HHQ Inhibits the bacterial growth or biofilm.

** Represents statistically significative results (0.05< *p* <0.001).

3.2.3 Anti-growth and anti-biofilm characteristics of HHQ analogues

A panel of 39 synthetically created analogues derived from HHQ, with specific structure modifications on the aromatic rings and on the alkyl-chain (Table 3), were tested for antimicrobial and antibiofilm activity against the same members of the Vibrionaceae family from UCC collection (Table 2). Analogues were initially tested against representative strains of HHQ tolerance (*V.parahaemolyticus* RIMD 2210633) and HHQ susceptibility (*V.fischeri* SR5), to understand if the molecule modifications have the capacity to maintain, increase or decrease the HHQ activity.

The effect of HHQ analogues on *V.fischeri* and *V.parahaemolyticus* growth is presented on Figure 17. Controls, which are represented as untreated (only media + culture), methanol and DMSO (solvents used in analogues synthesis) showed all similar absorbance results, discarding any external effect on the results obtained.

None of the molecules tested had an inhibitory effect on *V.parahaemolyticus*. On contrary, *V.fischeri* growth (Figure 17), was negatively and significantly affected by A5, A12, D3, EI and G11 HHQ analogues ($p<0.001$). A12, D3 and G11 had the higher impact with results similar to the ones of the HHQ positive control. B4, F1, G9 and G10 induced a marginal (not significant) reduction of bacterial growth. Curiously, H1 analogue increased the bacterial growth of both *V.fischeri* and *V.parahaemolyticus* ($p<0.01$ and $p<0.001$). Also, A12 and DMSO (on *V.parahaemolyticus* graph) significative increased *the* growth ($p<0.001$).

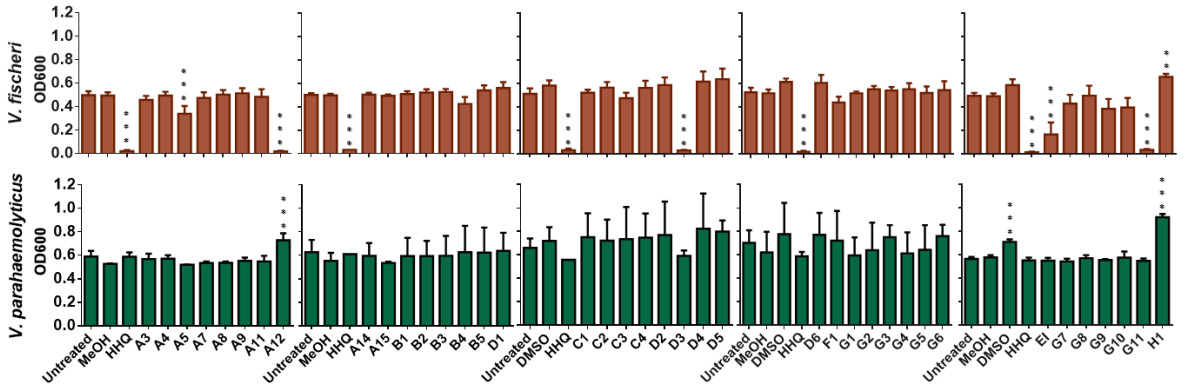


Figure 17. Growth inhibition effect in *V.fischeri* SR5 and *V.parahaemolyticus* RIMD 2210633 on 96-well plates containing 10 µM of HHQ synthetically created analogues. Negative controls were included (Untreated, MeOH, DMSO). HHQ was used as a positive control. All the wells were filled with 100 µl of indicator bacteria (OD_{600} ~0.05). On MeOH, DMSO wells, 1 µl of 100% MeOH and DMSO were respectively added. On HHQ and analogues wells, 1 µl of 10 mM HHQ and 10 mM analogues were respectively added. All the strains were grown on optimal growth conditions (Table 2). Data are the average of three independent experiments, each constituting three technical replicates. Error bars represent standard deviation of the means. Significative differences were determined by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. Asterisks represent statistically significant differences of treated *Vibrio* species relative to the control untreated: ** - p<0.01, *** - p<0.001. HHQ reduces *V.fischeri* growth in all the assays. *V.fischeri* growth was significative reduced by A5, A12, D3, EI and G11. H1 increased *V.fischeri* growth. None of the analogues reduced *V.parahaemolyticus* growth. A12, and H1 analogues and DMSO (on the last graph) significative increased *V.parahaemolyticus* growth.

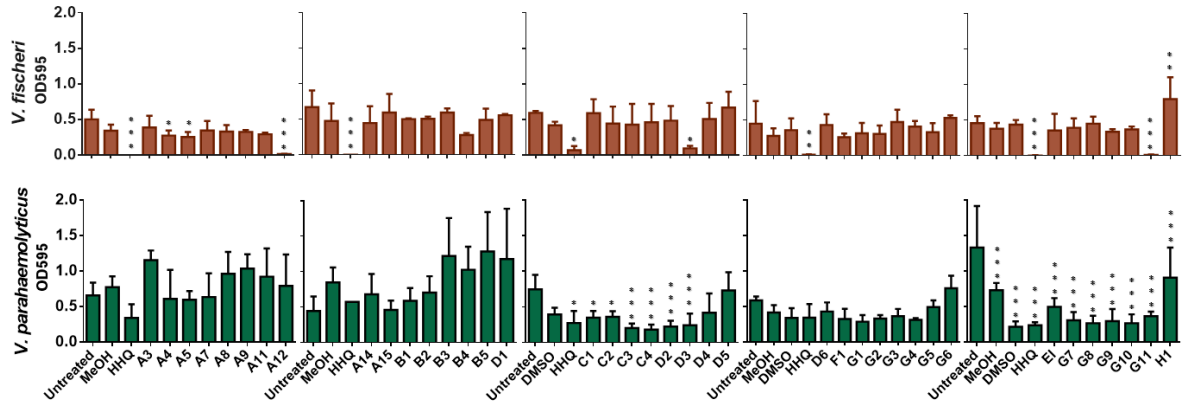


Figure 18. **Biofilm inhibition effect in *V.fischeri* SR5 and *V.parahaemolyticus* RIMD 2210633 on 96-well plates containing 10 µM of HHQ synthetically created analogues**. Negative controls were included (Untreated, MeOH, DMSO). HHQ was used as a positive control. All the wells were filled with 100 µl of indicator bacteria (OD_{600} ~0.05). On MeOH, DMSO wells, 1 µl of 100% MeOH and DMSO were respectively added. On HHQ and analogues wells, 1 µl of 10 mM HHQ and 10 mM analogues were respectively added. All the strains were grown on optimal growth conditions (Table 2). Data are the average of three independent experiments, each constituting three technical replicates. Error bars represent standard deviation of the means. Significative differences were determined by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. Asterisks represent statistically significant differences of treated *Vibrio* species relative to the control untreated: * - p<0.05, ** - p<0.01, *** - p<0.001. HHQ reduces *V.fischeri* biofilm in all the assays. *Vibrio fischeri* biofilm was reduced by A4, A5, A12, D3 and G11. H1 increased *V.fischeri* biofilm. The third *V.parahaemolyticus* graph showed that HHQ, C1, C2, C3, C4, D2 and D4 had a significative reduction on biofilm. The last V.*parahaemolyticus* graph showed that MeOH, DMSO, HHQ, EI, G7, G8, G9, G10, G11 and H1 had a significative reduction on biofilm.

Regarding biofilm inhibition effect of HHQ analogues (Figure 18), *V.fischeri* biofilm was significative reduced by A4, A5, A12, D3 and G11 ($0.001<p<0.05$). These results of A12, D3 and G11 were very similar with the ones obtained for HHQ positive control. EI, although showing a reduction on *V.fischeri* bacterial growth, seemed not to have an effect on its biofilm formation. Similarly, to what was observed on growth, B4, G9 and G10 induced a marginal (not significant) reduction of *V.fischeri* biofilm formation. Curiously, as on growth results, H1 analogue increased the biofilm formation of both *V.fischeri* ($p<0.01$). *V.parahaemolyticus* biofilm was significative reduced by, C1, C2, C3, C4, D2, EI, G7, G8, G9, G10, G11 and H1 ($p<0.001$). On the third *V.parahaemolyticus* graphic HHQ significative inhibit *V.parahaemolyticus* ($p<0.05$) and on the last graph showed that MeOH, DMSO and HHQ also had a significative reduction on biofilm ($p<0.001$).

Once determined the 4 analogues with best activity (similar to HHQ) against the representative strains of HHQ tolerance (*V.parahaemolyticus*) and HHQ susceptibility (*V.fischeri),* analogues, A12, D3, EI and G11 were tested against the initial 6 *Vibrio* strains from UCC collection (*V.parahaemolyticus* RIMD 2210633, *V.fischeri* SR5, *Vibrio* 1.19, V.*litoralis* DSM 17657, *V.scophthalmi* DSM 19140 and *V.gallaecicus* DSM 23502) to comprehend if a pattern of activity was present (Figure 19). *V.parahaemolyticus* and *Vibrio* 1.19 were not affected by any of the 4 HHQ analogues tested. *V.fischeri* and *V.gallaecicus* were inhibited by A12, D3, EI and G11. *V.fischeri* growth was significative reduced by A12, D3, EI, G11 ($p<0.001$). *V.gallaecicus* growth was significative reduced by D3, EI and G11 ($p<0.05$), but A12 wasn't statistically relevant. *V.scophthalmi* growth was inhibited by D3 and G11 but the results weren't also statistically relevant. It's important to note that the analogues A12 and EI had less impact on inhibiting *V.fischeri* growth when comparing with D3 and G11. A12 showed less impact on inhibiting *V.gallaecicus* than D3, EI and G11. *V.litoralis* growth was significative increased by A12 ($p<0.05$). Also, the inhibition effect of analogues was very similar with the obtained for HHQ positive control.

The negative controls, which are represented as untreated (only media + culture), methanol and DMSO (solvents of HHQ analogues) showed similar absorbance results as expected, although a small (not significant) increase on *V.fischeri* and *V.parahaemolyticus* growth could be seen on DMSO. These results are summarized on Table 8.

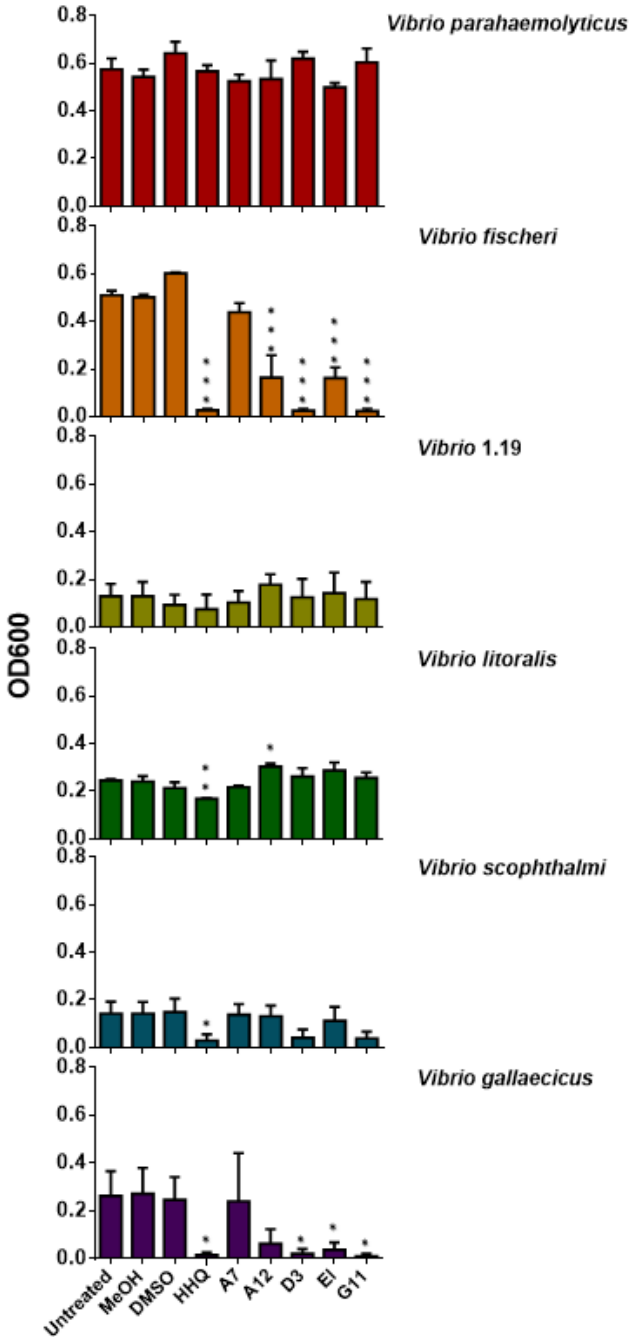
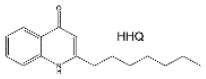
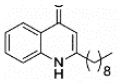
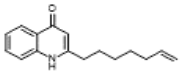
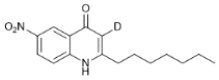
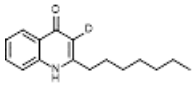


Figure 19. Growth inhibition effect in *Vibrio* species from UCC collection (*V.parahaemolyticus* RIMD 2210633, *V.fischeri* SR5, *Vibrio* 1.19, *V.litoralis* DSM17657, *V.scophthalmi* DSM 19140 and *V.gallaecicus* DSM 23502) on 96-well plates containing 10 µM of the 4-best bioactive (similar to HHQ) HHQ synthetically created analogues. Negative controls were included (Untreated, MeOH, DMSO, A7). HHQ was used as a positive control. All the wells were filled with 100 µl of indicator bacteria (OD_{600} ~0.05). On MeOH, DMSO wells, 1 µl of 100% MeOH and DMSO were respectively added. On HHQ and analogues wells, 1 µl of 10 mM HHQ and 10 mM analogues were respectively added. All the strains were grown on optimal growth conditions (Table 2). Data are the average of three independent experiments, each constituting three technical replicates. Error bars represent standard deviation of the means. Significative differences were determined by one-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. Asterisks represent statistically significant differences of treated *Vibrio* species relative to the control untreated: * - $p<0.05$, ** - $p<0.01$, *** - $p<0.001$. *Vibrio fischeri* growth was significative reduced by A12, D3, EI, G11. *V.litoralis* growth was increased by A12. *V.scophthalmi* growth was reduced by D3 and G11 the results weren't statistically relevant. *V.gallaecicus* growth was reduced by A12, D3, EI and G11. However, A12 wasn't statistically relevant. HHQ reduced *V.fischeri, V.litoralis, V.scophthalmi* and *V.gallaecicus*. None of the analogues had reduction effect in *V.parahaemolyticus* and *Vibrio* 1.19 growth.

Table 8. Growth of different *Vibrio* species in the presence of HHQ analogues.

Compound name and Structure	*V.parahaemolyticus* RIMD 2210633	*V.fischeri* SR5	*Vibrio* 1.19	*V.litoralis* DSM17657	*V.scophthalmi* DSM19140	*V.gallaecicus* DSM23502
HHQ 	+	-	+	+	-	-
A12 	+	-*	+	+	+	-*
D3 	+	-	+	+	-	-
EI 	+	-*	+	+	+	-
G11 	+	-	+	+	-	-

* These analogues have less effect than the others on the respective bacteria.

3.2.4 *V.scophthalmi* generates tolerant mutants to HHQ

As previously described, *V.scophthalmi* DSM 19140 was inhibited by HHQ supplementation on agar plates during 2 days, but after 6 days, resistant colonies started to appear. (Figure 14). To understand the real impact of HHQ on *V.scophthalmi* growth, a time-kill curve in liquid medium supplemented with HHQ, was done for both *V.scophthalmi* DSM 19140 (wildtype) and *V.scophthalmi* DSM 19140 mutant 1 (Table 2). By analysing the growth curves of *V.scophthalmi* and its mutant 1 without HHQ-supplementation it is clear that both have similar growth curves although, mutant 1 shows lower optical density values (absorbance measured at 600nm) (Figure 20). Both, *V.scophthalmi* and Mutant 1 grew exponentially until ~ 6h.

The more impacting differences can be seen with HHQ supplementation, where *V.scophthalmi* is highly inhibited by this compound during the 52 hours of experiment (with a not significative increasing after 24 hours), while the same does not happens for mutant 1 (Figure 20). When mutant 1 is exposed to HHQ, it behaves in a similar way to the wildtype strain during the first 24 hours of the assay. However, after a 52 hours analysis, is clear an increase in bacterial growth of mutant 1, with a peak that reaches the same OD600, that mutant 1 shows when grown without HHQ-supplementation. This observation clearly indicates a capacity of *V.scophthalmi* to overlap HHQ antimicrobial activity, confirming the results obtained on agar plates (Figure 14 and 15), that *V.scophthalmi* generates tolerant mutants to HHQ.

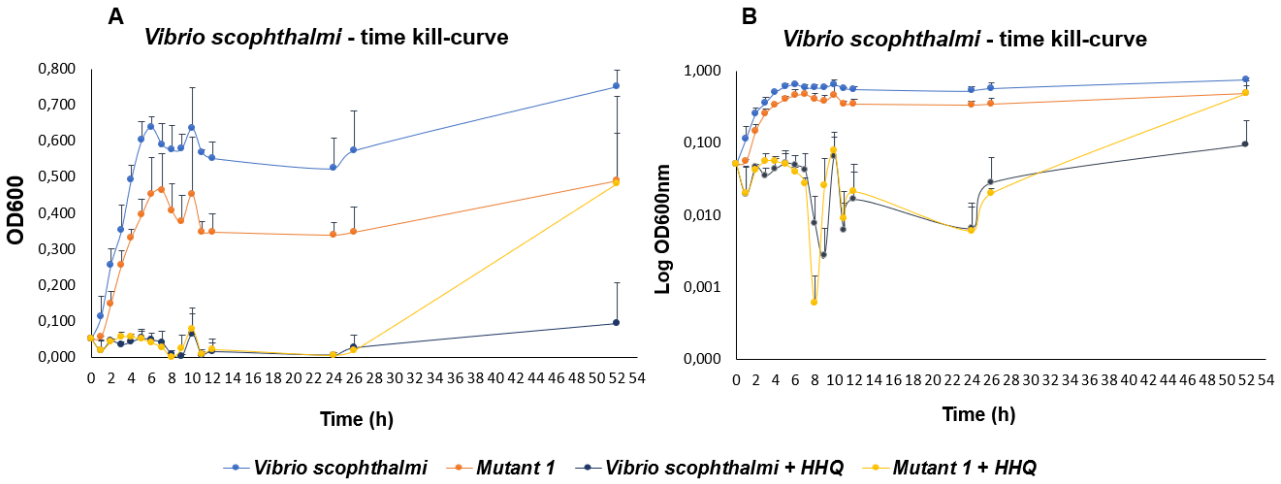


Figure 20. *V.scophthalmi* DSM 19140 (*V.scophthalmi*) and *V.scophthalmi* DSM 19140 m1 (Mutant 1) time kill-curve in absence and in presence of 10 µM of HHQ. (A) OD600 readings as function of time. (B) log OD_{600nm} as function of time. *V.scophthalmi* was grown on MA supplemented with 10 µM HHQ and incubated at 23ºC, 150 rpm. Optical density measures were taken for 52 hours period. Data are the average of three independent experiments. Error bars represent standard deviation of the means. *V. scophthalmi* and Mutant 1 grew exponentially until ~ 6h. With HHQ supplementation *V.scophthalmi* (*V.scophthalmi* + HHQ) and Mutant 1 (Mutant 1 + HHQ) growth were strongly reduced until 24 hours. After 24h and until 52 hours *V.scophthalmi* + HHQ showed slight increasing in the growth. Mutant 1 + HHQ showed a strong increasing in growth, reaching the untreated Mutant 1 optical density after 52 hours. Mutant 1 showed to create tolerance to HHQ.

To check that the tolerant colonies were indeed *V.scophthalmi* spontaneous mutants, and not a contamination by a different resistant species, the full length 16S rRNA gene of both wildtype *V.scophthalmi* DSM 19140 and its mutant 1, was amplified by a colony-PCR, and sequenced. PCR products were checked for correct size and purity by electrophoresis on a 1% agarose gel (Figure 21), confirming that both have the expected size of approximated 1500bp. Sequencing results, were compared to the NCBI 16S rRNA database, using BLAST (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Input sequence lengths varied from 900 to 1,300 bp.

A sequence alignment of nucleotide was done to identify similarity between the two sequenced 16S rRNA regions. Both wildtype mutant colonies showed a 100% similarity between them (*V.scophthalmi* DSM 19140 and *V.scophthalmi* DSM 19140 mutant 1) (Figure 22). The phylogenetic tree shows us 100% identity with of both with *V.scophthalmi* LMG 1958 which corresponds to *V.scophthalmi* DSM 19140. The closest relatives of the isolates are presented in Figure 23.

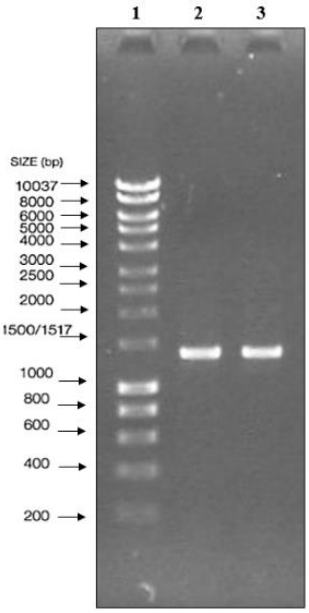


Figure 21. Amplification of 16S rRNA gene region on *V.scophthalmi* DSM 19140 and *V.scophthalmi* DSM 19140 m1. The amplifications were made by colony PCR. The primers (63F and 1387R) used to the amplifications are represented on Table 4. (1) HyperLadder™ 1kb (BIOLINE, 11-BIO-33053); (2) 16S rRNA *V.scophthalmi* DSM 19140; (3) 16S rRNA *V.scophthalmi* DSM 19140 m1.

```
CLUSTAL O(1.2.4) multiple sequence alignment

AR1_ADW0022399      --ATGACGAGCGGCGGACGGGTGAGTAATGCCTGGGAATATGCCTTGATGTGGGGGATAA      58
AR2_ADW0022399      AGATGACGAGCGGCGGACGGGTGAGTAATGCCTGGGAATATGCCTTGATGTGGGGGATAA      60
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AR1_ADW0022399      CCATTGGAAACGATGGCTAATACCGCATAATGCCTACGGGCCAAAGAGGGGGATCTTCGG      118
AR2_ADW0022399      CCATTGGAAACGATGGCTAATACCGCATAATGCCTACGGGCCAAAGAGGGGGATCTTCGG      120
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AR1_ADW0022399      ACCTCTCGCGTCAAGATTAGCCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACC      178
AR2_ADW0022399      ACCTCTCGCGTCAAGATTAGCCCAGGTGGGATTAGCTAGTTGGTGAGGTAATGGCTCACC      180
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AR1_ADW0022399      AAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGAGACACGG      238
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AR2_ADW0022399      GCATTTGAAACTGGCAGACTAGAGTACTGTAGAGGGGGGTAGAATTTCAGGTGTAGCGGT      600
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AR1_ADW0022399      GAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTG      658
AR2_ADW0022399      GAAATGCGTAGAGATCTGAAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAGATACTG      660
                    ************************************************************

AR1_ADW0022399      ACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG      718
AR2_ADW0022399      ACACTCAGATGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCG      720
                    ************************************************************

AR1_ADW0022399      TAAACGATGTCTACTTGGAGGTTGTGGCCTTGAGCCGTGGCTTTCGGAGCTAACGCGTTA      778
AR2_ADW0022399      TAAACGATGTCTACTTGGAGGTTGTGGCCTTGAGCCGTGGCTTTCGGAGCTAACGCGTTA      780
                    ************************************************************

AR1_ADW0022399      AGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCC      838
AR2_ADW0022399      AGTAGACCGCCTGGGGAGTACGGTCGCAAGATTAAAACTCAAATGAATTGACGGGGGCCC      840
                    ************************************************************

AR1_ADW0022399      GCACAAGCGGTGGAGCATGTGGTTTAATTCGATGCAACGCGAAGAACCTTACCTACTCTT      898
AR2_ADW0022399      GCACAAGCGGTGGAGCATGT----------------------------------------      860
                    ********************

AR1_ADW0022399      GACATCCAGAGAAGCCAGCGGAGACG  924
AR2_ADW0022399      -------------------------  860
```

Figure 22. Nucleotide sequence alignment of both sequenced *V.scophthalmi* DSM 19140 (AR1_ADW0022399) and *V.scophthalmi* DSM 19140 mutant 1 (AR2_ADW0022399). Both wildtype mutant colonies showed a 100% similarity between them. No gaps in the pairing sequences were found.

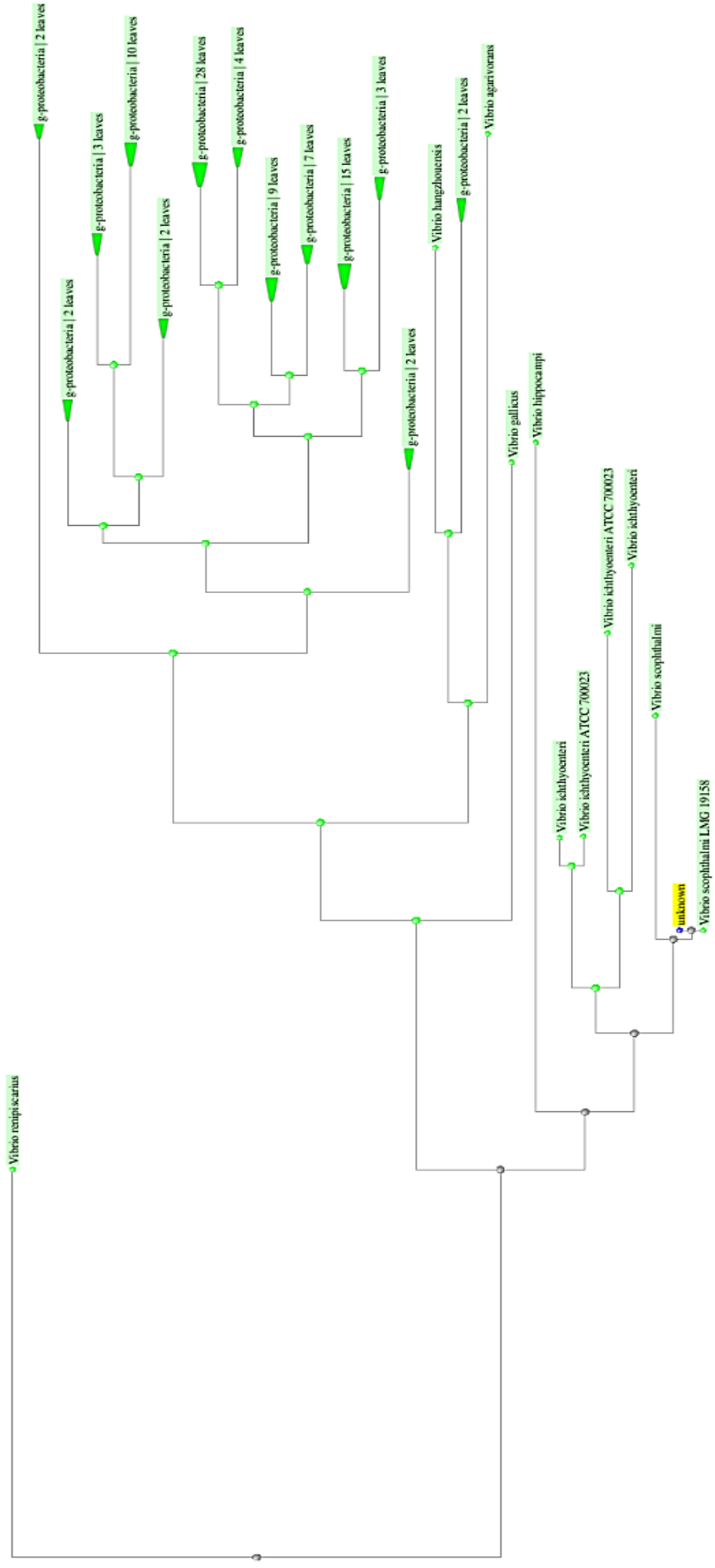


Figure 23. Phylogenetic tree on the basis of 16S rRNA gene sequenced. The tree was constructed using BLAST pairwise alignment after a nucleotide BLAST of the sequenced *V.scophthalmi* DSM 19140 and *V.scophthalmi* DSM 19140 mutant 1. Results showed to be equal for both sequenced regions. The sequences had 100% identity with *V.scophthalmi* LMG 1958 which corresponds to *V.scophthalmi* DSM 19140.

3.2.5 *cydAB* operon might be responsible for development of HHQ-tolerant mutants in the Vibrionaceae

A comparative genomic approach was used to further investigate the HHQ tolerance observed in *V.parahaemolyticus* and in other HHQ tolerant *Vibrio* strains, including the *V.scophthalmi* spontaneous mutants. Previous works have related microevolutions on the *cydAB* operon (cytochrome bd quinol oxidase responsible for oxidizing ubiquinol and reducing oxygen as part of the electron transport chain) with the capacity of different non-pathogenic Staphylococcal strains to resist molecules (pyocyanin and cyanide) released by *P.aeruginosa* (Voggu et al., 2006).

Thus, with the objective of verifying the presence of *cydAB* analogues on *Vibrio* species a comparative analysis of *cydAB* genes was done using protein-protein BLAST by comparing *V.parahaemolyticus* RIMD 2210633 with *V.alginolyticus, V.diabolicus, V.antiquarius, V.fischeri* SR5*, V.litoralis* DSM 17657*, V.scophthalmi* DSM 19140*, V.cholerae* O395, *V.anguillarum, Staphylococcus carnosus* subsp*. carnosus* TM300*, S.aureus* NCTC 8325 and *P.aeruginosa* (Table 9). From this analysis it was possible to find that all *Vibrio* strains have one identic copy of *cydA* (538aa) and *cydB* (378aa) genes on chromosome 1 (Table 9). However, *V.parahaemolyticus* has a second copy of both *cydA* (454) and *cydB* (335) on chromosome 2 that is not present in the *Vibrio* species used in this study and present on *P.aeruginosa* (Table 9). Although amplification of *cydAB* operon was possible from *V.scophthalmi* DSM 19140 (Data not shown), using the primers described on Table 4, the comparative genome analysis did not reveal the presence of such genes on the sequenced genome.

By doing a second protein-protein BLAST and comparing *V.parahaemolyticus* RIMD 2210633 with Vibrionaceae family, *V.alginolyticus, V.diabolicus* and *V.antiquarius* were the only species (excluding *P.aeruginosa*) containing the second copy of these genes (Table 9). These novel c*ydA* (454aa) and *cydB* (335aa) genes showed high similarity with *P.aeruginosa cioAB* (cyanide-insensitive oxidase*)* and correspond to genes VPA1137 and VPA1138 of *V.parahaemolyticus* RIMD 2210633 genome (chromosome 2). After this a third comparative genome analysis of chromosome 2 was done using protein-protein BLAST for *V.parahaemolyticus* RIMD 2210633 versus *V.fischeri* SR5*, V.litoralis* DSM 17657*, V.scophthalmi* DSM 19140*, V.cholerae* O395 and *V.anguillarum.* From this analysis it was possible to find that the DNA region containing those novel c*ydA* (VPA1137) and *cydB* (VPA1138) genes with 3409bp long are between

VPA1116 and VPA1158 region (Data not shown), only present in *V.parahaemolyticus* RIMD 2210633 but absent from all other *Vibrio* species.

To further investigate the contribution of these novel genes to the observed HHQ tolerance, the c*ydAB* operon (2401bp) and *cydB* gene *(*1008bp) alone were amplified by PCR, using oligonucleotide primers described on Table 4, and resolved by electrophoresis on a 1% agarose gel (Figure 24). Initially, three different annealing temperatures (53ºC, 55ºC and 58ºC) were used to determine the ideal annealing temperature for both *cydAB* and *cydB*. As shown in Figure 24 both *cydAB* (lanes 2, 3 and 4 of Figure 24) as well as *cydB* (lanes 5, 6 and 7 of Figure 24) PCR products were efficiently amplified with the correct sizes at all three temperatures.

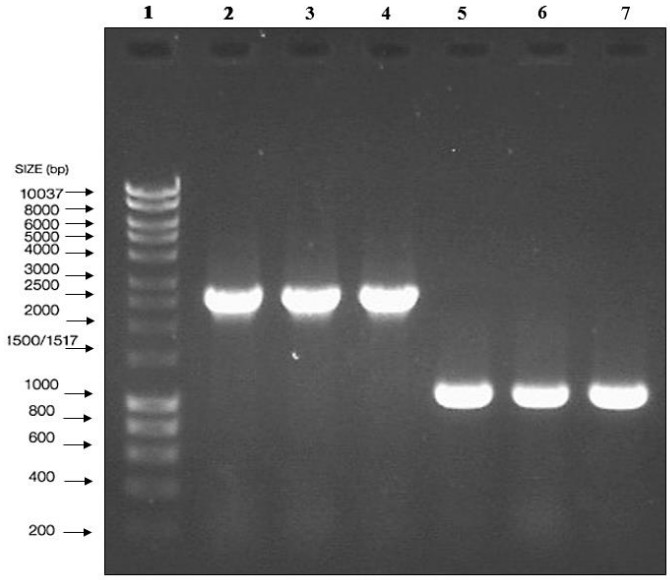


Figure 24. Amplification of *cydAB* and *cydB* gene region of *V.parahaemolyticus* RIMD 2210633. The amplifications were made by colony PCR. The 2401bp (*cydAB*) and 1008bp (*cydB*) fragments were amplified using the primers (cydA forward, cydB reverse; cydB forward) present on Table 4. (1) HyperLadder™ 1kb (BIOLINE, 11-BIO-33053); (2) – (4) *cydAB* amplified in three different annealing temperatures (53ºC, 55ºC and 58ºC)*;* (5) – (7) *cydB* amplified in three different annealing temperatures (53ºC, 55ºC and 58ºC).

Next a novel PCR, with a proofreading enzyme was performed (Figure 25) and the PCR product digested and ligated to plasmid vector pBBR1MCS-2 digested (Figure 26) with same enzymes (Figure 27) The ligation and transformation into *Escherichia coli* CH3-Blue competent cells was successful for both *cydAB* (1 colony) and *cydB* (3 colonies) genes and transformants isolated on selective media (LB supplemented with 50µg/ml Kanamycin). There were no visible colonies on the control plates carrying the empty plasmid and growth of more than 100 colonies was observed when the control vector DNA (pUC19) was used.

After plasmid extraction from *cydAB* and *cydB* transformants, a PCR was done to check for presence of correct inserts, that were resolved by electrophoresis on a 1% agarose gel (Figure 28 and Figure 29). It is possible to see that Lanes 2, 3, 4, that correspond to the PCR products of *cydB* carrying transformants have the expected size

of approximately 1000bp, consistent with *V.parahaemolyticus cydB*. On contrary, there were no PCR amplicons from *cydAB* transformants (figure 29, Lane 2), indicating that this transformation wasn't successful. Plasmid carrying the *cydB* gene was named pAR1.

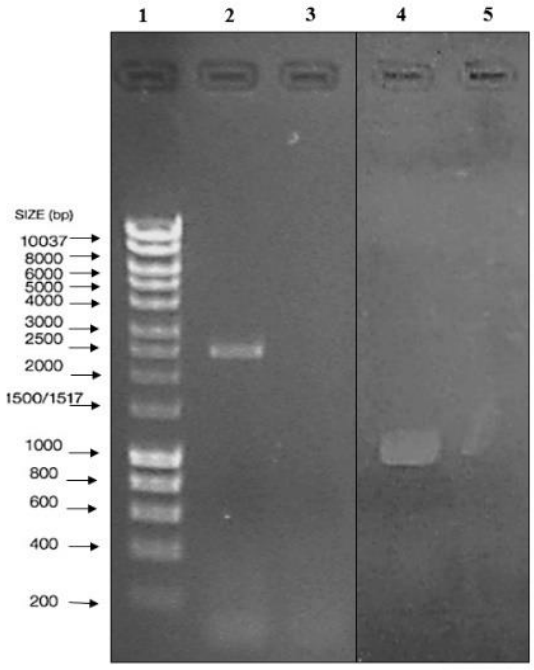


Figure 25. Amplification of *cydAB* and *cydB* gene region of *V.parahaemolyticus* RIMD 2210633 using Q5™ High fidelity PCR Kit, for cloning purposes. The amplifications were made by colony PCR. The 2401bp (*cydAB*) and 1008bp (*cydB*) fragments were amplified using the primers (cydA forward, cydB reverse; cydB forward) present on Table 4. (1) HyperLadder™ 1kb (BIOLINE, 11-BIO-33053); (2) *V.parahaemolyticus* RIMD 2210633 *cydAB;* (4) *V.parahaemolyticus* RIMD 2210633 *cydB;* (3) and (5) negative controls.

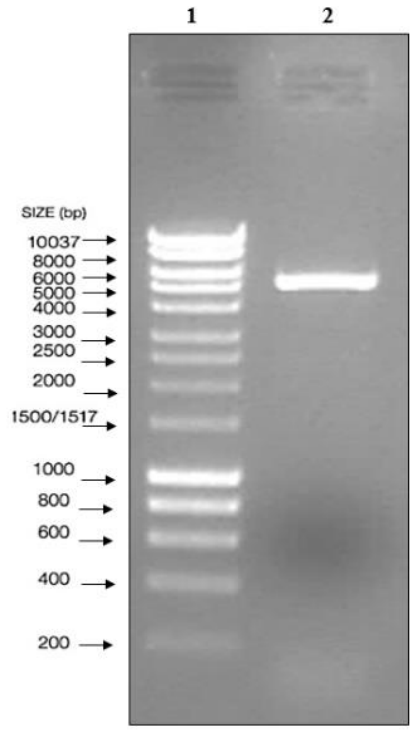


Figure 26. Restricted plasmid vector pBBR1MCS-2 on a 1% agarose gel electrophoresis. Plasmid vector pBBR1MCS-2 was previously digested with *Xho*I and *BamH*I restriction enzymes (Roche applied Science). (1) HyperLadder™ 1kb (BIOLINE, 11-BIO-33053); (2) pBBR1MCS-2.

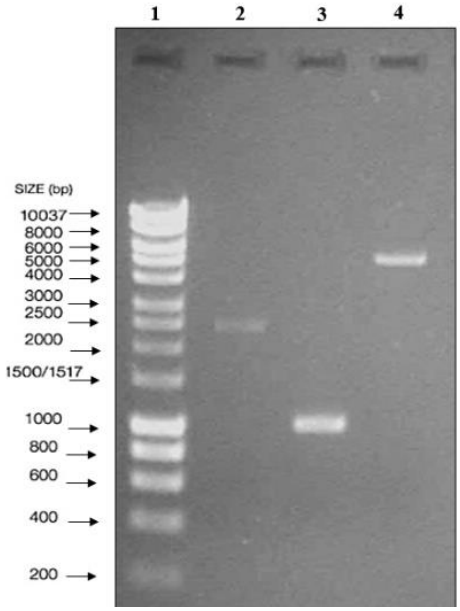


Figure 27. ***V.parahaemolyticus*** **RIMD 2210633** ***cydAB*** **and** ***cydB*** **gene, and plasmid vector pBBR1MCS-2 DNA purified fragments on a 1% agarose gel electrophoresis.** (1) HyperLadder™ 1kb (BIOLINE, 11-BIO-33053); (2) *V. parahaemolyticus* RIMD 2210633 *cydAB;* (3) *V.parahaemolyticus* RIMD 2210633 *cydB;* (4) pBBR1MCS-2.

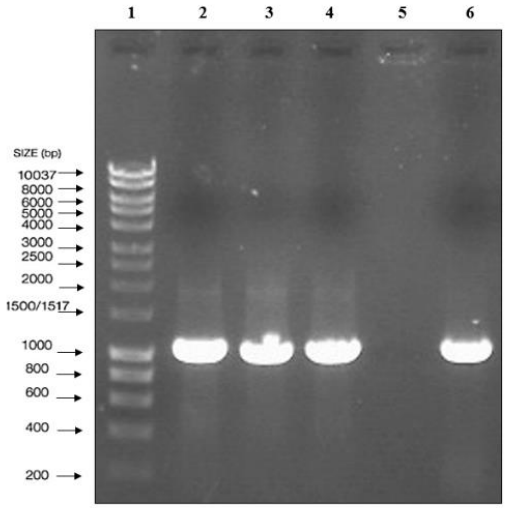


Figure 28. **Amplification of** ***cydB*** **from the extracted plasmid after transformation of** ***E.coli*** **competent cells.** The 2401bp (*cydAB*) and 1008bp (*cydB*) fragments were amplified using the primers (cydB reverse and cydB forward) present on Table 4. (1) HyperLadder™ 1kb (BIOLINE, 11-BIO-33053); (2) – (4) 3 different colony PCR of *cydB E. coli* transformants; (5) negative control; (6) positive control.

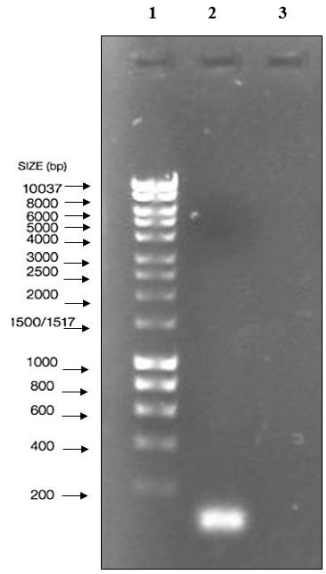


Figure 29. Amplification of ***cydAB*** **from the extracted plasmid after transformation of** ***E. coli*** **competent cells**. The 2401bp (*cydAB*) and 1008bp (*cydB*) fragments were amplified using the primers (cydA forward and cydB reverse) present on Table 4. (1) HyperLadder™ 1kb (BIOLINE, 11-BIO-33053); (2)1 colony PCR of *cydAB E. coli* transformants; (3) negative control.

Table 9. Comparative genomic analysis (BLAST) for *V.parahaemolyticus* RIMD 2210633 *cydA* (VP1053) and *cydB* (VP1054) on chromosome 1 and for *V.parahaemolyticus* RIMD 2210633 *cydA* (VPA1137) and *cydB* (VPA1138) on chromosome 2 versus other *Vibrio* and *Staphylococcus* strains and *P. aeruginosa,* to understand these genes distribution.

	Chromosome 1		**Chromosome 2**	
	V.parahaemolyticus RIMD 2210633 **VP1053** (*cydA*) 538aa	*V.parahaemolyticus* RIMD 2210633 **VP1054** (*cydB*) 378aa	*V.parahaemolyticus* RIMD 2210633 **VPA 1137** (*cydA*) 454aa	*V.parahaemolyticus* RIMD 2210633 **VPA 1138** (*cydB*) 335aa
Strains	Ident %	Ident %	Ident %	Ident %
V.alginolyticus	99	98	99	98
V.diabolicus	99	98	99	98
V.antiquarius	99	98	99	98
V.fischeri SR5	87	74	30	27
V.litoralis DSM 17657	84	78	30	29
V.scophthalmi DSM 19140 / LMG 19158	95	84	30	26
V.cholerae O395	89	85	47	38
V.anguillarum	92	86	45	38
S.carnosus subsp. *carnosus* TM300	25	**NR**	30	**NR**
S.aureus NCTC 8325	30	**NR**	31	**NR**
P.aeruginosa	74*	53**	74***	59****

* GenBank: RFJ63962.1 cytochrome bd-I ubiquinol oxidase subunit c*ydA* (521aa);

** GenBank: ASD11937.1 cytochrome d ubiquinol oxidase subunit II *cydB* (378aa);

*** NCBI Reference Sequence: WP_033988782.1 Cytochrome ubiquinol oxidase subunit I - *CioA* (488aa) present in various *Pseudomonas* strains. REGION: PA3930. e.g. *PAO1;*

**** GenBank: PPB14364.1 cytochrome d ubiquinol oxidase subunit II - *cioB* (335aa) LOCUS: PA3929.

75-100% Ident
50-75% Ident
<50% Ident
NR (No results)

4. Discussion

The main focus of this research was to explore the bioactive potential of microbial small molecules for the development of next generation antimicrobials, to be used against aquaculture pathogens. This is of particular importance because of the negative impact fish diseases have on aquaculture economy, and of the lack of effective solutions that can inhibit aquaculture relevant pathogens.

Initially the study was focused on 67 *Bacillus* strains isolated from the gut of several aquaculture fish species, that were previously identified and characterized as putative probiotics at NUTRIMU group. *Bacillus* are gram-positive spore-former and important producers of several secondary metabolites (4% of its genome) such as lipopeptides, polypeptides, macrolactones, fatty acids and bacteriocins, some with antimicrobial activities (Earl, Losick, & Kolter, 2008; Olmos, 2014; Sansinenea & Ortiz, 2011). Previous research demonstrated that *Bacillus* spp. have antimicrobial activity against members of Vibrionaceae (B. Vaseeharan, 2003), and Aeromonadaceae (Kong et al., 2017) families. Accordingly, in this study, NUTRIMU probiotic *Bacillus* strains were shown to have antimicrobial activity against a panel of the most important aquaculture fish pathogens. The growth of Vibrionaceae family species was highly affected by *Bacillus* extracellular molecules, being FI314, FI330 and FI442 the strains that showed higher inhibition zones. *S.sonnei* and *E.tarda* growth could also be diminished, although to a lower extent. *V.anguillarum* was the strain inhibited by more cell-free supernatants, in a total of 6 *Bacillus* strains, followed by *V.parahaemolyticus* and *P.damselae* subsp.*damselae* inhibited by 4 and *V.harveyi* and *V.vulnificus* inhibited by 3. *Aeromonas* strains were not affected by the *Bacillus* probiotics, although previous research done at NUTRIMU showed that some isolates could inhibit biofilm formation of *A.salmonicida*, suggesting the ability of these strains to inhibit bacterial surface attachment and/or disrupt attached biofilms (Miquel, Lagrafeuille, Souweine, & Forestier, 2016).

Because pathogens in general, and *Vibrio* species in particular, regulate their gene expression and virulence through quorum-sensing (QS), the quorum-quenching (antagonization of quorum-sensing; QQ) potential of the same *Bacillus* probiotic isolates was also evaluated. From the 67 *Bacillus* strains tested, only two (FI314 and FI376) showed quorum-quenching activity when using a AI1 (AHL type QS) biosensor (Weiland-Brãuer et al., 2015). This observation suggests that these 2 *Bacillus* strains produce

nontoxic QS-interfering molecules (that does not allow the expression of the lethal gene *ccdB*), against AHL quorum-sensing molecules of Gram-negative bacteria.

Such results, that highlight the potential of fish gut *Bacillus* probiotic strains on targeting *Vibrio* pathogens merit further investigations, namely by characterizing the nature of the observed antimicrobial/ anti-biofilm/ anti-QS activities and to understand if the molecules responsible are common to each phenomenon or diverse. A biochemical approach, trying to isolate/purify/identify and characterize the responsible molecules, would be a good starting point. A genomic approach, to map the responsible genes and identify novel biochemical pathways would also be of utmost interest.

While looking for molecules with anti-*Vibrio* activity, research done at BIOMERIT shown that PQS (Pseudomonas quinolone signal) and its precursor HHQ (2-heptyl-4-quinolone), which are important QS signal molecules involved in *P.aeruginosa* cell–cell communication, have antimicrobial and antibiofilm activity against gram-positive and gram-negative bacteria, including the pathogenic *V.vulnificus* (Reen et al., 2011). Curiously, an interkingdom impact, exhibiting antibiofilm activity against the clinically important fungal pathogens *Candida albicans* and *Aspergillus fumigatus* was also detected (Jerry Reen et al., 2016; Reen et al., 2011, 2016). Although the mechanisms through which HHQ mediate their species-specific targets remain unknown, it is noteworthy to say that *P.aeruginosa* and some of these *Vibrio* species may share the same environmental niche and could acquire mechanisms to adapt to the presence of these released molecules. Besides the interest for aquaculture, due to the relevance *Vibrio* species have as fish-pathogens, this observation might also impact human health, since *P. aeruginosa* is known by its morbidity and mortality in Cystic Fibrosis (CF) patients (Lund-Palau et al., 2016; Park et al., 2004).

In this study, the HHQ antimicrobial and antibiofilm activity was tested against different members of the Vibrionaceae family, to understand if this molecule has species-specific ways of targeting *Vibrio* species and to characterise further the nature of this important bioactivity. Distinct groups of HHQ response were observed with the initial antimicrobial assays done on agar plates: i) an HHQ-resistant group including *V.parahaemolyticus, V.litoralis* and *Vibrio* 1.19*,* whose growth was not affected by HHQ presence in the culture medium; ii) an HHQ-sensitive group, whose growth was completely inhibited by the presence of HHQ, that included *V.fischeri* and *V.gallaecicus*; iii) a group, composed by *V.cholerae* and *V.anguillarum,* that experienced diminished growth in the presence of HHQ; iv) and *V.scophthalmi* characterized by creating tolerant

mutants, few days after cultivation. These results clearly suggest the existence of a species-specificity HHQ-action in the Vibrionaceae, with tolerant and intolerant species. Importantly, there must be a mechanism or mechanisms to allow the HHQ-tolerant strains such as *V.parahaemolyticus*, as well as the spontaneous mutants of *V.scophthalmi,* to resist to the compound. Similar results were obtained when testing *V.parahaemolyticus*, *V.fischeri*, *Vibrio* 1.19, V.*litoralis*, *V.scophthalmi* and *V.gallaecicus* on liquid medium sustaining our conclusions of HHQ species-specificity.

Because *Vibrio* species inhabit a wide variety of aquatic environments and are exposed to fluctuations of several conditions as temperature, salinity, oxygen availability, plankton, tidal flushing and osmotic stress, bacteria have a constant challenge and environmental pressure to adapt (Depaola et al., 2003; Naughton et al., 2009; Reen et al., 2006). Previous research showed that *V.parahaemolyticus* has homologues of *ectABC*, four betaine/carnitine/choline transporters (BCCTs) and two ProU compatible solute transporters (encoded by *proVWX*) showing higher amount of osmoadaptation systems than the identified in other *Vibrio* species such as *V.cholerae*, *V.vulnificus* and *V.fischeri* (Naughton et al., 2009). There are other *Vibrio* strains that also encode *ectABC*, (e.g. *V.alginolyticus*) but they were not investigated in this study (Naughton et al., 2009). Thus, in this research, the contribution to HHQ-tolerance of these homologues of specialized systems previously shown to play an important role on the response to the osmotic stress on *V.parahaemolyticus* RIMD 2210633, was examined by knock-outing genes such as *betAD*, *proVWX*, *ectABC*, and *bcct.* These are part of operons that encode compatible solute systems (synthesis and uptake of compatible solutes) and contribute to protecting the cells from changes in turgor pressure or outside pressure (Reen et al., 2006). None of the *V.parahaemolyticus* deleted genes were responsible for HHQ tolerance (Figure 12), indicating that the mechanism of resistance of this bacterium must be associated with another genetic source.

As exemplified to *V.parahaemolyticus,* other species are exposed to fluctuations of several environmental conditions, being the scarcity of nutrients one of the most common. *V.cholerae* is one of the bacterial species that evolved and acquired mechanisms to overcome this problem (Mulligan et al., 2011). Thus, knockout mutants of *V.cholerae* with deletions in DNA regions such as Tripartite ATP-independent periplasmic (TRAP) transporters systems were also tested. TRAP are from the family of substrate-binding protein (SBP)-dependent secondary transporters that use electrochemical gradients across the membrane to collect low concentrations of nutrients in a more efficient way (Mulligan et al., 2011, 2012). These tests were done to investigate

the effect of gene loss and to understand if these genes were associated with the mechanisms of *V.cholerae* partial tolerance to HHQ. Although these mechanisms are very important for bacterial adaptation, in this particular case, there is no correlation between TRAP and *V.cholerae* partial tolerance to HHQ (Figure 13).

To further understand the specificities of the observed HHQ effect, a series of HHQ synthetic analogues, carrying modifications on the anthranilate ring and on the alkyl chain length (Table 3) were created and tested for their antimicrobial and anti-biofilm activity. None of the changes in the molecule structure increased HHQ bioactivity, but some of the changes made the molecule loose its activity (e.g. A7, A9, B2, G1). Some of the analogues, respectively A12, D3, EI and G11, were shown to have high significative capacity ($0.001<p<0.05$) (Identical to HHQ) to inhibit the growth of the sensitive group, namely *V.fischeri* and *V.gallaecicus,* and had no effect on the tolerant group including *V.parahaemolyticus, V.litoralis* and *Vibrio* 1.19*. V.scophthalmi* was an exception since it was only inhibited by D3 and G11. This observation might be connected to the fact that this species has the ability to develop HHQ-spontaneous tolerant mutants. The connection between being targeted by only two out of the four active analogues that showed similar results to HHQ, merits further investigations, since the specific molecule modifications might give insights on the mechanisms of tolerance development.

The effect of HHQ synthetic analogues on biofilm formation was very similar to the one observed on growth for *V.fischeri*, however *V.parahaemolyticus* biofilm was significative reduced by some analogues, an interesting result since the same molecules didn't shown any activity in what concerns to growth. However, these results can't be considered as the controls were also significative reduced. These results showed us important insight that will allow us to understand the molecule changes impact on further investigation.

Curiously, H1 analogue showed interesting results on both growth and biofilm assays since it was the only analogue that significantly increased the bacterial growth of *V.fischeri* and *V.parahaemolyticus.* Typically, HHQ has 16 carbons, 9 of them being part of the aromatic structure, however on H1 (that has 22 total carbons) 17 are included on this aromatic structure. This happening, together with the growth increasing could indicate the use of H1 analogue as a source of carbon and energy. Interestingly, previous tested compounds including aromatic rings, as phenanthrene, a polycyclic aromatic hydrocarbons (PAHs), showed to be degraded by some *Vibrio* strains (e.g. *V.parahaemolyticus* and *V.fluvialis*) (Hedlund & Staley, 2001; West, Okpokwasili,

Brayton, Grimes, & Colwell, 1984). Also, *V.cyclotrophicus* can use phenanthrene and naphthalene as a sole carbon and energy (Hedlund & Staley, 2001). As a future perspective, it would be interesting to understand if these *Vibrio* species are able to use H1 as a sole carbon source.

From these results, HHQ molecule seems to have a very specific way to target *Vibrio* species as some changes, specifically the ones incorporating decorations on the anthranilate ring on C3, C5, C6, C7 and C8 positions, would affect their actual antimicrobial activity. Previous research showed that the hydroxylation of HHQ molecule leads to complete loss of the PqsR (Pqs Receptor) antagonistic activity, however the installation of a blocking group (carboxamide) in C3 would restore its antagonism and demonstrated to reduce mortality by *P.aeruginosa* (Lu, Maurer, Kirsch, Steinbach, & Hartmann, 2014). Interestingly, the two analogues D1 and D2 carrying carboxamide group couldn't target any of the *Vibrio* species tested. This statement not only show impact of this specific modification on different bacterial species but also prove us the specific requirements of this molecular structure. Another study, now carried by Hartmann and co-workers showed that analogues of HHQ with strong electron-withdrawing groups at C6 (e.g. N_2O and NC) were antagonistic towards PqsR (Kamal, Petrera, Eberhard, & Hartmann, 2017). Interestingly, our research showed a loss of activity in all the 14 created analogues that included N_2O in the same structural position, with exception of EI.

In fact, only isolated changes in the length or structure of the alkyl chain, as introduction of a double bond (D3) and increasing in the length (A12) maintained the bioactivity of the molecule. Also, the addition of deuteron on C3 position (G11) and deuteron on C3 position combined with N_2O group on C6 position (EI) also maintained this bioactivity. Thus, analogues activity is comparable to the parent HHQ molecule, providing important insights into the structure function relationship underpinning the suppression. So, it would be interesting to understand the source of this interesting specificity, why they target specifically strains and which mechanism is related with the tolerance of others.

The observed specificity of the HHQ molecule could be related with the PqsH protein in *P. aeruginosa*. PqsH catalyzes the last step and transition of HHQ to PQS in the presence of O_2 and NADH, and previous studies showed that the alkyl side chain length is important for its activity, being the ideal size-chain length between 7 and 9 carbons, specifically HHQ and C9-HHQ (Schertzer et al., 2010). This is consistent with

our analogue's specificity as the four analogues that maintained an activity equal to HHQ had the alkyl side chain length between these values.

One of the most interesting findings in this research was the appearance of *V.scophthalmi* tolerant mutant colonies on HHQ testing plates (Figures 14 and 15) and on HHQ supplemented liquid growth medium (Figure 20). This assay was repeated three times, to confirm the tolerant mutant colonies appearing and the absence of contaminations. HHQ deterioration was excluded as it was always stored on cold conditions and didn't compromised any other strain results. Also, 16S rRNA sequencing results confirmed the identity of spontaneous mutant colonies as *V.scophthalmi*.

The emergence of such HHQ-tolerant mutants of *V.scophthalmi*, adding to the observation that some *Vibrio* species are HHQ-resistant, raised several questions: what is protecting these *Vibrio* species from being inhibited by HHQ? Could the mechanism be the same that protects *P.aeruginosa* from its own signaling molecule? How do/did *Vibrio* species acquire the mechanism?

Previous studies showed that several non-pathogenic staphylococci are resistant to respiratory toxins released by *P.aeruginosa* as pyocyanin, hydrogen cyanide, and even a mixture of quinoline N-oxides, due to alterations (microevolutions) in the *cydAB* genes (pyocyanin and cyanide insensitive cytochrome bd quinol oxidase) (Hotterbeekx, Kumar-Singh, Goossens, & Malhotra-Kumar, 2017; Voggu et al., 2006). Thus, the question that appear was if not only *V.scophthalmi* but all the *Vibrio* species that showed to be resistant to HHQ could have *cydAB* genes. If positive, this could be an interesting finding to the development of new ways of targeting important *Vibrio* species.

A BLAST analysis revealed the presence of *cydAB* genes homologues in *V.parahaemolyticus* RIMD 2210633. Then, a comparative genomic analysis for this strain versus other *Vibrio* strains was done to understand these genes distribution (Attachments, 1 and 2). The genomic comparisons identified that all sequenced *Vibrio* strains used in this study have one identic copy of *cydA* (VP1053; 528aa) and *cydB* (VP1054; 378aa) genes on chromosome 1. Surprisingly, a second copy of both *cydA* (VPA1137; 454aa) and *cydB* (VPA1138; 335aa) on chromosome 2 are unique to *V.parahaemolyticus* and showed high similarity to *P.aeruginosa cioA* (PA3930; 454aa) and *cioB* (PA3929; 335aa) gene. Interestingly by doing a second protein-protein BLAST and comparing now with the Vibrionaceae family, there were three strains (*V.alginolyticus, V.diabolicus* and *V.antiquarius*) containing the second copy of these genes (as in *P.aeruginosa*). A future investigation, to test HHQ activity against these

strains and verify if they are also tolerant to HHQ molecule would be interesting and a way of confirming *cydAB* contribution to HHQ tolerance in these species.

cioA and *cioB* genes (CIO) are homologous to subunits I and II of cytochrome bd-I from *E.coli* and slight differences in sequence and structure of the *cioB* subunit are responsible for cyanide resistance (Edalat, 2012). In light of this, the next question that arose was if this *cioB* gene present in *P.aeruginosa* could be responsible for HHQ-tolerance in *Vibrio* species?

Genomic comparisons also have identified a large DNA region (44683bp) located between genes VPA1116 and VPA1158 [that includes the novel *cydA*B (VPA1137; VPA1138) operon described earlier], that is unique to *V.parahaemolyticus* RIMD 2210633 and absent from almost all the other *Vibrio* species*,* suggesting the existence of a genomic island. Genomic islands are portions of the genome that evidence the existence of horizontal gene transfer, a phenomenon common on bacterial genomes, as they contribute to the diversification and adaptation of microorganisms and to genome evolution under certain environmental conditions (Juhas et al., 2009). Therefore, we speculate that, because *V.parahaemolyticus* species share the same environmental niche of *P.aeruginosa*, there might have been a selective pressure on some *Vibrio* strains to acquire these genes, and therefore evolve to a resistant phenotype to adapt to the presence of HHQ signaling molecule. As a future perspective, it would be interesting to verify if this genomic island, containing *cydAB,* is also present in the three species (*V.alginolyticus, V.diabolicus* and *V.antiquarius*) that already contain the *cydAB* second copy.

Adding to this, *V.scophthalmi* time inhibition curves (Figure 20) have shown that the emergence of the HHQ-tolerant spontaneous mutants appeared after 52 hours and this time matches perfectly with the stationary phase, which is known to have a microaerobic environment characterized by low oxygen concentration solved in the culture media. Curiously, *P.aeruginosa* has a preference to grow in a microaerobic environment even under aerobic conditions (Arai, 2011; Sabra et al., 2002) which is correlated with the enhanced production of several compounds, as hydrogen cyanide, elastase and pyocyanin (Sabra et al., 2002). Hydrogen cyanide is an important virulence factor and a respiratory chain inhibitor characterized for being produced in environments with lower oxygen availability conditions (Arai, 2011; Williams et al., 2007). To avoid this respiratory chain inhibitor, *P.aeruginosa* has CIO genes (*cioAB* operon), which encode cyanide insensitive oxidases that protect the bacteria from this virulence factor. Previous

research on other species, namely on *E.coli,* shown that the expression and membrane content of cytochrome bd (encoded by *cydAB*) is also increased by low O_2 concentrations (stationary phase) and by the presence and increase of other compounds as cyanide (Edalat, 2012).This observations open the possibility that, similarly to *V.parahaemolyticus*, *V.scophthalmi* might possess analogues of these operons, that are turned-on when low oxygen conditions (in the case of liquid medium assay) or other environmental insults, such as lack of nutrients (in the case of agar-plates assays) are present, leading to the development of spontaneous mutants. Indeed, amplification of *cydAB* operon was possible from *V.scophthalmi* DSM 19140, using the primers described on Table 4, but, the comparative genome analysis did not reveal the presence of such genes on the sequenced genome. This is clearly something that merits further investigation, namely by sequencing the PCR product obtained to verify its identity.

To further investigate the role of the novel *cydAB* operon on the HHQ-tolerance phenotype observed in *Vibrio* species, an insertion of both *cydA* and *cydB* and *cydB* alone on a susceptible bacterium such as *V.fischeri* was planned, and the initial steps performed in this work. Although both the entire operon as well as *cydB* gene alone were successfully amplified, only the ligation of *cydB* alone to vector pBBR1MCS-2 succeed, generating plasmid pAR1. No *E.coli* positive transformants were obtained for the *cydAB* insert. To understand the role of *cydB* on HHQ tolerance, plasmid pAR1 was tentatively inserted by conjugation onto *V.fischeri* genome. This approach would allow to understand if this strain becomes tolerant to HHQ after the insertion, but the transformation was not successful, and the final tests could not be done. Nevertheless, further conjugations attempts should be performed as well as try other cloning methods, such as electroporation, as it was shown to be a good method to do insertion in members of the Vibrionaceae (Gonzales, Brooks, Pukatzki, & Provenzano, 2013; Hamashima, Nakano, Tamura, & Arai, 1990; Visick & Ruby, 1996; Zeaiter, Mapelli, Crotti, & Borin, 2018). Also, knockout *cydAB* in *V.parahaemolyticus* and *cioAB* in *P.aeruginosa* would be an interesting approach to understand if these strains become sensitive, thus proving the contribution of these genes to HHQ tolerance.

This research led us to newly discovers on the HHQ molecule specificity on targeting *Vibrio* species which was never described previously. Also, it was a step forward to the understanding of *P.aeruginosa* mechanisms of protection from its own signalling molecules, suggesting the implication of *cioAB* as a potential ‘’immunity factor’’ for PQS/HHQ. This work future perspectives might open new scenarios on finding ways to target and create next generation antimicrobials.

5. Conclusion

By exploring the bioactive potential of microbial small molecules derived from both the *Bacillus* isolates and *P.aeruginosa* quorum-sensing molecular-based signaling molecules, it was achieved important discoveries, that proved their potential on targeting specifically *Vibrio* pathogens. Also, this study brought us important insights into the HHQ molecular structure function relationship underpinning the suppression. These discoveries could in the future provide the aquaculture sector with new natural compounds with potential to be used as novel disease prevention strategies.

With a combination of microbiologic, bioinformatic, molecular genetics and biochemical analyses we could achieved these findings that allow us to show also the diverse bioactivity against the most important fish pathogens, to fortify the idea of QS importance, the incredibly complexity of important pathways and the impact of interactions between different species in the environment.

Thus, we should also keep in mind that there are many source bioactivities and mechanisms that remains unknown and that require further investigations for clear understanding of how this sophisticated bacterial regulatory mechanisms works, that surely will lead to unexpected interesting findings and creation of next generation antimicrobials.

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